0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(9):3985–3994

doi: 10.1210/en.2003-0293

Protein Kinase B Is Obligatory for Follicle-Stimulating Hormone-Induced Granulosa Cell Differentiation

ANTHONY J. ZELEZNIK, DEEKSHA SAXENA, AND LYNDA LITTLE-IHRIG

Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Although FSH receptors are linked to the cAMP second messenger system, additional intracellular signaling pathways appear to be required for the induction of aromatase and the LH receptor during granulosa cell differentiation. We employed adenovirus vectors to modulate specific intracellular signaling systems in undifferentiated granulosa cells to identify the signaling pathway(s) that may be involved in the FSH-mediated induction of aromatase and the LH receptor. Expression of either the constitutively activated human LH receptor D578H or the constitutively active human $G_s\alpha$ Q227L resulted in increased cAMP production without increasing aromatase activity or mRNA levels for the LH receptor. To explore the contributions of other pathways, we expressed the constitutively activated forms MAPK kinase (MEK) and protein kinase B (PKB). Neither MEK nor PKB alone increased estrogen or progesterone production by undifferentiated granulosa cells. Stimulation of granulosa cells by FSH in the presence of the constitutively active PKB, but not MEK, led to an amplification of FSH-induced aromatase and LH receptor mRNA levels, whereas a dominant negative PKB vector completely abolished the actions of FSH. The expression of the constitutively active PKB in combination with the constitutively active LH receptor D578H, the constitutively active $G_s\alpha$ Q227L, or 8-bromo-cAMP led to an induction of aromatase as well as LH receptor mRNA comparable to that seen in cells stimulated with FSH alone. These results demonstrate that PKB is an essential component of the FSH-mediated granulosa cell differentiation and that both PKB and $G_s\alpha$ signaling pathways are required. (*Endocrinology* 144: 3985–3994, 2003)

ALTHOUGH IT IS unequivocal that FSH is the primary stimulus for the growth and differentiation of the preovulatory follicle, the intracellular signaling systems used by the FSH receptor that are responsible for the coordinated induction of steroidogenic enzymes, the LH receptor, and cell cycle regulatory proteins remain uncertain. It is well documented that FSH stimulates adenylyl cyclase with a resultant increase in cAMP synthesis (1, 2). However, it is unclear whether activation of the cAMP-protein kinase A signaling pathway alone is sufficient to account for the expression pattern of the many genes that are activated during FSH-dependent follicular maturation (3). Indeed, studies have shown that in addition to stimulating the cAMP pathway, FSH stimulates the MAPK pathway (4), protein kinase B (PKB) (5), and intracellular free Ca^{2+} (6). However, it is not known the extent to which each of these pathways, individually or together, participates in the control of granulosa cell proliferation and steroidogenesis. With respect to the latter, it is equally uncertain whether the regulation of genes involved in estrogen production are controlled by the same signaling pathway(s) that regulates the genes required for the production of progesterone. For example, β-adrenergic agents stimulate progesterone production, but not estrogen production, by FSH-primed granulosa cells, whereas FSH stimulates both estrogen and progesterone production by these cells (7). Likewise, although both FSH and vasoactive intestinal peptide stimulate cAMP and progesterone production by granulosa cells, FSH is more effective in stimulating LH receptors than vasoactive intestinal peptide (8). A major obstacle in addressing the contributions of individual signaling pathways involved in granulosa cell proliferation and differentiation is the ability to selectively manipulate each of the many pathways that may be involved in these processes. Although pharmacological inhibitors of signaling pathways are available, issues of specificity and cross-talk between pathways may confound the interpretation of results (9).

We have adopted the use of replication-defective adenovirus vectors to explore the signaling pathways involved in granulosa cell function. Because these vectors infect granulosa cells and direct the expression of proteins with high efficiency (10), it is possible to express constitutively activated as well as dominant-negative signaling proteins and directly assess their influences on granulosa cell steroidogenesis and cAMP production as well as the expression of mRNAs that encode for differentiation and proliferation-associated proteins. Previously, with this approach we expressed both the wild-type and a constitutively active human LH receptor in undifferentiated granulosa cells (*i.e.* no prior exposure to FSH) and found that although activation of FSH receptors and LH receptors produced comparable responses in stimulating progesterone production and increasing mRNA levels for α-inhibin and 3β-hydroxysteroid dehydrogenase (3β-HSD), activation of FSH receptors was more effective than both the wild-type and the constitutively active LH receptor in stimulating mRNA levels for aromatase and the endogenous granulosa cell LH receptor (11). Collectively, these observations suggest that the expression of aromatase and

Abbreviations: 8Br-cAMP, 8-Bromo-cAMP; CMV, cytomegalovirus; 8CTP-2Me-cAMP, 8-(4-chloro-phenylthio)-2′-*O*-methyl-cAMP; Epac, guanine exchange factor directly activated by cAMP; FBS, fetal bovine serum; 3β-HSD, 3β-hydroxysteroid dehydrogenase; MEK, MAPK kinase; PCNA, proliferating cell nuclear antigen; PI-3 kinase, phosphoinositol 3-kinase; PKB, protein kinase B.

the LH receptor during FSH-induced granulosa cell differentiation may be controlled by signaling pathways preferentially used by the FSH receptor. In the present study we continue our use of adenovirus vectors to further elucidate the signaling pathways involved in FSH-stimulated granulosa cell differentiation and proliferation.

Materials and Methods

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). 8-(4-Chloro-phenylthio)-2′-*O*-methyl-cAMP (8CTP-2Me-cAMP) was purchased from BIOLOG Life Science Institute (Bremen, Germany). Human FSH (AFP-4161-B; 3205 IU Second International Reference Preparation of FSH/mg, 225 IU Second International Reference Preparation of LH/mg), and antiserum to cAMP (lot CV-27) were provided by the National Hormone and Pituitary Program (NIDDK, NIH).

Adenovirus vectors

Preparation of the constitutively activated human LH receptor adenovirus vector Ad-RSV-LHrD578H was described previously (11). Adenovirus vectors that direct the expression of a constitutively active PKB (Ad-myrAKT) and a dominant negative PKB (Ad-dnAKT) under the control of a cytomegalovirus (CMV) promoter were obtained from Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA) (12). A constitutively active MAPK/ERK [MAPK kinase (MEK)] adenoviral vector (Ad-caMEK) was provided by Dr. N. Mitsuda (Osaka University, Osaka, Japan) (13). An adenovirus that directs the expression of a β-galactosidase (Ad-βgal) was provided by Dr. Joseph Alcorn (University of Texas Medical School, Dallas, TX). An adenovirus vector that directs the expression of a constitutively activated $G_s\alpha$ (Ad-$G_s\alpha$Q227L) was constructed using the AdMax system (Microbix Biosystems, Inc., Toronto, Canada). A 1195-bp fragment from plasmid GNAOSLOOCO (Guthrie Research Institute, Sayre, PA) corresponding to the coding sequence of human $G_s\alpha$Q227L was excised and subcloned into the adenovirus shuttle vector pDC316. Five micrograms of the pDC316 $G_s\alpha$Q227L shuttle vector were cotransfected with 5 μg of the shuttle vector pBHGloxΔE,3Cre into the human embryonic kidney cell line 293 in 60-mm culture dishes using the Superfect reagent according to the manufacturer's directions (QIAGEN, Inc., Valencia, CA). Transfected cells were maintained in DMEM containing 4.5 g/liter glucose (Life Technologies, Inc., Gaithersburg, MD) and 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcorss, GA) at 37 C in 5% CO_2. Approximately 14 d after transfection when the viral cytopathic effect was observed, the cells were collected and frozen on dry ice, thawed three times, then further propagated in 293 cells. When the cells exhibited a complete viral cytopathic effect, the cells were collected, resuspended in PBS, frozen and thawed on dry ice three times, and then centrifuged (1000 × *g*, 4 C, 10 min) to remove cellular debris. Aliquots of virus stocks were diluted 50- and 100-fold in lysis solution [0.1% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA] and incubated for 10 min at 56 C in a shaking water bath. The OD of the samples was measured at 260 nm, and the value obtained was used to calculate virus content using the relationship 10^{12} virus particles/ml/OD 260 U (14).

Granulosa cell culture

All procedures were approved by the University of Pittsburgh institutional animal care and use committee. Immature female rats (23–25 d old) were purchased from Hilltop Lab Animals (Scottsdale, PA). Granulosa cells were collected from the ovaries by puncturing follicles with a 25-gauge hypodermic needle, and cells were expressed into medium 199 (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum. Granulosa cells were seeded into 6-well (~10^6 cells/well) or 24-well (~2×10^5 cells/well) tissue culture plates and allowed to attach overnight. The next morning, medium and unattached cells were removed, and the granulosa cell monolayers were exposed to adenoviruses and stimulatory agents as described in the figure legends. At the end of the experiment, tissue culture medium was collected, boiled for 10 min to inactivate phosphodiesterases, and stored at −20 C for subsequent RIAs. Where indicated, total RNA was prepared from the cell monolayers using RNA-Bee (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's directions.

mRNA analysis

Samples of total RNA (1–5 μg) were analyzed for mRNAs for cytochrome P450 aromatase, 3β-HSD, the α-subunit of inhibin, and the LH receptor by ribonuclease protection assay according to the instructions provided by the supplier (Ambion, Inc., Austin, TX). Antisense RNA probes were prepared using [α-^{32}P]CTP (PerkinElmer Life Sciences, Boston, MA) from the following cDNA inserts: P450 aromatase (bp 1034–1295) (15), rat LH receptor (bp 1–622) (16), α subunit of inhibin (bp 694-1095) (17), 3β-hydroxysteroid dehydrogenase (bp 453–932) (18), proliferating cell nuclear antigen (PCNA; bp 204–456) (19), and cyclophylin (bp 34–142) (20). After electrophoresis (5% acrylamide containing 8 M urea), gels were dried and exposed to x-ray film for 16–96 h.

Aromatase assay

Granulosa cells were plated in 12-well dishes and stimulated with FSH, 8-bromo-cAMP (8Br-cAMP), and adenovirus vectors as described above. After stimulation, cells were washed twice with PBS and incubated for 2 h in 1 ml medium 199 containing 30 pmol 1β-[^{3}H]androstenedione (PerkinElmer Life Sciences) and 120 pmol unlabeled androstenedione for 2 h at 37 C in 5% CO_2. After incubation, the medium was transferred to an extraction tube containing 0.5 ml 30% trichloroacetic acid, and 2 ml chloroform were added. The tubes were mixed vigorously for 15 sec and centrifuged for 5 min at 3000 rpm, after which 1.0 ml of the aqueous upper phase were transferred to a 12 × 75-mm glass tube. At 4 C, 1 ml dextran/charcoal (5.0 g Norit plus 0.5 g dextran in 100 ml H_2O) was added to each tube and incubated for 20 min. The samples were centrifuged at 3500 rpm for 30 min, and 1.0 ml of the supernatants was transferred and counted for ^{3}H radioactivity in a liquid scintillation counter. Resultant counts were corrected for background and extraction efficiency, and cell monolayers were analyzed for protein content by Bradford assay.

RIA

Estradiol and progesterone concentrations in culture medium were determined by RIAs as described previously (21). cAMP concentrations in culture medium were analyzed by RIA using [^{125}I]cAMP-2-0′ monosuccinlyl cAMP tyrosine methyl ester (22) and anti-cAMP in accordance with the instructions provided by the National Hormone and Pituitary Program.

Western immunoblotting

Granulosa cells were scraped into cold PBS, centrifuged at 14,000 × *g* for 10 min, and resuspended in TE buffer [50 mM Tris-HCl (pH 7.4) and 1.0 mM EDTA] supplemented with 20 μg/ml phenylmethylsulfonylfluoride and 0.5 μg/ml leupeptin, 0.2 mM sodium vanadate, and 100 nM microcystin (10). Whole cell lysates were separated on 12% SDS-discontinuous polyacrylamide gels, and the resolved proteins were electrophoretically transferred to nitrocellulose membranes. Anti-PKB immunoblotting was performed using a polyclonal Anti-PKB (no. 9272, Cell Signaling, Beverly, MA) at a final concentration of 1 μg/ml. Chemiluminescent detection was accomplished using the BM Chemiluminescence Western Blotting Kit (Roche, Indianapolis, IN) with the appropriate horseradish peroxidase-conjugated secondary antibody diluted to 1:2000 according to the manufacturer's directions. Membranes were stripped and probed with an anti-CREB antibody (SC-186, Santa Cruz Biotechnology, Santa Cruz, CA) at a concentration of 1 μg/ml to verify equivalent sample loading and analyzed as described above.

Statistics

Where indicated, results were assessed for statistical significance by ANOVA, followed by comparison of group means with Fisher's least

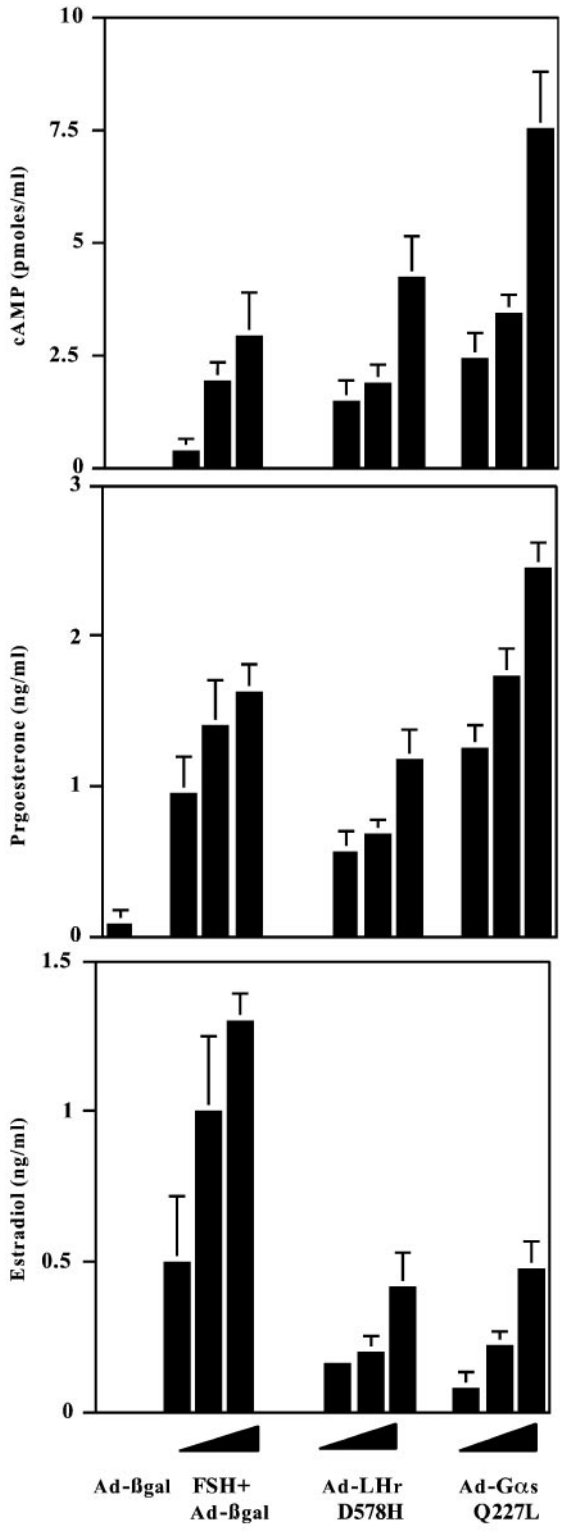

FIG. 1. The production of cAMP, progesterone, and estrogen by undifferentiated rat granulosa cells in response to FSH stimulation, the expression of constitutively active LH receptors or constitutively active $G_s\alpha$. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to Ad-βgal at a concentration of 5×10^{10} particles/ml; Ad-LHrD578H at concentrations of 1.33, 2.5, and 5.0×10^{10} particles/ml; or Ad-GαSQ227L at concentrations of 1.25, 1.33, and 2.5×10^{10} particles/ml for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without human FSH at concentrations of 10, 50, and 100 ng/ml. Forty-eight hours later medium was collected and analyzed for estradiol and progesterone content by RIA. Results show the mean ± 1 SEM of four separate groups of granulosa cells.

significant difference analysis (StatView version 4.5, Abacus Concepts, Berkeley, CA).

Results

Comparison of the effects of FSH, Ad-RSV-LHrD578H, and Ad-CMV-GαSQ227L on estrogen, progesterone, and cAMP production by undifferentiated granulosa cells

Primary cultures of rat granulosa cells collected from immature female rats were stimulated by FSH or infected with adenovirus vectors that express the constitutively activated human LH receptor D578H (Ad-LHrD578H) or the constitutively activated $G_s\alpha$Q227L (Ad-GαSQ227L). Samples of culture medium were collected 48 h after FSH stimulation and 72 h after virus infection and were analyzed for estrogen, progesterone, and cAMP content by RIA. Figure 1 illustrates that each of the stimuli resulted in dose-dependent increases in cAMP, progesterone, and estrogen production. The absolute amounts of progesterone produced were comparable among the cells stimulated by FSH, Ad-LHrD578H, and Ad-GαSQ227L, whereas the production of estrogen, an index of aromatase activity, was greater in cells stimulated by FSH than in cells stimulated by Ad-LHrD578H and Ad-GαSQ227L ($P < 0.01$). It can be seen in Fig. 1 that cAMP production rates were similar in response to 100 ng/ml FSH, Ad-LHrD578H at 5×10^{10} particles/ml, and Ad-GαSQ227L at 1.3×10^{10} particles/ml. These concentrations of FSH and adenoviral vectors were used in subsequent studies to achieve comparable levels of cAMP with the different treatments to diminish the likelihood that any differences in granulosa cell responses would be due to differences in cAMP production.

Figure 2 illustrates the time course of progesterone production and aromatase activity as assessed by the conversion of [^{3}H]androstenedione to ^3H_2O in response to FSH, Ad-LHrD578H, and Ad-GαSQ227L. At selected time intervals, progesterone production in response to each stimulus was comparable. However, similar to the estrogen production rates shown in Fig. 1, aromatase activity was significantly elevated by FSH at 48 and 72 h compared with activity in response to both Ad-LHrD578H and $G_s\alpha$Q227L ($P < 0.01$).

Figure 3 illustrates the expression of selected mRNAs by granulosa cells stimulated by FSH, Ad-LHrD578H, and Ad-GαSQ227L. As reported previously (11), activation of the FSH receptor and the LH receptor produced similar increases in mRNAs for 3β-HSD and α-inhibin, whereas activation of the FSH receptor was more effective in inducing mRNA for the LH receptor and aromatase. In the current study the effects of Ad-GαSQ227L were similar to those of Ad-LHrD5788H, in that both stimulated 3β-HSD and α-inhibin, but were not as effective as FSH in stimulating mRNAs for aromatase and the LH receptor. Col-

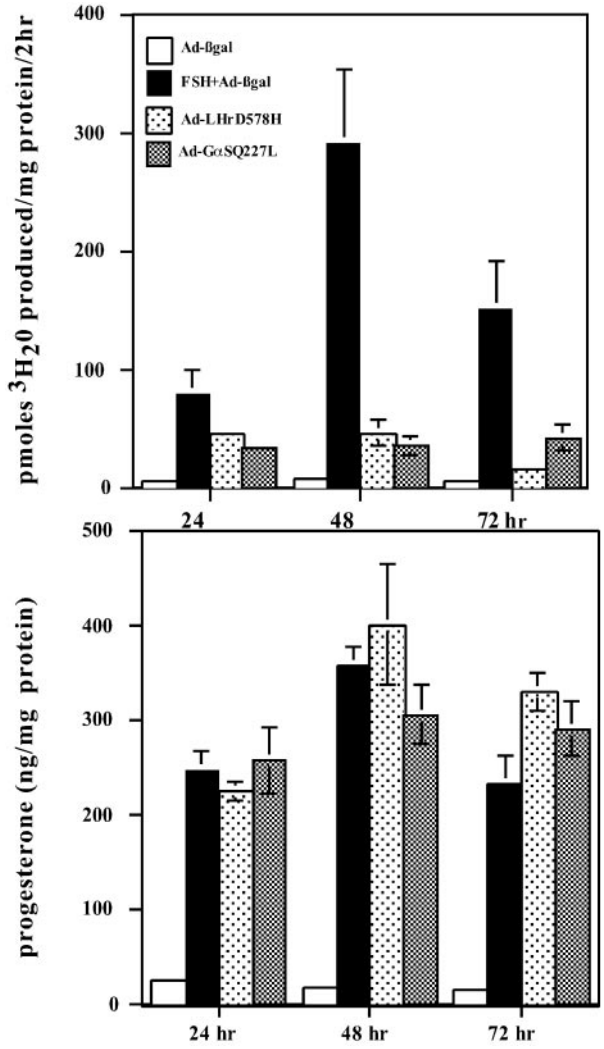

FIG. 2. Time course of progesterone production and aromatase activity by undifferentiated rat granulosa cells in response to FSH stimulation, expression of constitutively active LH receptors, or constitutively active $G_s\alpha$. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to Ad-βgal at a concentration of 5×10^{10} particles/ml, Ad-LHrD578H at a concentration of 5.0×10^{10} particles/ml or Ad-GαSQ227L at a concentration of 1.33×10^{10} particles/ml for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199 containing 30 ng/ml testosterone with or without FSH at a concentration of 100 ng/ml. After 24, 48, and 72 h, medium was collected, and aromatase activity was measured as described in *Materials and Methods*. Results show the mean ± 1 SEM of six separate groups of granulosa cells.

lectively, the results presented in Figs. 1–3 confirm our earlier results that activation of the FSH receptor is more effective than activation of the LH receptor with respect to the induction of aromatase and the induction of mRNA for the LH receptor. In addition, the current results show that Ad-GαSQ227L exerted effects similar to those of Ad-LHrD578H on aromatase and LH receptor mRNA, suggesting that the optimal induction of aromatase and the LH receptor by FSH relies on other intracellular signaling pathways in addition to $G_s\alpha$.

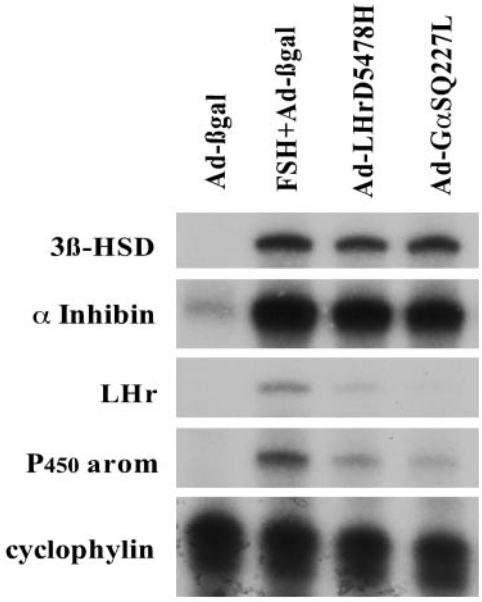

FIG. 3. Ribonuclease protection analysis of mRNA levels for α-inhibin, LH receptor, P450aromatase, and 3β-hydroxysteroid dehydrogenase in the undifferentiated rat granulosa cell and granulosa cells infected with either the wild-type LH receptor or the constitutively activated D578H LH receptor. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% fetal bovine serum. The next morning media and unattached cells were removed, and monolayers were exposed to Ad-βgal at a concentration of 5×10^{10} particles/ml, Ad-LHrD578H at concentrations of 5.0×10^{10} particles/ml, or Ad-GαSQ227L at concentrations of 1.33 particles/ml for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without human FSH at a concentration of 100 ng/ml. Forty-eight hours later total RNA was prepared from the monolayers and analyzed for mRNAs by ribonuclease protection assay. Results shown are representative of four separate groups of granulosa cells.

PKB is an obligatory component of FSH-mediated granulosa cell differentiation and may participate with the $G_s\alpha$ signaling pathway in the induction of aromatase and the LH receptor

To explore the contributions of other signaling pathways, we employed replication-defective adenovirus vectors that direct the expression of constitutively activated PKB (Ad-myrPKB) and MEK (Ad-caMEK), as it has previously been shown by others that FSH stimulates both PKB and MAPK (4, 5). Figure 4 presents data from a pilot study to explore this possibility. As expected, FSH plus testosterone stimulated the production of estrogen and progesterone. Although neither Ad-myrPKB nor Ad-caMEK in the presence of testosterone stimulated estrogen or progesterone production, Ad-myrPKB amplified the stimulatory effects of FSH plus testosterone on both estrogen and progesterone production. Ad-caMEK appeared to suppress FSH-stimulated estrogen and progesterone production; however, this observation was not pursued further in the current study. Figure 5 illustrates an immunoblot analysis to document that infection of granulosa cells with Ad-myrPKB leads to enhanced expression of PKB.

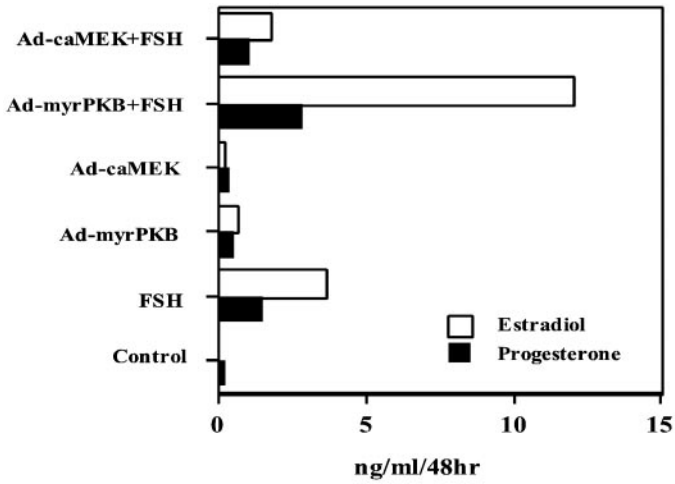

FIG. 4. Effect of expression of a constitutively active MEK or a constitutively active PKB on basal and FSH-stimulated estrogen and progesterone production. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to an adenoviral vector that directs the expression of a constitutively active PKB (Ad-myrPKB) or an adenoviral vector that directs the expression of a constitutively active MEK (Ad-caMEK) at concentrations of 5×10^{10} particles/ml for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without human FSH at a concentration of 100 ng/ml. Forty-eight hours later medium was collected and analyzed for estradiol and progesterone content by RIA. Results show the means of two groups of granulosa cells.

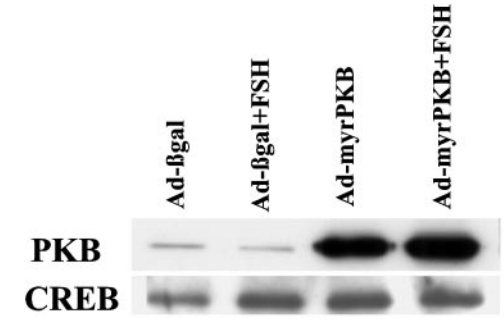

FIG. 5. Immunoblot analysis of granulosa cells infected with Ad-βgal or Ad-myrPKB. Granulosa cells were infected with 5×10^{10} particle/ml of either Ad-βgal or Ad-myrPKB for 2 h after which the virus-containing media was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without human FSH (100 ng/ml). Forty-eight hours later cells were collected and processed for Western immunoblotting with anti-PKB, followed by anti-CREB to verify equivalent loading of samples. Results are representative of three separate groups of cells.

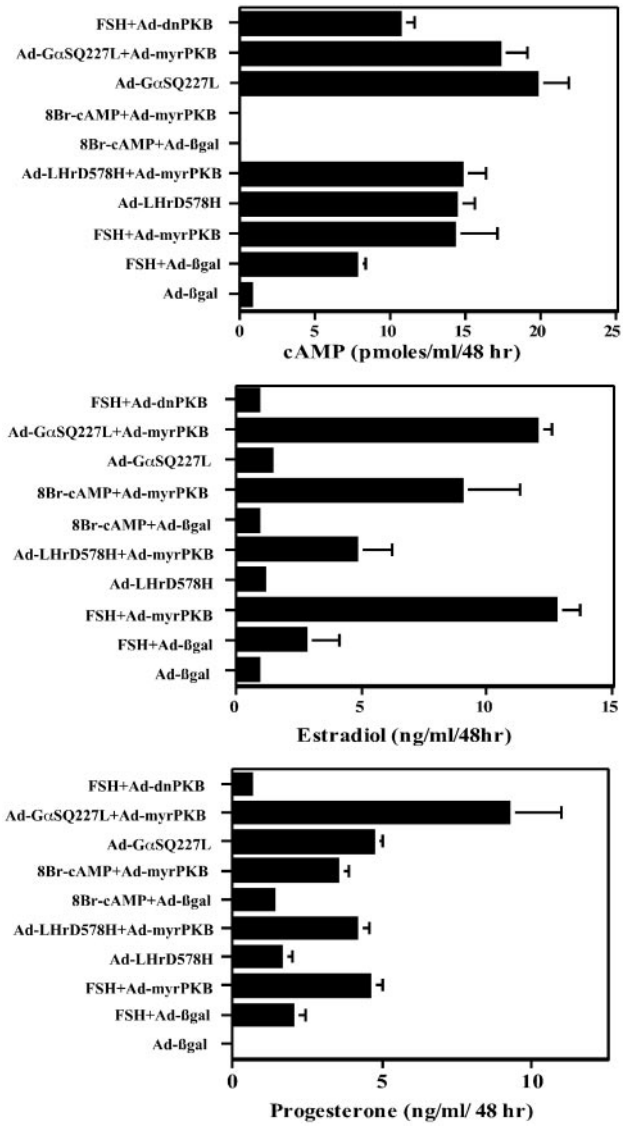

FIG. 6. Effect of expression of a constitutively active and a dominant-negative PKB on estrogen and progesterone production by undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to Ad-βgal at a concentration of 5×10^{10} particles/ml or adenoviral vectors as indicated on the y-axes: Ad-LHrD578H, 5.0×10^{10} particles/ml; Ad-GαSQ227L, 1.33 particles/ml; Ad-myrPKB, 5×10^{10} particles/ml; Ad-dnPKB, 5×10^{10} particles/ml for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without human FSH (100 ng/ml) or 8Br-cAMP (0.5 mM). Forty-eight hours later medium was collected and analyzed for estradiol, progesterone, and cAMP content by RIA. Results show the mean ± 1 SEM of three groups of granulosa cells.

To further explore the contribution of PKB in granulosa cell differentiation, we assessed cAMP, estradiol, and progesterone production by undifferentiated granulosa cells stimulated by FSH, Ad-LHrD578H, Ad-GαSQ227L, and 8Br-cAMP in the presence and absence of the constitutively activated PKB (Ad-myrPKB). As shown in Fig. 6, Ad-myrPKB amplified the stimulatory effects of FSH on both estrogen and progesterone production. In addition, neither Ad-LHrD578H, Ad-GαSQ227L, nor 8Br-cAMP alone stimulated estrogen production to the extent seen with FSH, although each, in combination with Ad-myrPKB, stimulated estrogen production comparable to that seen in cells stimulated with FSH. An obligatory role for PKB in FSH-stimulated granulosa cell differentiation was revealed by cells stimulated with FSH after being infected with an adenovirus vector that expresses a dominant-negative mutant of PKB (Ad-dnPKB).

In these cells, FSH-stimulated production of estrogen and progesterone was inhibited without any reduction in FSH-stimulated cAMP production. The lack of effect of Ad-dnPKB on FSH-stimulated cAMP production indicates that its inhibition of steroidogenesis was probably not due to a toxic effect of viral infection. Assessment of aromatase activity using the tritiated water production assay yielded results identical to those obtained by measurement of estradiol by RIA (data not shown). Figure 6 also illustrates that Ad-myrPKB enhanced FSH-stimulated cAMP production, but did not appear to elevate cAMP production in response to Ad-LHrD578H or Ad-GαSQ227L. Table 1 shows that Ad-myrPKB also slightly elevated basal cAMP production by undifferentiated granulosa cells.

PKB amplifies markers of FSH-stimulated differentiation, but not proliferation

FSH stimulation of granulosa cells is associated with both differentiation and proliferation. We demonstrated previously that activin synergizes with FSH to increase mRNA levels for two proliferation-associated proteins, cyclin D2 and PCNA (19). We performed ribonuclease protection assays to determine whether PKB participates in FSH stimulation of both differentiation- and proliferation-associated mRNAs. Figure 7 shows that the expression of constitutively activated PKB amplified the expression of the differentiation-associated mRNAs for 3β-HSD, α-inhibin, P450 aromatase, and the LH receptor in response to FSH, Ad-LHrD578H, 8Br-cAMP, and Ad-GαSQ227L. However, neither mRNA for cyclin D2 nor PCNA was amplified by the expression of the constitutively activated PKB. Furthermore, whereas the dominant-negative mutant of PKB (Ad-dnPKB) abrogated the FSH stimulation of differentiation-associated mRNAs, it did not reduce the basal expression of mRNAs for cyclin D2 or PCNA.

Constitutively activated PKB overcomes wortmannin inhibition of FSH-induced differentiation

Figure 8 demonstrates that the phosphoinositol 3-kinase (PI-3 kinase) inhibitor wortmannin (100 nM) blocked FSH-stimulated increases in estrogen and progesterone production by granulosa cells. Infection with Ad-myrPKB overcame the wortmannin-induced inhibition, indicating that the enhancement of FSH-stimulated granulosa cell differentiation by PKB is probably mediated through PI-3 kinase.

TABLE 1. cAMP production by undifferentiated granulosa cells stimulated with FSH and Ad-myrPKB

Treatment	cAMP (pmol/48 h)[a]
Ad-βgal (5×10^{10} particles/ml)	<0.06
Ad-myrPKB (5×10^{10} particles/ml)	0.35 ± 0.04
FSH (100 ng/ml) + Ad-βgal (5×10^{10} particles/ml)	0.99 ± 0.13
FSH (100 ng/ml) + Ad-myrPKB (5×10^{10} particles/ml)	2.45 ± 0.40

[a] Mean ± 1 SEM of three groups of granulosa cells.

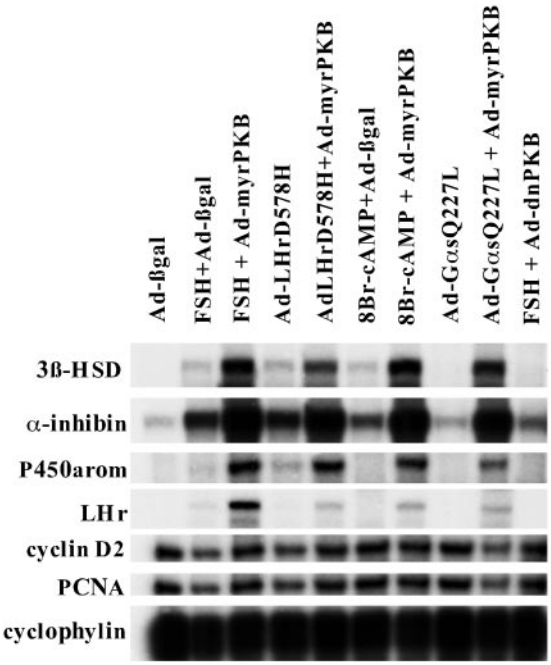

FIG. 7. Effect of expression of a constitutively active and a dominant negative PKB on mRNA levels associated with differentiation and proliferation in undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to Ad-CMV-β-gal at a concentration of 5×10^{10} particles/ml, Ad-LHrD578H at 5.0×10^{10} particles/ml, Ad-$G_s\alpha$Q227L at 1.33 particles/ml, Ad-myrPKB at 5.0×10^{10} particles/ml, or adeno-dnAKT at 5.0×10^{10} particles/ml for 2 h as indicated in the figure, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without human FSH (100 ng/ml) or 8Br-cAMP (0.5 mM). Forty-eight hours later total RNA was prepared from the monolayers and analyzed for mRNAs by ribonuclease protection assay. Results shown are representative of three separate groups of granulosa cells.

The guanine exchange factor directly activated by cAMP (Epac)-selective cAMP analog 8CTP-2Me-cAMP fails to substitute for PKB in augmenting granulosa cell steroidogenesis

Like granulosa cells, thyroid cells respond to their trophic hormone (TSH) with a cAMP-dependent stimulation of proliferation and differentiation-associated gene expression (23, 24). The response to TSH appears to be regulated in part by Rap1-regulated PKB activity through cAMP-dependent guanine nucleotide exchange factors (25). To determine whether the cAMP-dependent guanine nucleotide exchange factor Epac is involved in the selective induction of aromatase by FSH, we treated granulosa cells with the cAMP analog 8CPT-2Me-cAMP, which selectively activates Epac over PKA (26). Results shown in Fig. 9 indicate that this Epac-selective cAMP analog failed to increase estrogen production alone or in combination with either FSH or Ad-GαSQ227L.

Constitutively active PKB is unable to overcome H-89 inhibition of estrogen production

Figure 10 illustrates that inhibition of protein kinase A by 10 μM H-89 abolished FSH-stimulated induction of

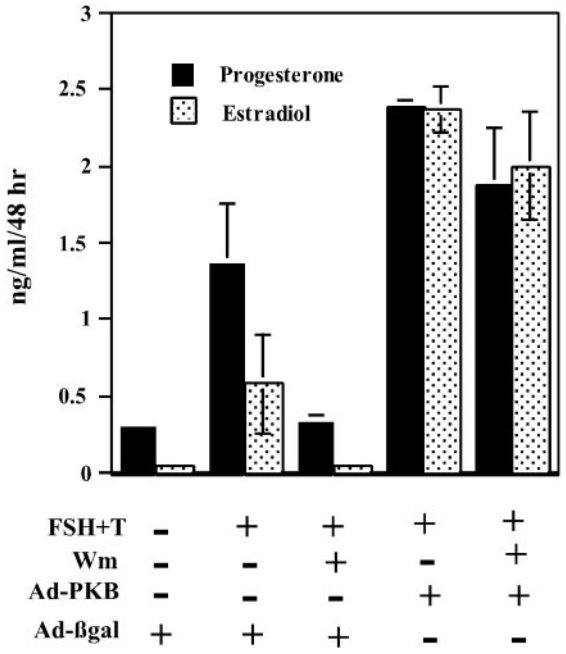

FIG. 8. Constitutively active PKB overcomes inhibition of granulosa cell steroidogenesis by wortmannin. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to medium alone or Ad-myrPKB (5.0×10^{10} particles/ml) for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh Medium 199 containing 30 ng/ml testosterone ± wortmannin (Wm; 100 nM). One hour later, human FSH (100 ng/ml) was added as indicated in the figure. Forty-eight hours later medium was collected and analyzed for estradiol and progesterone content by RIA. Results show the mean ± 1 SEM of three groups of granulosa cells.

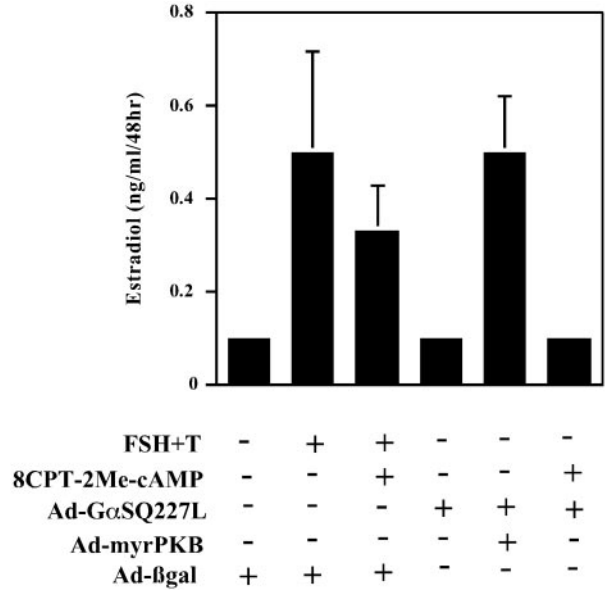

FIG. 9. The Epac-selective cAMP analog 8CTP-2Me-cAMP fails to substitute for PKB in modulating estrogen and progesterone production by undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to Ad-CMV-GαSQ227L (1.33×10^{10} particles/ml) for 2 h as indicated, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without FSH (100 ng/ml) and/or 8CTP-2Me-cAMP (0.1 mM) as indicated. Forty-eight hours later medium was collected and analyzed for estradiol and progesterone content by RIA. Results show the mean ± 1 SEM of three groups of granulosa cells.

aromatase in undifferentiated granulosa cells, as assessed by measurement of estrogen production. Although Ad-myrPKB augmented FSH-stimulated estrogen production, it did not overcome the inhibition imposed by H-89. These results indicate that PKB is not downstream of PKA and that optimal induction of aromatase requires both PKA and PKB.

Discussion

These results support our previous findings that although both activation of the FSH receptor and activation of the LH receptor stimulate cAMP production, there appears to be additional signaling pathways used by FSH in the induction of the LH receptor and aromatase during granulosa cell differentiation. In the current study we show that an adenoviral vector that directs the expression of constitutively active $G_s\alpha$Q227L can stimulate cAMP production to an extent equivalent to or greater than that of FSH. This vector was effective in stimulating progesterone production and increasing mRNA levels for α-inhibin and 3β-HSD comparable to that of FSH, but was less effective than FSH in stimulating aromatase activity and the expression of mRNA for the LH receptor. In this regard, the cellular responses to Ad-GαSQ227L were similar to those of Ad-LHrD578H and those resulting from the direct stimulation of granulosa cells by 8Br-cAMP. These observations therefore indicate that activation of the $G_s\alpha$ signaling pathway and its resultant stimulation of cAMP production alone are not sufficient for the optimal induction of aromatase and the LH receptor by FSH. Interestingly, Laven *et al.* (27) recently investigated ovarian function in a women with McCune-Albright syndrome, a sporadic disease caused by an activating mutation of the GNAS1 gene, which in this patient encodes for a constitutively active $G_s\alpha$R201H. As a result of this mutation, numerous follicular cysts were present in the affected ovary, and analysis of follicular fluid estrogen and progesterone concentrations in smaller follicles of the affected ovary revealed a disproportionate increase in the progesterone to estrogen ratio, similar to the steroidogenic profile seen in our current study of granulosa cells infected with the adenoviral vector which directs the expression of the constitutively active $G_s\alpha$Q227L.

To identify other interacting pathways, we infected undifferentiated granulosa cells with adenoviral vectors that direct the expression of constitutively active mutants of PKB and MEK. Our findings indicated that neither PKB nor MEK alone was sufficient to stimulate estrogen or progesterone production. However, when constitutively activated PKB was expressed in combination with FSH

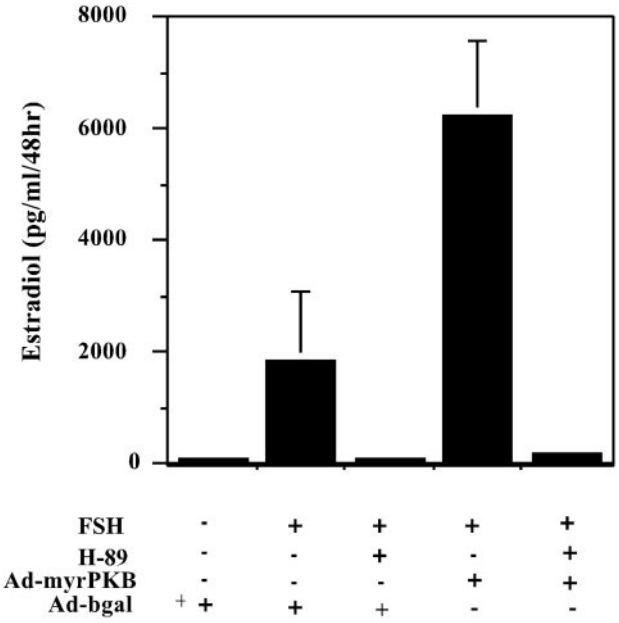

FIG. 10. Constitutively active PKB fails to overcome the inhibition of estrogen production by H-89. Undifferentiated rat granulosa cells were plated overnight in medium 199 containing 10% FBS. The next morning media and unattached cells were removed, and monolayers were exposed to medium alone, Ad-βgal, or Ad-myrPKB (5.0×10^{10} particles/ml) for 2 h, after which the virus-containing medium was removed and replaced with fresh medium 199. After 24 h, medium was replaced with fresh medium 199 containing 30 ng/ml testosterone with or without H-89 (10 μM). One hour later, human FSH (100 ng/ml) was added as indicated in the figure. Forty-eight hours later medium was collected and analyzed for estradiol and progesterone content by RIA. Results show the mean $\pm$ 1 SEM of three groups of granulosa cells.

treatment, we observed a dramatic amplification of estrogen production and, to a lesser extent, progesterone production compared with cells that were stimulated by FSH alone. Moreover, the expression of a dominant-negative mutant of PKB blocked FSH-stimulated estrogen production and aromatase activity without interfering with FSH-stimulated cAMP production. By contrast, the constitutively activated MEK did not synergize with FSH to stimulate estrogen or progesterone production. In fact, this preliminary study suggests that MEK may be antagonistic to granulosa cell steroidogenesis, as was recently reported by others (28).

The constitutively activated PKB also amplified progesterone production by granulosa cells either stimulated by FSH or 8Br-cAMP or infected with an adenoviral vectors that directs the expression of constitutively activated LH receptors and $G_s\alpha$. More importantly, however, although neither the constitutively active LH receptor, the constitutively active $G_s\alpha$, nor 8Br-cAMP was effective in stimulating estrogen production, aromatase activity, or mRNAs for aromatase and the LH receptor, each in combination with activated PKB, became effective in stimulating these hallmarks of FSH-stimulated granulosa cell differentiation. The finding that neither 8Br-cAMP nor $G_s\alpha$Q227L optimally stimulated the expression of aromatase is somewhat surprising in view of fact that the aromatase promoter contains a cAMP response element (29). However, the results shown in Fig. 10 demonstrate that inhibition of PKA by H-89 completely blocks the stimulation of estrogen production by FSH, indicating that PKA (and presumably cAMP response element-binding protein activation) is also required for induction of aromatase. Moreover, the finding that Ad-myrPKB cannot overcome the inhibition of aromatase imposed by H-89 indicates that both the PKA and PKB signaling pathways are required. Elucidation of the targets downstream of PKB and PKB that underlie this synergism is probably the key in understanding granulosa cell differentiation.

Surprisingly, infection of granulosa cells with Ad-myrPKB amplified FSH-stimulated cAMP production, but did not appear to augment cAMP production in response to Ad-LHrD578H or Ad-GαSQ227L. At the present time, the mechanism by which this occurred is not known. Whether PKB regulates the activity of G proteins and/or phosphodiesterases in granulosa cells remains a question for future investigation. It is also possible that there is cellular compartmentalization of the FSH signaling system (3), and that local changes in phosphodiesterase activity might selectively affect FSH-stimulated cAMP production, but not that stimulated by Ad-LHrD578H or Ad-GaSQ227L.

The synergism between the PKB and the $G_s\alpha$ signaling pathways in regulating granulosa cell differentiation is not confined to aromatase and the LH receptor, as mRNAs for α-inhibin and 3β-HSD were also amplified in the presence of PKB. Likewise, FSH-stimulated progesterone production was amplified by PKB. This raises the question of whether PKB exerts a global effect on granulosa cell function such as modifications of cellular metabolism that could indirectly amplify gene expression. This does not appear to be the case because, as shown in Fig. 7, PKB did not amplify the expression of the proliferation-associated mRNAs PCNA and cyclin D2, and the dominant-negative PKB did not reduce the levels of expression of these mRNAs. In this regard, we demonstrated previously that activin synergizes with both FSH and forskolin to regulate the expression of PCNA and cyclin D2 in granulosa cells (19). It would therefore appear that a number of individual signaling factors, including PKB, Smad, and androgens (30), may converge upon the $G_s\alpha$ pathway to regulate granulosa cell differentiation and proliferation.

The activation of PKB is complex and can occur in a PI-3 kinase-dependent and independent manner (31). Although we have not examined directly the activation of PKB in the current studies, the finding that the PI-3 kinase inhibitor wortmannin blocked FSH-stimulated estrogen and progesterone production and that this inhibition was completely reversed by the constitutively active PKB (Fig. 8) provides indirect evidence for a link between PI-3 kinase and PKB in FSH-stimulated granulosa cell differentiation. In support of this, Gonzalez-Robayna *et al.* (5) demonstrated that FSH stimulates the phosphorylation and activation of PKB which is blocked by wortmannin but not the PKA inhibitor H-89. These authors suggest that phosphorylation of PKB could be mediated by activation of PI-3 kinase through cAMP-dependent guanine nucleotide exchange factors (cAMP-GEF). However, our preliminary observation that 8CPT-2Me-cAMP, a cAMP ana-

log that selectively activates the cAMP-GEF Epac1 (26), does not stimulate estrogen and progesterone production (Fig. 9) alone or in combination with constitutively active $G_s\alpha$ indicates that the cAMP-Epac-Rap1/Rap2 pathway is not a principal participant in granulosa cell differentiation, at least as assessed by estrogen and progesterone production under the tissue culture conditions in which these studies were conducted. Alternate mechanisms for PKB activation could also include either stimulation of PI-3 kinase by IGF-I (32), which, in turn, could augment the responsiveness of granulosa cells to FSH (33) or direct stimulation of PI-3 kinase by a G protein $\beta\gamma$ heterodimer (34). However, a caveat to this hypothesis is that Gonzalez-Robayna *et al.* (5) also demonstrated that 8Br-cAMP stimulates PKB phosphorylation in granulosa cells. This would indicate that both the cAMP and PKB pathways would be concurrently activated by 8Br-cAMP, yet in our studies 8Br-cAMP did not optimally induce aromatase or mRNA for the LH receptor (Figs. 5–7). At the present time, we have no explanation for this other than the possibility that the extent to which PKB is activated by FSH and 8Br-cAMP could differ either as a function of time or as absolute activity.

In summary, our current results demonstrate that PKB is obligatory to FSH-stimulated granulosa cell differentiation, as the effect of FSH on differentiation-associated mRNAs is completely abrogated by the overexpression of a dominant-negative PKB. Moreover, because the expression of PKB with constitutively active $G_s\alpha$ mimics the effect of FSH on aromatase and LH receptor mRNA, it appears that both the PKB and $G_s\alpha$ pathways are required for the expression of these hallmarks of granulosa cell differentiation. However, because the constitutively active PKB did not amplify mRNA levels for PCNA or cyclin D2, and the dominant-negative PKB did not reduce the expression of these mRNAs, it appears that PKB may not be required for the proliferative effects of FSH.

Acknowledgments

We acknowledge Drs. Deborah Segaloff, Ian Mason, and Stephen G. Hillier for providing cDNAs for this study. We also acknowledge Dr. Talal El-Hefnawy for subcloning the $G_s\alpha$ cDNA into the adenovirus shuttle vector; Drs. J. Alcorn, Kenneth Walsh, and N. Mitsuda for providing us with adenovirus vectors; and Dr. Carole Mendelson for providing us with a protocol for the aromatase assay.

Received March 5, 2003. Accepted May 29, 2003.

Address all correspondence and requests for reprints to: Anthony J. Zeleznik, Ph.D., Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, 830 Scaife Hall, 3500 Terrace Street, Pittsburgh, Pennsylvania 15261. E-mail: zeleznik@pitt.edu.

This work was supported by NIH Grant HD-16842.

References

1. **Hunzicker-Dunn M, Birnbaumer L** 1976 Adenylyl cyclase activities in ovarian tissues. II. Regulation of responsiveness to LH, FSH, and PGE1 in the rabbit. Endocrinology 99:185–197
2. **Zeleznik AJ, Keyes PL, Menon KM, Midgley Jr AR, Reichert Jr LE** 1977 Development-dependent responses of ovarian follicles to FSH and hCG. Am J Physiol 233:E229–E234
3. **Conti M** 2002 Specificity of the cyclic adenosine 3′,5′-monophosphate signal in granulosa cell function. Biol Reprod 67:1653–1661
4. **Das S, Maizels ET, DeManno D, St Clair E, Adam SA, Hunzicker-Dunn M** 1996 A stimulatory role of cyclic adenosine 3′,5′-monophosphate in follicle-stimulating hormone-activated mitogen-activated protein kinase signaling pathway in rat ovarian granulosa cells. Endocrinology 137:967–974
5. **Gonzalez-Robayna IJ, Falender AE, Ochsner S, Firestone GL, Richards JS** 2000 Follicle-stimulating hormone (FSH) stimulates phosphorylation and activation of protein kinase B (PKB/Akt) and serum and glucocorticoid-induced kinase (Sgk): evidence for A kinase-independent signaling by FSH in granulosa cells. Mol Endocrinol 14:1283–1300
6. **Flores JA, Leong DA, Veldhuis JD** 1992 Is the calcium signal induced by follicle-stimulating hormone in swine granulosa cells mediated by adenosine cyclic 3′,5′-monophosphate-dependent protein kinase? Endocrinology 130: 1862–1866
7. **Adashi EY, Hsueh AJ** 1981 Stimulation of β_2-adrenergic responsiveness by follicle-stimulating hormone in rat granulosa cells *in vitro* and *in vivo*. Endocrinology 108:2170–2178
8. **Davoren JB, Hsueh AJ** 1985 Vasoactive intestinal peptide: a novel stimulator of steroidogenesis by cultured rat granulosa cells. Biol Reprod 33:37–52
9. **Davies SP, Reddy H, Caivano M, Cohen P** 2000 Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105
10. **Somers JP, DeLoia JA, Zeleznik AJ** 1999 Adenovirus-directed expression of a nonphosphorylatable mutant of CREB (cAMP response element-binding protein) adversely affects the survival, but not the differentiation, of rat granulosa cells. Mol Endocrinol 13:1364–1372
11. **Bebia Z, Somers JP, Liu G, Ihrig L, Shenker A, Zeleznik AJ** 2001 Adenovirus-directed expression of functional luteinizing hormone (LH) receptors in undifferentiated rat granulosa cells: evidence for differential signaling through follicle-stimulating hormone and LH receptors. Endocrinology 142:2252–2259
12. **Fujio Y, Walsh K** 1999 Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. J Biol Chem 274:16349–16354
13. **Mitsuda N, Ohkubo N, Tamatani M, Lee YD, Taniguchi M, Namikawa K, Kiyama H, Yamaguchi A, Sato N, Sakata K, Ogihara T, Vitek MP, Tohyama M** 2001 Activated cAMP-response element-binding protein regulates neuronal expression of presenilin. J Biol Chem 276:9688–9698
14. **Mittereder N, March L, Trapnell BC** 1996 Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. J Virol 70:7498–7509
15. **Hickey GJ, Krasnow JS, Beattie WG, Richards JS** 1990 Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3′,5′-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5′ genomic DNA. Mol Endocrinol 4:3–12
16. **McFarland KC, Sprengel R, Phillips HS, Kohler M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH** 1989 Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science 245:494–499
17. **Esch FS, Shimasaki S, Cooksey K, Mercado M, Mason AJ, Ying SY, Ueno N, Ling N** 1997 Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Mol Endocrinol 1: 388–396
18. **Lorence MC, Naville D, Graham-Lorence SE, Mack SO, Murry BA, Trant JM, Mason JI** 1991 3β-Hydroxysteroid dehydrogenase/delta 5–4-isomerase expression in rat and characterization of the testis isoform. Mol Cell Endocrinol 80:21–31
19. **El-Hefnawy T, Zeleznik AJ** 2001 Synergism between FSH and activin in the regulation of proliferating cell nuclear antigen (PCNA) and cyclin D2 expression in rat granulosa cells. Endocrinology 142:4357–4362
20. **Danielson PE, Forss-Petter S, Brow MA, Calavetta L, Douglass J, Milner RJ, Sutcliffe JG** 1998 p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 7:261–267
21. **Zeleznik AJ, Resko JA** 1980 Progesterone does not inhibit gonadotropin-induced follicular maturation in the female rhesus monkey. Endocrinology 106:1820–1826
22. **Steiner AL, Parker CW, Kipnis DM** 1972 Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides. J Biol Chem 247:1106–1113
23. **Cass LA, Summers SA, Prendergast GV, Backer JM, Birnbaum MJ, Meinkoth JL** 1999 Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol 19:5882–5891
24. **Ribeiro-Neto F, Urbani J, Lemee N, Lou L, Altschuler DL** 2002 On the mitogenic properties of Rap1b: cAMP-induced G_1/S entry requires activated and phosphorylated Rap1b. Proc Natl Acad Sci USA 99:5418–5423
25. **Lou L, Urbani J, Ribeiro-Neto F, Altschuler DL** 2002 cAMP inhibition of Akt is mediated by activated and phosphorylated Rap1b. J Biol Chem 277:32799–32806
26. **Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG, Doskeland SO, Blank JL, Bos JL** 2002 A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol 4:901–906
27. **Laven JS, Lumbroso S, Sultan C, Fauser BC** 2001 Dynamics of ovarian function in an adult woman with McCune-Albright syndrome. J Clin Endocrinol Metab 86:2625–2630

28. **Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss 3rd JF, Amsterdam A** 2001 The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. J Biol Chem 276:13957–13964
29. **Young M, McPhaul MJ** 1998 A steroidogenic factor-1-binding site and cyclic adenosine 3′,5′-monophosphate response element-like elements are required for the activity of the rat promoter in rat Leydig tumor cell lines. Endocrinology 139:5082–5093
30. **Zeleznik AJ, Hillier SG, Ross GT** 1979 Follicle stimulating hormone-induced follicular development: an examination of the role of androgens. Biol Reprod 21:673–681
31. **Chan TO, Rittenhouse SE, Tsichlis PN** 1999 AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu Rev Biochem 68:965–1014
32. **Chakravorty A, Joslyn MI, Davis JS** 1993 Characterization of insulin and insulin-like growth factor-I actions in the bovine luteal cell: regulation of receptor tyrosine kinase activity, phosphatidylinositol-3-kinase, and deoxyribonucleic acid synthesis. Endocrinology 133:1331–1340
33. **Eimerl S, Orly J** 2002 Regulation of steroidogenic genes by insulin-like growth factor-I and follicle-stimulating hormone: differential responses of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, and 3β-hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. Biol Reprod 67:900–910
34. **Brock C, Schaefer M, Reusch HP, Czupalla C, Michalke M, Spicher K, Schultz G, Nurnberg B** 2003 Roles of G $\beta\gamma$ in membrane recruitment and activation of p110γ/p101 phosphoinositide 3-kinase γ. J Cell Biol 160:89–99