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3β-hydroxy-5-ene Steroid Dehydrogenase Gene Expression Regulation in Porcine Granulosa Cells

Differential Effect of FSH and LH on Gene Transcription

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The objective of this study was to investigate the effect of the tumor-promoting phorbol ester phorbol 12-myristate 13-acetate (PMA) on FSH- and LH-induced 3β-HSD-gene expression in cultured porcine granulosa cells. FSH and LH induced a dose dependent increase in the accumulation of 3β-HSD mRNA, measured by Northern blot. A 1.6- to 1.8-fold increase ($p < 0.01$) was observed with 10 ng/mL of FSH or LH. Maximal levels of 2.5- to 2.9-fold increases, relative to control, were reached at 30 and 100 ng/mL of the gonadotropins. When granulosa cells were treated with PMA (100 n*M*) just before the addition of FSH, the 3β-HSD rnRNA levels induced by 10 or 30 ng/mL of FSH were inhibited or partially inhibited, respectively. PMA did not inhibit elevated levels of 3β-HSD mRNA induced by FSH at concentrations of 100, 300, and 1000 ng/mL. Alternatively, PMA added just before LH, inhibited LH-stimulated 3β-HSD mRNA levels at all doses of LH tested (10, 30, 100, 300, and 1000 ng/mL). The protein kinase A-stimulators, dibutyryl-cAMP (cAMP) (0.5 m*M*) and forskolin (10 n*M*), also elevated the 3β-HSD-gene transcription, 3.5- and 4.0-fold respectively. PMA prevented the stimulation of the 3β-HSD-gene transcription when it was added just before cAMP or forskolin. We concluded that stimulation of PKC by PMA appears to have inhibited the gonadotropin-induced increase in 3β-HSD mRNA levels by preventing cAMP-activated 3β-HSD-gene transcription. The data also suggest that the effect of PMA appears to be more specific for regulation of LH-stimulated intracellular signals than those of FSH. This effect may indicate a site of differential regulation of FSH and LH on the stimulation of 3β-HSD-gene transcription.

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Introduction

The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), stimulate granulosa cell steroidogenesis by binding to cell membrane receptors coupled to adenylate cyclase (Segaloff and Ascoli, 1993). Gonadotropin binding is followed by activation of adenylate cyclase, which leads to an increase in the intracellular levels of cAMP, stimulation of protein kinase-A (PKA), and the initiation of a cascade of reactions that act on the genome targeting the genes encoding for the steroidogenic enzymes (Richards et al., 1994). Several laboratories have generated data suggesting that gonadotropins also stimulate phospholipase-C (Dimino et al., 1987). This reaction is followed by an increase in the phosphoinositide turnover within the plasma membrane, resulting in the synthesis of the second messengers diacylglycerol, which activates protein kinase-C (PKC), and inositol 1,4,5-triphosphate (IP3) (Davis et al., 1987; Guderman et al., 1992). Activation of PKC by phorbol esters has been reported to mediate inhibition of steroid hormone biosynthesis in porcine granulosa cells (Veldhuis et al., 1986; Flores et al., 1993). These observations raised the question of whether or not intracellular pathways that respond to second messengers other than cAMP play a role in gonadotropin stimulation of steroidogenesis.

The results of our studies, conducted in cultured porcine granulosa cells, show that gonadotropins elevate the mRNA levels of 3β-5-hydroxy-5-ene steroid dehydrogenase (3β-HSD), the enzyme that converts pregnenolone into progesterone (Chedrese et al., 1990a). Granulosa cells cultured in the presence of the PKC-activator phorbol 12-myristate 13-acetate (PMA) lose the capacity to respond to FSH and LH (Chedrese et al., 1990b). The mechanism by which PKC activation mediates inhibition of the 3β-HSD mRNA has not been thoroughly investigated. In a recent

report, we have shown that the 3β-HSD-gene is highly regulated by FSH and LH through a mechanism involving cAMP-mediated transcriptional activation (Chedrese et al., 1995). In the present report, we have extended these studies to investigate the effect of PhC stimulation with PMA on the FSH- and LH-mediated regulation of 3β-HSD-gene expression in cultured porcine granulosa cells.

Results

The effect of increased concentrations of FSH or LH on the steady-state levels of 3β-HSD mRNA in granulosa cells is shown in Fig. 1. FSH and LH induced a dose-dependent increase in the accumulation of 3β-HSD mRNA. An increase ($p < 0.01$) of 1.6- to 1.8-fold was observed at the concentration of 10 ng/mL with a maximum increase of 2.5- to 2.9-fold at concentrations of 30–100 ng/mL of gonadotropins (Fig. 1A,B). When granulosa cells were treated with PMA (100 n*M*) just before the addition of gonadotropins, the increase of 3β-HSD mRNA levels induced by 10 ng/mL FSH was inhibited (Fig. 1A). Addition of PMA partially inhibited ($p < 0.05$) the effect of 30 ng/mL FSH, but did not show any inhibitory effect at concentrations of 100, 300, or 1000 ng/mL (Fig. 1A). Alternatively, PMA was able to inhibit the LH-stimulated 3β-HSD mRNA levels at all the doses tested in the present study (10, 30, 100, 300, and 1000 ng/mL) (Fig. 1B). Figure 2 shows the effect of PMA when added to granulosa cells after an 8-h exposure to gonadotropins. Under these conditions, addition of PMA did not change the elevated levels of 3β-HSD mRNA induced by FSH (Fig. 2A) or LH (Fig. 2B), at any of the times tested (2, 4, and 8 h after PMA addition). No change in the levels of GAPDH mRNA were observed by treatment of cultured granulosa cells with PMA, FSH, and LH, or their combinations (data not shown).

Figure 3 shows the effect of PMA, cholera toxin (CT) (10 ng/mL), and forskolin (FK) (10 n*M*) on 3β-HSD-gene transcription rate in granulosa cells, as measured by nuclear run-on assay. No changes were observed on the basal rate of 3β-HSD-gene transcription when PMA alone was added to the cultures. CT and FK increased 3β-HSD-gene transcription to 163 ppm (3.5-fold) and 175 ppm (3.8-fold), respectively, from 46 ppm in the control group. When PMA was added just before this additon of CT or FK, the 3β-HSD-gene transcription rate was reduced to 68 ppm (~1.5-fold) and 55 ppm (~1.2-fold), respectively (Fig. 3). The effect of the combination of PMA with dibutyryl- cAMP (cAMP) on 3β-HSD-gene transcription rate in granulosa cells is illustrated in Fig. 4A. Addition of PMA to granulosa cells did not change the basal rate of 3β-HSD-gene transcription. Treatment with 0.5 m*M* cAMP activated the 3β-HSD-gene transcription rate from 27 ppm in the control group to 53 ppm in the treated group (1.96-fold). Addition of PMA just before cAMP inhibited the activation of the 3β-HSD-gene transcription, which theoretically would have been induced by

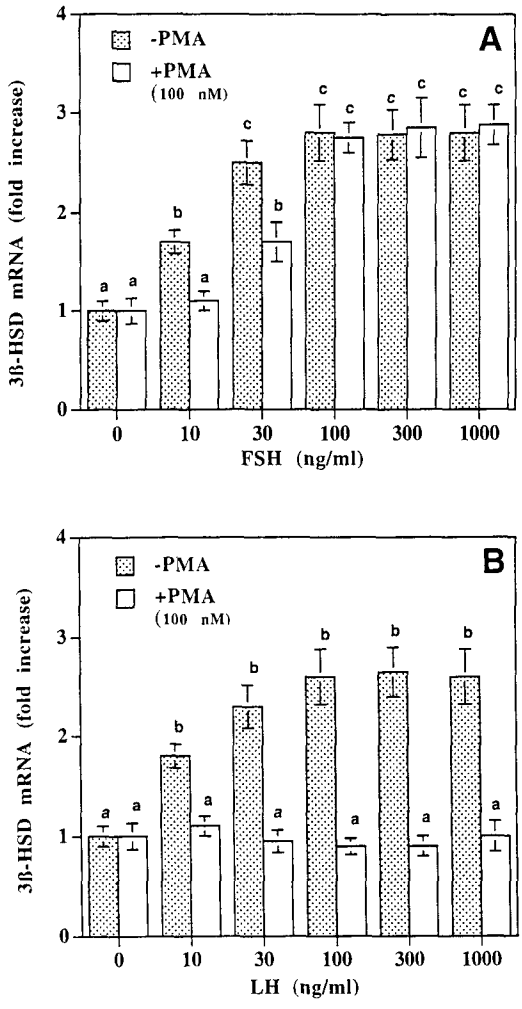

Fig. 1. Effect of FSH **(A)** and LH **(B)** on 3β-HSD mRNA levels in cultured porcine granulosa cells. Granulosa cells were cultured in the absence (–PMA) or presence (+PMA) of PMA (100 n*M*) with increasing concentrations of FSH (A) or LH (B) (0, 10, 30, 100, 300, and 1000 ng/mL). After a 6-h incubation, cells were collected for RNA extraction. Samples of total RNA (5 μg) were analyzed by Northern blot for 3β-HSD and GAPDH mRNAs. mRNA levels were quantitated using scanning densitometry and 3β-HSD levels were corrected for hybridization to GAPDH mRNA. The means ± SEM of three separate experiments are expressed relative to the basal 3β-HSD mRNA level in the absence of gonadotropins or PMA.

cAMP. Alternatively, PMA added after cAMP did not change the cAMP-induced activation of the 3β-HSD-gene transcription. The effects of the different combinations of PMA with cAMP on the 3β-HSD-gene transcription rate in granulosa cells previously treated with PMA for 24 h are illustrated in Fig. 4B. Under these conditions, granulosa cells responded to cAMP by activating 3β-HSD-gene transcription rate (~2-fold). Re-addition of PMA, before or after the addition of cAMP, did not change the cAMP-activated transcription of the 3β-HSD-gene. No changes in the GAPDH-gene transcription rates were observed by treatment of cultured granulosa cells with PMA, cAMP, CT or FK, or their combinations (Figs. 3 and 4).

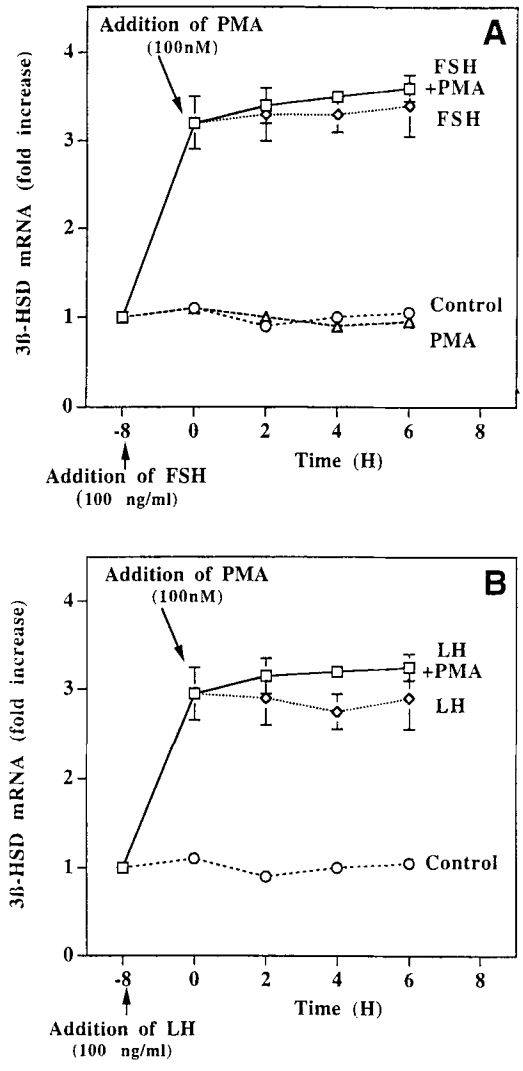

Fig. 2. Effect of PMA on the levels of 3β-HSD mRNA after stimulation with FSH **(A)** or LH **(B)**. Granulosa cells were cultured in the absence (control) or presence of FSH or LH (100 ng/mL). After an 8-h incubation, cells were treated with PMA (100 n*M*). At the indicated periods of time, granulosa cells were collected for total RNA extraction and analyzed by Northern blot for 3β-HSD and GAPDH mRNAs. The means ± SEM of three separate experiments are expressed relative to the basal 3β-HSD mRNA level in the control group.

The effect of FSH and LH on the generation of cAMP by granulosa cells is shown in Fig. 5. When granulosa cells were incubated with FSH or LH, a dose-dependent increase in the levels of cAMP was observed. An increase ($p < 0.01$), with respect to control values, was observed at the concentration of 10 ng/mL of FSH or LH. Maximal stimulation of 2.3- and 4.4-fold, compared to control values, was respectively observed for FSH or LH at the concentrations of 30, 100, and 300 ng/mL.

Discussion

In this report, we have examined the effect of PMA on the FSH- and LH-induced regulation of 3β-HSD-gene expression in cultured porcine granulosa cells. These

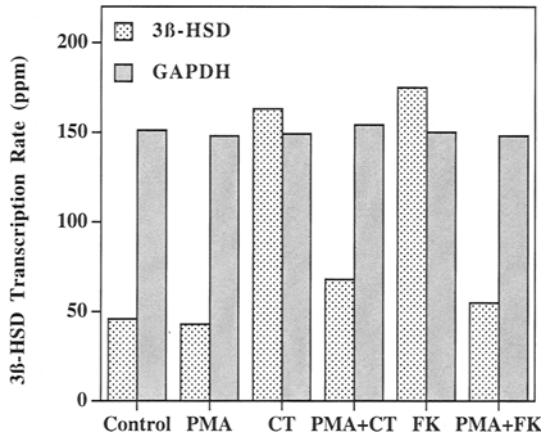

Fig. 3. Effect of PMA, cholera toxin (CT) and forskolin (FK) on 3β-HSD-gene transcription. Granulosa cells were cultured in the absence (control) or presence of PMA (100 n*M*) with or without CT (10 ng/mL) or FK (10 n*M*)). After a 4-h incubation, granulosa cells were collected and the nuclei were isolated. Transcription rates were determined by nuclear run-on assays. Results are expressed in parts per million (ppm) after subtracting background hybridization from the plasmid DNA (pGEM) and correction for the efficiency of hybridization. Data are the mean of duplicates from a representative experiment.

experiments were prompted by the observation that activation of PKC with PMA negatively regulates 3β-HSD mRNA levels in cultured granulosa cells (Chedrese et al., 1990). We have now shown that the effect of PMA is dependent on the type and concentration of gonadotropin used in the experiment. The results could be interpreted to mean that the effects of FSH and LH on 3β-HSD mRNA levels are differentially inhibited by stimulation of PKC.

The ability of PMA to inhibit the increase in 3β-HSD-gene transcription by CT and FK suggests that the effect of PMA occurs after the generation of cAMP. The mechanism of differential regulation between FSH and LH cannot be attributed to a better capability of FSH in generating cAMP. The data shown in Fig. 5 were interpreted to mean that, when granulosa cells were cultured in the presence of MIX, LH was at least three times more potent than FSH in stimulating intracellular levels of cAMP. We were unable to detect any significant effect of FSH or LH on the levels of cAMP when granulosa cells were cultured in the absence of MIX (data not shown). This result indicates that the activity of the phosphodiesterase(s) is very high in granulosa cells. Although regulation of phosphodiesterase activity by several protein kinases has been recently suggested (Conti et al., 1995), no indication of FSH- or LH-specific phosphodiesterases has apparently been reported in granulosa cells. Whether or not MIX, nonspecific inhibitor of phosphodiesterases (Conti et al., 1995), could be masking a differential effect of FSH or LH on cAMP action by inhibiting a specific phosphodiesterase remains unknown.

We have shown previously (Chedrese et al., 1995) that the transcription-inhibiting antibiotic, actinomycin D, low-

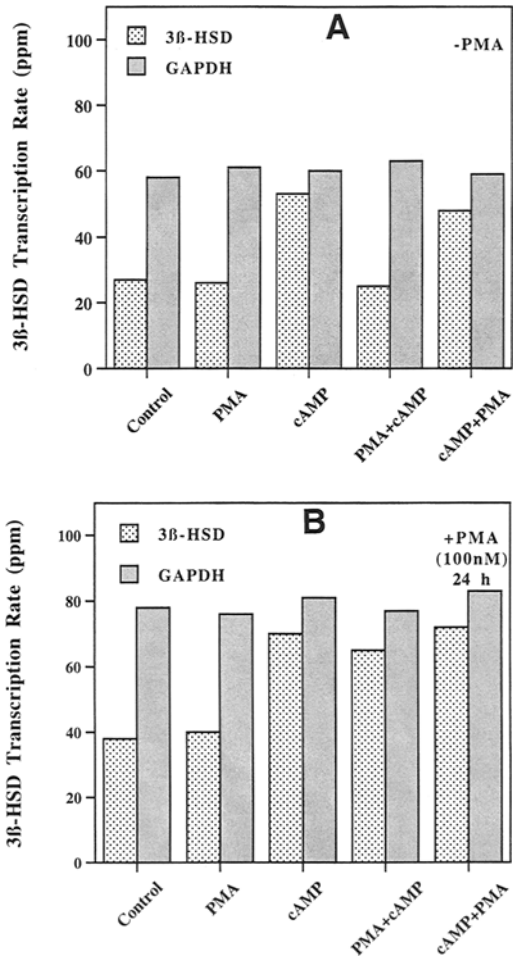

Fig. 4. Effect of PMA and cAMP on 3β-HSD-gene transcription. Granulosa cells were cultured in the absence **(A)** or presence of PMA (100 n*M*) **(B)**. After a 24-h exposure to PMA, cells were washed extensively and treated with medium containing PMA or cAMP (0.5 m*M*). Sequences of PMA added first and then cAMP (PMA+cAMP) or cAMP added first and then PMA (cAMP+PMA) were utilized. After a 4-h incubation, granulosa cells were collected, nuclei isolated, and analyzed as described in the legend to Fig. 3. Data are the mean of duplicates from a representative experiment.

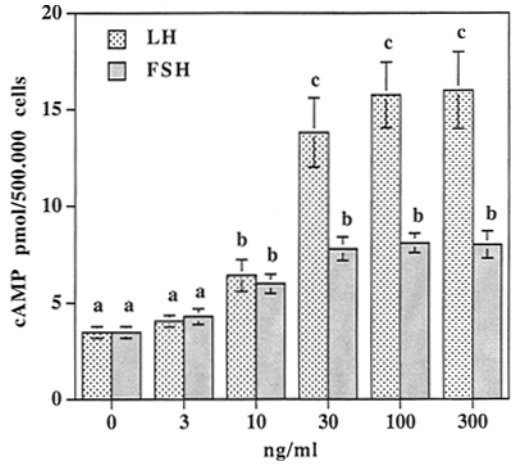

Fig. 5. Effect of FSH and LH on the levels of cAMP. Granulosa cells were cultured in the absence (control) or presence of increasing doses of FSH or LH (0 to 300 ng/mL) in the presence of MIX (0.1 m*M*). After a 1-h incubation, cells were collected for cAMP assay. Data are the means ± SEM of four determinations. Results are representative of three experiments.

ered the basal levels of 3β-HSD mRNA. In the same study we also demonstrated that addition of actinomycin D to cells exposed to FSH or LH for 6 h induced a rapid decay of the FSH- and LH-elevated levels of 3β-HSD mRNA. In the present study, we used the same experimental paradigm in order to test the effect of PMA on basal and gonadotropin-stimulated 3β-HSD mRNA levels. No change in the level of 3β-HSD mRNA was observed after addition of PMA alone. Addition of PMA after a 6-h incubation with FSH or LH did not change the elevated levels of 3β-HSD mRNA induced by either gonadotropin. These observations indicated that PKC stimulation inhibited the gonadotropin-activated expression of the 3β-HSD-gene, not its basal rate. Moreover, the inhibitory mechanism induced by PMA must be triggered before the gonadotropin activation of the 3β-HSD-gene.

The results, which indicated that PMA inhibited the elevation of the 3β-HSD-gene transcription, were confirmed by nuclear run-on assays. These assays also indicated that exposure of granulosa cells to PMA for 24 h abolished any further inhibitory effect of PMA on 3β-HSD-gene transcription (Fig. 4B). After 24 h of incubation with PMA, cAMP was able to elevate the transcription rate of the 3β-HSD-gene whether it was added before or after a new addition of PMA. This result could be interpreted to suggest that prolonged treatment with PMA abolishes PKC activity. It is known that a dose-dependent increase in PKC activity occurs after 3–6 h exposure to phorbol esters, but a progressive decline in PKC activity appears after a 12–24-h treatment (Rodriguez-Pena and Rozengurt, 1984). A 24-h exposure to PMA has been reported to result in PKC-deficient luteal cells, while other functions remained intact (Wiltbank et al., 1989). It has been suggested that the down-regulation of PKC produced by PMA is probably the result of a more rapid proteolytic degradation of the enzyme (Mizuguchi et al., 1988).

The collective data consistently show that the PMA inhibition of 3β-HSD-gene expression was present whether the source of cAMP elevation was FSH, LH, CT, FK, or an exogenously added cAMP analog. We had previously observed an identical inhibitory effect on the LH-elevated levels of 3β-HSD mRNA when PKC was activated by the diterpene, nonphorbol ester, mezerein (Chedrese et al., 1991). The fact that the phorbol ester 4αPDD, which does not activate PKC, did not elicit an inhibitory effect further supports a role for PKC as a mediator of the inhibition of cAMP-induced 3β-HSD mRNA levels (Chedrese et al., 1990b). Taken together, the findings indicate that this phenomenon is a consequence of PKC activation, which then

interferes with the mechanisms leading to cAMP-activated 3β-HSD-gene transcription.

The reported results could be interpreted to suggest that PKC mediates an LH-specific inhibition of the steroidogenic pathway at a point distal to cAMP generation. However, the FSH-mediated stimulation of the 3β-HSD-gene only exhibits this PKC inhibitory mechanism at low concentrations of FSH; it is ineffective at higher concentrations (Fig. 1). We conclude that the 3β-HSD-gene is one of the targets where the PKA and PKC second messenger pathways differentially interact to regulate steroidogenesis in granulosa cells.

There are indications that neither PMA nor the gonadotropins had any significant effects on the expression of the GAPDH-gene in our in vitro experimental conditions. This is in agreement with our previous observations in which neither gonadotropin nor PMA had any detectable effect on the levels of γ-actin mRNA in cultured porcine granulosa cells (Chedrese et al., 1991). We concluded that the stimulatory effect of gonadotropins or the inhibitory effect of PMA on 3β-HSD-gene expression did not appear to be attributed to changes in the general gene expression of granulosa cells.

The molecular mechanism by which cAMP stimulates and PMA inhibits transcription of the 3β-HSD-gene remains unknown. At this time we do not know of any studies addressing the functionality of its promoter in the ovary. We have conducted a computer-aided DNA sequence analysis of the human 3β-HSD II (Lachance et al., 1991; data not shown). This study does not indicate any evidence of the presence of a phorbol ester responsive element (TRE) (Landschultz et al., 1988), or AP2, a regulatory element described in some cAMP and phorbol ester-responsive genes (Imagawa et al., 1987). Several Sp1 and Sp1-like element-binding sites were found in the 5' flanking region preceding the first exon. This observation is particularly interesting, as stimulation by FK and inhibition by phorbol esters are mediated by a GA box element that binds Sp1 or an Sp1-like protein in the cytochrome P450 side-chain cleavage (P450scc) gene (Begeot et al., 1993). Since it has been reported that SP1 can be phosphorylated by DNA-dependent protein kinases, its role in second messenger signals has become attractive (Jackson et al., 1990). An interesting possibility is raised by the recent description of the transcription factor steroidogenic factor 1 (SF-1; also called adrenal 4 binding protein, Ad4BP), which regulates the expression of a number of cytochrome P450 steroidogenic enzymes in adrenal as well as gonadal tissue (Lynch et al., 1993). The analysis of the DNA sequence of the human 3β-HSD II promoter also reveals a putative SF-1 consensus sequence TCAAGGTAA at −64 to −56 bp. Recent evidence has been reported that SF-1 can interact with the human 3β-HSD II promoter, suggesting that this transcription factor may play a role in regulating ovarian progesterone synthesis (Leers-Sucheta et al., 1995).

The physiological implications of the PKC-mediated inhibition of 3β-HSD-gene transcription are unknown at the present time and the natural agonist of PMA remains to be elucidated. Despite the fact that phorbol esters have been extensively used in the study of second messenger signaling pathways, their actions remain complex and contradictory. Short term incubation (4–8 h) of porcine granulosa cells with PMA has been shown to have an additive effect with FSH on cylochrome P450 cholesterol side-chain cleavage (P450scc) mRNA stimulation (Lahav et al., 1995). Alternatively, long-term incubation (24–48 h) of granulosa cells with PMA has been reported to suppress the intracellular accumulation of insulin-stimulated P450scc mRNA and to inhibit low-density lipoprotein (LDL) metabolism, including the LDL receptor number, their internalization and degradation (Flores et al., 1993). It has been reported recently that PKC activation antagonizes the FSH-induced morphological modification and accumulation of insulin-like growth factor-I mRNA, which is characteristic of the in vitro differentiation of granulosa cells (Hatey et al., 1995). Whether or not gonadotropins and/or growth factors coordinate their actions by differential modulation of the PKA and PKC pathways mediating stimulatory or inhibitory signals to the 3β-HSD-gene during granulosa cell differentiation remains to be determined.

In summary, we concluded that the 3β-HSD-gene is one of the targets of the negative regulation of steroidogenesis by PKC activation. Cyclic AMP elevates, whereas PMA prevents 3β-HSD-gene transcription. The ability of FSH, but not LH, to overcome the PKC inhibitory signal on the expression of the 3β-HSD-gene would suggest competitive and noncompetitive inhibitory mechanisms for the effects of PMA/PKC on the actions of FSH and LH, respectively. We have developed the hypothesis that there exists a PKC-mediated differential inhibition of the FSH- and LH- stimulated 3β-HSD-gene transcription in porcine granulosa cells.

Materials and Methods

Reagents

Dulbecco's MEM, antibiotic-antimycotic mixture, fetal calf serum, trypsin, and reagents used for RNA preparations and Northern analyses were obtained from Gibco-BRL (Burlington, Ontario). FK, CT, dibutyryl-cAMP, 3-isobutyl-1-methyl-xanthine (MIX), PMA, bovine serum albumin (BSA), 2'-O-monosuccinyladenosine 3':5'-cyclic monophosphate tyrosyl methyl ester (succinyl-cyclic nucleotide tyrosine methyl ester), sodium metabisulfite, sodium phosphate, sodium acetate, materials for cAMP acetylation and chloramine T were purchased from Sigma (St. Louis, MO). $Na^{125}I$ (100 mCi/mL) was purchased from Dupont Canada (Mississauga, Ontario) and Sephadex G-10 was obtained from Pharmacia Biotech (Quebec). cAMP antibody was a gift from Joel Linden of the University of Virginia, School of Medicine (Charlottesville, VA). FSH

(NIADDK-oFSH) and LH (NIADDK-oLH) were obtained as a gift from the National Hormone and Pituitary Program (Rockville, MD).

Granulosa Cell Culture

Ovaries of prepubertal gilts were obtained from a local abattoir. Granulosa cells were collected from medium-sized nonatretic follicles (4–6 mm) and washed three times in Dulbecco's MEM containing 100 IU/mL penicillin, 100 μg/mL streptomycin and 1 μg/mL fungizone (DMEM), as previously described (Chedrese et al., 1987). Viable granulosa cells were plated in 100-mm plastic cell culture plates (Falcon, Lincoln Park, NJ) at a density of 5×10^6 viable cells/well. Cell cultures were maintained in a CO_2 incubator (Forma Scientific, Marietta, OH) at 37°C with a water saturated atmosphere containing 95% air and 5% CO_2. Cells were initially cultured in serum-containing (10% FCS) DMEM for 48 h to allow attachment onto the plates. Cells were then cultured for an additional 48 h in serum-free DMEM. At the end of this period, the experiments were initiated by replacing the culture media with serum-free DMEM containing the treatments.

Studies of 3β-HSD mRNA Steady States Levels

In the first set of experiments, granulosa cells were cultured in the absence (–PMA) or presence of PMA (100 n*M*) (+PMA) with increasing concentrations of FSH or LH (0, 10, 30, 100, 300, and 1000 ng/mL) for 6 h. Granulosa cells were also cultured in the absence (control) or presence of PMA (100 n*M*) with or without CT (10 ng/mL) or FK (10 n*M*) for 6 h.

In a second set of experiments, granulosa cells were cultured in the absence (control) or presence of FSH or LH (100 ng/mL) and incubated for 8 h. After this period, cells were treated with PMA (100 n*M*) and incubated for 2, 4, and 8 h.

Northern Blot Analyses

Granulosa cells were collected using 2 mL of 1% sodium dodecyl sulfate (SDS) and 10 m*M* EDTA, pH 7.0, solution. RNA was isolated by acid phenol/chloroform extraction (Liu, 1994). Samples of total RNA (5 μg) were denatured, size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel, and transferred onto a nylon membrane (Hybond-N, Amersham Canada, Oakville, Ontario) by diffusion blotting. RNA was crosslinked to a membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). cDNAs complementary to mRNAs encoding 3β-HSD (Luu-The et al., 1989) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al., 1985) were used as probes. cDNAs were labeled by random primer synthesis (Feinberg et al., 1983) with [α^{32}P] dCTP (>3000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of $1.5–3.0 \times 10^9$ dpm/μg of DNA. Membranes were hybridized for 16 h at 65°C in a solution containing 1*M* NaCl, 10% dextran sulfate, and 1% SDS. After hybridization, membranes were washed twice for 15 min at room temperature in a 2X SSC and 0.5% SDS solution and twice in a 1X SSC and 0.5% SDS solution at 65°C (20X SSC contained 3*M* NaCl and 0.3*M* Na_3 citrate). Hybridization was first performed with labeled 3β-HSD cDNA. Radioactive labeling was removed by incubating the filters in 10 m*M* Tris-10 m*M* EDTA for 30 min at 90°C before probing with labeled GAPDH cDNA. Northern blot autoradiograms were quantitated by computer-aided scanning densitometry using a ScanJet IIp Hewlett Packard scanner and analyzed with a digital image processing program (NIH Image 1.41). Optical density data were corrected for variability in loading by calculation of the ratio of 3β-HSD to GAPDH mRNAs. GAPDH mRNA levels were unaffected by treatment with FSH, LH, or PMA and therefore were useful as control values.

Transcription Run-on Assays

Granulosa cells were cultured in the absence (A) or presence (B) of PMA (100 n*M*) for 24 h. After this period, cells were washed extensively and treated with medium containing: PMA; cAMP (0.5 m*M*); PMA added first and then cAMP (PMA+cAMP); or cAMP added first and then PMA (cAMP+PMA). After 4 h of incubation, granulosa cells were collected and homogenized in a solution of 10 m*M* Tris-Cl, pH 7.4, 3 m*M* $CaCl_2$, 3 m*M* $MgCl_2$, and 0.5% Nonidet P-40 using a Dounce homogenizer. Nuclei were collected by centrifugation at 500*g* for 10 min and resuspended in 200 μL of storage buffer (50 m*M* Tris-Cl pH 8.3, 40% glycerol, 5 m*M* $MgCl_2$, and 0.1 m*M* EDTA) and maintained in liquid nitrogen.

Newly synthesized mRNA transcripts were analyzed using a modification of the procedure described by McKnight and Palmiter (1979). Isolated nuclei (5×10^7 nuclei in 200 μL) were mixed with 200 μL of transcription buffer (10 m*M* Tris-Cl, pH 8.0, 5 m*M* $MgCl_2$, and 0.3 KCl) containing 0.5 m*M* each of ATP, GTP, and CTP, and 100 μCi [α-^{32}P]UTP (3000 Ci/mmol; New England Nuclear). After incubation at 30°C for 30 min, labeled RNA was isolated by digestion with DNAse I (35 U) and proteinase K (100 μg). This step was followed by extraction with 1% SDS and acid phenol (Liu et al., 1994) using 10 μg *E. coli* tRNA carrier. Labeled RNA (~5×10^7 cpm) was hybridized to membranes containing excess (5 μg) of 3β-HSD or GAPDH cDNAs and incubated at 65°C for 72 h. The pGEM-3 cloning vector DNA was used as a control for background hybridization. We performed DNA excess filter hybridization to estimate the hybridizable radioactivity in 3β-HSD mRNA as described previously (Chedrese et al, 1994). Radioactivity in the membranes was quantified by liquid scintillation spectrometry. Synthesis rates for mRNAs were calculated from the radioactivity values of [^{32}P]RNA bound to specific cDNA-containing membranes minus the value of pGEM-3 containing membranes. The data were expressed as parts per million (ppm) for the levels of 3β-HSD-gene transcription after correction for the efficiency of hybridization.

Determination of cAMP

For testing the effect of FSH and LH on cAMP production, 5×10^5 granulosa cells were plated and cultured in Falcon 24-well plastic plates (1 mL of medium/well). The medium was replaced by fresh serum-free medium containing the phosphodiesterase inhibitor MIX (0.1 m*M*) along with the following treatments: medium alone (control); FSH (100 ng/mL); or LH (100 ng/mL). After 1 h of incubation, media was removed and cAMP was extracted from the cells by adding 0.5 mL ethanol. The ethanol was evaporated and cell extracts were resuspended in 200 µL of assay buffer (0.05*M* sodium acetate, pH 6.2). Samples were acetylated (Harper and Broker, 1975) and cAMP was determined by radioimmunoassay according to the method described by Steiner et al. (1972). Succinyl-cyclic nucleotide tyrosine methyl ester was used as a tracer and was iodinated using the method described by Hunter and Greenwood (1962). Approximately 2–4 µg of the derivative (in 50 µL water) was added to 40 µL 0.5*M* sodium phosphate buffer, pH 7.5. After the addition of 0.5 to 1.0 µCi of ^{125}I, 50 µL of a solution of chloramine-T (35 µg/10 mL phosphate buffer) was added and the reaction was run for 45 s. The iodine was then reduced by the addition of 100 µL of sodium metabisulfite (24 mg/10 mL 0.05*M* sodium phosphate buffer). The iodinated cyclic nucleotide derivatives were purified by column chromatography on a Sephadex G-10 column (0.9 × 9 cm), previously washed with 1 mL of 3% BSA in phosphosaline buffer (0.15*M* NaCl and 0.01*M* sodium phosphate). In the same experiment, cells exposed to identical treatments were dissociated with 0.1% trypsin and collected for counting with a hemocytometer to determine the number of cells per well after culture. Levels of cAMP were expressed as pmol/5×10^5 cells.

Statistical Analyses

Data were subjected to two-way analysis of variance. In the presence of significant F values, means were compared using Duncan's multiple range test (Steele and Torrie, 1980).

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