0888-8809/07/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 21(8):1940–1957

doi: 10.1210/me.2007-0020

Follicle-Stimulating Hormone Induces Multiple Signaling Cascades: Evidence that Activation of Rous Sarcoma Oncogene, RAS, and the Epidermal Growth Factor Receptor Are Critical for Granulosa Cell Differentiation

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FSH regulates ovarian granulosa cell differentiation not only by activating adenylyl cyclase and protein kinase A (PKA) but also by other complex mechanisms. Using primary rat granulosa cell cultures, we provide novel evidence that FSH rapidly activates two small GTP-binding proteins RAP1 and RAS. FSH activation of RAP1 requires cAMP-mediated activation of exchange factor activated by cAMP/RAPGEF3 whereas FSH activation of RAS and downstream signaling cascades involves multiple factors. Specifically, FSH activation of RAS required Rous sarcoma oncogene (SRC) family tyrosine kinase (SFK) and epidermal growth factor receptor (EGFR) tyrosine kinase activities but not PKA. FSH-induced phosphorylation of ERK1/2 was blocked by dominant-negative RAS as well as by inhibitors of EGFR tyrosine kinase, metalloproteinases involved in growth factor shedding, and SFKs. In contrast, FSH-induced phosphorylation of protein kinase B (PKB/AKT) and the Forkhead transcription factor, FOXO1a occurred by SFK-dependent but RAS-independent mechanisms. The SFKs, c-SRC and FYN, and the SRC-related tyrosine kinase ABL were present and phosphorylated rapidly in response to FSH. Lastly, the EGF-like factor amphiregulin (AREG) activated RAS and ERK1/2 phosphorylation in granulosa cells by mechanisms that were selectively blocked by an EGFR antagonist but not by an SFK antagonist. However, AREG-mediated phosphorylation of PKB and FOXO1a required both EGFR and SFK activation. Moreover, we show that FSH induces AREG and that activation of the EGFR impacts granulosa cell differentiation and the expression of genes characteristic of the luteal cell phenotype. Thus, FSH orchestrates the coordinate activation of three diverse membrane-associated signaling cascades (adenylyl cyclase, RAS, and SFKs) that converge downstream to activate specific kinases (PKA, ERK1/2, and PKB/FOXO1a) that control granulosa cell function and differentiation. (*Molecular Endocrinology* 21: 1940–1957, 2007)

FSH IS A POTENT and essential regulator of granulosa cell proliferation and differentiation (1, 2). This pituitary gonadotropin acts exclusively by binding to the FSH receptor, a class A G protein-coupled receptor (GPCR) that is essential for later stages of follicular development (3–5). FSH binding to its cognate receptor (FSH receptor) leads to the rapid activation of many signaling molecules that impact diverse effects in granulosa cells (2, 3). One primary and well-characterized signaling cascade regulated by the liganded FSH receptor is the activation of adenylyl cyclase and the production of cAMP (6). cAMP, in turn, activates cAMP-dependent protein kinase [protein kinase A (PKA)], leading to the phosphorylation of key substrates one of which is CREB (cAMP-regulatory element binding protein) (7, 8). In granulosa cells this transcription factor binds cAMP regulatory elements present within the proximal promoter regions of specific genes such as aromatase (*Cyp19a*) (9, 10) and inhibin *a* (*Inhba*) (11, 12) to facilitate transcription.

More recent studies indicate that the signaling events controlled by FSH activation of its receptor in granulosa cells are more complex and that some molecular events,

First Published Online May 29, 2007

Abbreviations: AG1478, [4-(3chloroanilino)-6,7dimethoxy quinozoline; AREG, aregulin; 6-Bnz-cAMP, N^6-Benzoladenosine-3′,5′-cyclic monophosphate; CG, chorionic gonadotropin; CREB, cAMP regulatory element-binding protein; DN-RAS, dominant-negative form of RAS; E, estradiol; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; 8-MeOPT-cAMP, 8-(4-methoxyphenylthio)-2′-O-methyl-adenosine-3′,5′-cyclic monophosphate; MEK, MAPK kinase; NGS, normal goat serum; PI3K, phosphatidylinositol 3-kinase; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d)pyrimidine; PP3, 4-amino-7-phenylpyrazolo[3,4-d)pyrimidine; SDS, sodium dodecyl sulfate; SFK, SRC family tyrosine kinase; SRC, Rous sarcoma oncogene; TBS, Tris-buffered saline; TBS-T, TBS and 0.1% Tween 20.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

such as the phosphorylation of protein kinase B (PKB) and ERK1/2, can occur independently of PKA activation (2, 13, 14). These observations have prompted investigations to identify other potential signaling molecules that might mediate cAMP action. Of interest are the exchange proteins directly activated by cAMP (EPAC1/RAPGEF3 and EPAC2/RAPGEF4) that have been shown to bind cAMP and activate the small GTP-binding proteins RAP1/2 by exchanging bound GDP for GTP (15–18). Moreover, EPAC activates RAP1 independently of PKA (16, 17, 19) and EPAC-mediated RAP1 activation leads to PKB phosphorylation whereas activation of PKA suppresses PKB phosphorylation (19). EPAC1 localizes to specific intracellular domains in dividing and nondividing cells (17), binds scaffolding proteins and phosphodiesterase4D (20, 21), and possibly controls secretory events (22). Some of these effects relate to the cell-specific expression of EPAC. In rat neuroendoccrine AtT20 cells, EPAC1/2 was expressed and localized to the nuclear membrane whereas in rat phaeochromocytoma PC12 cells another exchange factor, C3G/RAPGEF1, was present and localized to the cytoplasm/plasma membrane (23), explaining the differential responses of these cells to cAMP. Because granulosa cells express mRNAs encoding *Rapgef3* and *Rapgef4*, we hypothesized that FSH via cAMP-dependent activation of EPAC might mediate PKA-independent phosphorylation of PKB and FOXO1A via the phosphatidylinositol 3-kinase (PI3K) cascade (14).

Other studies indicate that FSH potently activates both p38MAPK (MAPK14) and ERK1/2/ (MAPK3/1) pathways (13, 24–26). The mechanisms by which FSH mediates these effects have not been clearly defined. Because cAMP is a critical second messenger and because EPAC mRNA was expressed in granulosa cells, we reasoned that p38MAPK and ERK1/2 might also be direct or indirect downstream targets of EPAC and RAP1. Alternatively, the FSH receptor, like other GPCRs, might activate RAS and specific tyrosine kinase cascades, a pattern that has been established for GPCRs in other cells (27–34). That LH/human chorionic gonadotropin (CG) via its cognate receptor (LHCGR) can activate RAS in an MA10 mouse Leydig cell line and that activation of RAS induces phosphorylation of ERK1/2 via a receptor tyrosine kinase-dependent mechanism (35), provides support for the notion that gonadotropin receptors are also linked functionally to RAS and tyrosine kinase activities. Moreover, LH as well as FSH is a potent stimulator of p38MAPK and ERK1/2 phosphorylation in granulosa cells of preovulatory follicles (36), and FSH activates these kinases in cumulus cells and granulosa cells of ovulating follicles (24). Because these phosphorylation events in granulosa cells and cumulus cells of preovulatory follicles are sensitive to inhibition by the epidermal growth factor receptor (EGFR) tyroinse kinase blocker AG1478, they appear to be mediated, at least in part, by the rapid and marked activation and/or induction of the EGF-like factors amphiregulin (AREG), epiregulin, and betacellulin that have been shown to occur in these cells in response to gonadotropin (24, 37–40). In addition, a limited number of studies have shown that the effects of FSH and LH are sensitive to inhibitors of SRC family tyrosine kinases (SFKs) (26, 35), indicating that these tyrosine kinases may also be involved. Thus, we considered the possibilities that FSH might activate RAS and/or RAP1 and SFKs in immature granulosa cells isolated from small follicles of estradiol (E)-primed rats.

Based on these observations, we hypothesized that FSH impacts not just one but several signaling cascades to regulate the transcription of specific genes and the differentiation of granulosa cells. Therefore, we sought to determine the expression pattern of EPAC1 protein in the rodent ovary *in vivo* and the extent to which FSH and EPAC-specific agonists activate the small GTP-binding proteins RAP1 and/or RAS. Furthermore, we sought to determine which of these or other factors were critical for phosphorylation of the downstream kinases PKB/AKT, p38MAPK, and ERK1/2 as well as for the induction of specific genes. Our results show that FSH can rapidly activate the EPAC/RAP1 pathway as well as a SRC/RAS/EGFR/MAPK kinase (MEK) pathway. Whereas RAP1 is a relatively weak activator of p38MAPK, FSH-mediated activation of RAS by SFKs and EGFR tyrosine kinases is essential for phosphorylation of ERK1/2. RAS also impacts p38MAPK whereas SFKs appear to be more critical for FSH- and AREG-dependent PKB phosphorylation in these cells. In addition, our results show that FSH induces expression of *Areg* mRNA in these immature granulosa cells and that AREG activation of the EGFR accounts, in part, for FSH-mediated phosphorylation of ERK1/2, induction of specific genes, and granulosa cell differentiation.

RESULTS

EPAC/RAPGEF3 Is Expressed in and Mediates FSH Activation of RAP1 in Primary Rat Granulosa Cells

Because EPAC is specific target of cAMP, we hypothesized previously that this factor might mediate specific signaling cascades downstream of FSH in granulosa cells that were independent of PKA (14) (Fig. 1A). Therefore, we first determined whether EPAC protein was expressed in the rodent ovary. Western blot analysis of rat tissue protein extracts using an EPAC-specific antibody demonstrated that EPAC was expressed more abundantly in the immature rat ovary than in the pituitary, adrenal, or liver tissues (Fig. 1B). In the ovary, immunohistochemical analyses with the same EPAC-specific antibody used for Western analyses localized EPAC selectively to the granulosa cells and oocytes of the small preantral and antral follicles compared with theca/stroma cells (Fig. 1B). When granulosa cells from E-primed rats were cultured for

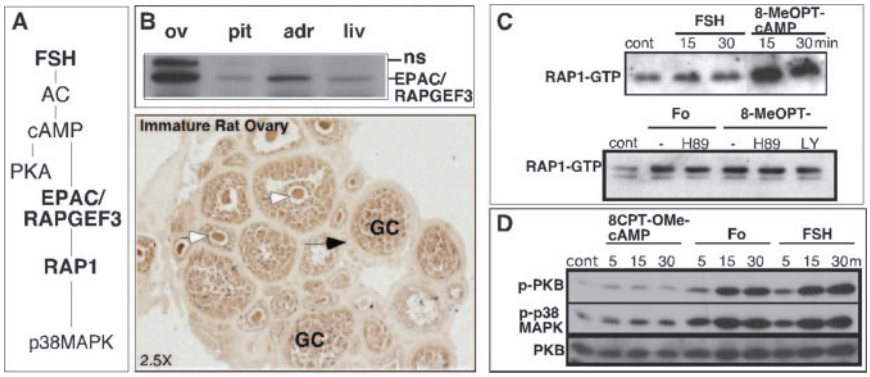


Fig. 1. FSH Activates EPAC/RAPGEF3 in Granulosa Cells

A, Schematic of FSH-mediated activation of PKA and EPAC/RAPGEF3 by cAMP- and EPAC-specific activation of RAP1. B, EPAC protein is expressed at high levels in the rat ovary. Western blot analysis: whole cell extracts (10 μg protein) were prepared from different rat tissues, resolved by SDS-PAGE, transferred to immobilon filters, and probed with an EPAC-specific antibody. EPAC protein is more abundantly expressed in the immature rat ovary (ov) than in the pituitary (pit), adrenal gland (adr), or liver (liv). Immunohistochemical analysis with the same EPAC-specific antibody demonstrates that EPAC is localized to granulosa cells (GC) and oocytes (*white arrows*) of the small preantral and antral follicles present in the immature rat ovary. EPAC does not appear to be present in theca cells (*dark arrow*). C, FSH, forskolin (Fo), and an EPAC-specific cAMP agonist activate RAP1 in rat primary granulosa cells. Western analysis of activated RAP1 purified by the pull-down method and detected with a RAP1-specific antibody. Rat primary granulosa cell cultures were grown in defined media overnight and then treated with FSH or 8-pMeOPT-2′-O-Me-cAMP for 15 or 30 min before lysis. Other granulosa cell cultures were pretreated with H89 or LY294002 (LY) for 1 h before stimulation with either forskolin (Fo) or 8-pMeOPT-2′-*O*-Me-cAMP for 15 min. D, Western blot analysis shows time-dependent increases in the phosphorylation of PKB and p38MAPK by FSH and Fo and increased levels of p-p38MAPK by the EPAC agonist. Granulosa cells were cultured overnight in defined medium and then stimulated with 8-pMeOPT-2′-*O*-Me-cAMP, Fo, or FSH for 5, 15, and 30 min. PKB was used as a loading control. AC, Adenylyl cyclase; cont, control; ns, not significant.

24–48 h with FSH, levels of EPAC protein were not affected by hormone treatment (data not shown).

Because EPAC1 is expressed abundantly in granulosa cells, we next sought to determine whether FSH via cAMP activated EPAC and if EPAC activated RAP1, a target of EPAC in other cells (Fig. 1C). To analyze this, granulosa cells were cultured overnight in serum-free medium and then exposed to FSH, forskolin, or 8-(-4-methoxyphenylthio)-2′-O-methyl-adenosine-3′,5′-cyclic monophosphate (8-MeOPT-2′-*O*-Me-cAMP), an EPAC-specific cAMP analog that has a 7.1-fold greater affinity for EPAC than does cAMP (41). Cell extracts were prepared and a RAL-GDS RAP1 binding domain pull-down assay was performed to isolate and measure GTP-bound RAP1 (16, 17). FSH activation of RAP1 [1.4 $\pm$ 0.2-fold ($P < 0.05$)] was significant relative to untreated controls (Fig. 2B). However, the response to 8-pMeOPT-2′-*O*-Me-cAMP was more pronounced [3.0 $\pm$ 0.9-fold ($P < 0.05$) relative to controls] (Fig. 2B). RAP1 activation occurred as early as 15 min after treatment with FSH or EPAC agonist exposure and remained elevated at 30 min. Forskolin also activated RAP1 2.4 $\pm$ 0.7-fold ($P < 0.05$) relative to controls. Neither this response nor RAP1 activation by 8-pMeOPT-2′-*O*-Me-cAMP was altered by pretreatment with the PKA inhibitor H89 or the PI3K inhibitor LY294002 (Fig. 1C), indicating that FSH activation of EPAC did not involve PKA or P13K pathways.

To determine whether activation of EPAC mediated FSH-induced phosphorylation of PKB or p38MAPK, a potential downstream target of RAP1, granulosa cells were cultured overnight in serum-free medium and then exposed to an EPAC agonist, forskolin, or FSH for 5, 15, and 30 min (Fig. 1D). As in previous studies (13), forskolin and FSH potently increased PKB phosphorylation whereas the EPAC agonist was ineffective at these time intervals. In these same samples, forskolin and FSH also increased levels of phospho-p38MAPK to a greater extent (3.5 $\pm$ 0.3; $P < 0.05$) than did the EPAC agonist.

FSH Activates RAS in Granulosa Cells

Because the phosphorylation of PKB and p38MAPK by EPAC was not as potent as that induced by FSH, despite the ability of EPAC agonists to activate RAP1, we sought other potential mediators of FSH action. Importantly, our results indicated that FSH and forskolin potently activated ERK1/2 in these granulosa cells by mechanisms that appeared to be largely independent of PKA and PI3K (13). Moreover, FSH has been shown recently to induce expression of the EGF-like factors AREG, epiregulin and betacellulin in cumulus cells (38). By binding and activating EGFRs, these factors are known to activate RAS and ERK1/2 phosphorylation in other cells (42). These observations, combined with reports that LH induces EGF-like factor expression in granulosa cells (38) and that LH can activate RAS in a mouse MA10 Leydig cell line (43), caused us to examine whether FSH might also be capable of activating RAS in granulosa cells (Fig. 2A). To

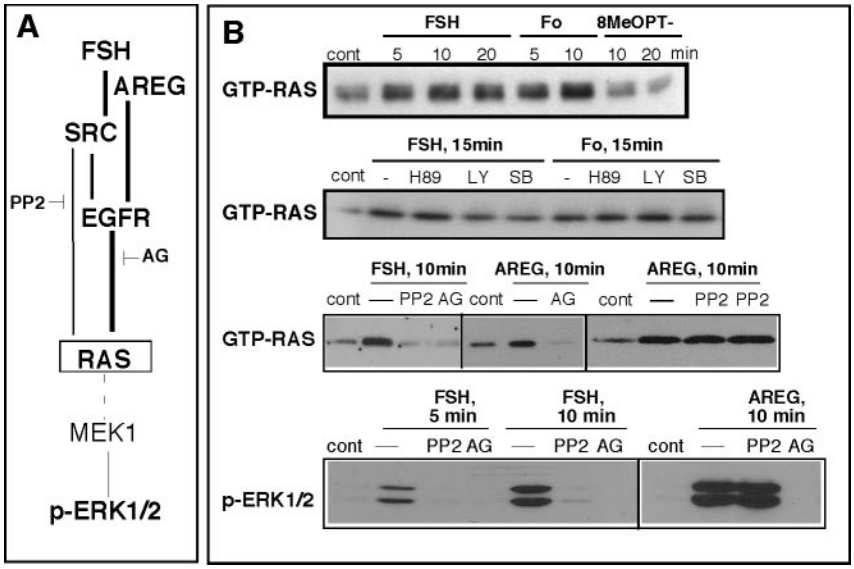


Fig. 2. FSH and AREG, but Not EPAC, Activate RAS by Mechanisms Independent of PKA, PKB, or P38MAPK

A, Schematic shows that FSH activation of RAS involves SFK activity and EGFR tyrosine kinase activation whereas AREG activates RAS predominantly via EGFR activity. (*Bold lines and arrows* indicate major pathways.) B, Western analysis of activated RAS purified by the pull-down method and detected with a RAS-specific antibody. Cells were cultured as in Fig. 1 and then treated with FSH, forskolin, or 8-pMeOPT-2′-*O*-Me-cAMP for 5, 10, or 20 min. Activation of RAS was also analyzed in cultured cells pretreated with specific inhibitors of PKA (H89; 10 μM), PKB (LY294002; LY, 25 μM), and p38MAPK (SB203580; SB, 20 μM) for 1 h before stimulation with agonists for 10 min. Activation of RAS as well as phosphorylation of ERK1/2 by FSH was compared with that of AREG using similar culture and assay conditions using selective inhibitors of SFK activity (PP2, 20 μM) or EGFR tyrosine kinase (AG1478; 10 μM) to block the relevant pathways. Each experiment has been repeated twice with similar results. Quantitative changes in the intensity of immunoreactive bands were obtained by densitometric analyses using a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA). AG, AG1478; Fo, forskolin.

analyze RAS activation, granulosa cells were cultured overnight in defined medium and then stimulated with FSH, forskolin, or 8-MeOPT-2′-*O*-Me-cAMP for 5–20 min. The levels of GTP-bound RAS were measured using the RAF1-GDS RAS binding domain pull-down assay. As shown in Fig. 2B, FSH and forskolin both rapidly increased the levels of GTP-RAS whereas the EPAC ligand had little or no effect. Furthermore, the ability of FSH and forskolin to activate RAS at 10 min was not altered by inhibitors of PKA (H89), PI3K (LY), or p38MAPK (SB203580). However, the FSH activation of RAS (3.7 $\pm$ 0.22 compared with control; $P < 0.001$; n = 5) was completely blocked by the presence of inhibitors of either the SFKs (PP2; $P < 0.001$, n = 5) or EGFR tyrosine kinase (AG1478; $P < 0.001$ n = 5) (Fig. 2B). AREG activation of RAS (4.7 $\pm$ 0.4 compared with control; $P <$ 0.001; n = 3) was undetectable in the presence of AG1478 ($P < 0.001$; n = 3) but was not affected by PP2 ($P < 0.03$; n = 5). Because PP2 effectively blocked RAS activation by FSH at 10 min, SFK activation by FSH appears to be essential for FSH activation of RAS. In contrast, RAS activation by AREG appears to be predominantly mediated by EGFR activation.

To analyze the ability of FSH and AREG to stimulate ERK1/2 phosphorylation, granulosa cells were cultured overnight in defined medium, pretreated for 1 h with either PP2 or AG1478, and then exposed to FSH or AREG. FSH stimulated a time-dependent increase in ERK1/2 at 5 and 10 min, a response that was blocked by either PP2 or AG1478. AREG-mediated phosphorylation of ERK1/2 was inhibited by AG1478 but not by PP2. These results document, for the first time, that FSH as well as AREG rapidly activate RAS in immature granulosa cells and that FSH-induced RAS activation is downstream of both SFKs and EGFR tyrosine kinase. Moreover, FSH-mediated phosphorylation of ERK1/2 is sensitive to inhibitors of both SFKs and EGFR tyrosine kinase activity.

FSH-Mediated Phosphorylation of ERK1/2 as Well as PKB and FOXO1a Are Dependent on FSH Activation of SFK

Because FSH-mediated phosphorylation of ERK1/2 appeared to be downstream of SFK and EGFR activation, we next sought to determine whether activation of other kinase cascades was regulated by SFKs and EGFR activation. For these studies, granulosa cells were cultured overnight in defined medium, exposed to specific inhibitors for 1 h, and then stimulated with FSH for 5, 10, and 30 min. As shown (Fig. 3A), FSH-mediated phosphorylation of ERK1/2 at each time interval was reduced by 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and [4-(3-chloroanilino)-6,7dimethoxy quinazoline (AG1478) as well as by the metalloprotease

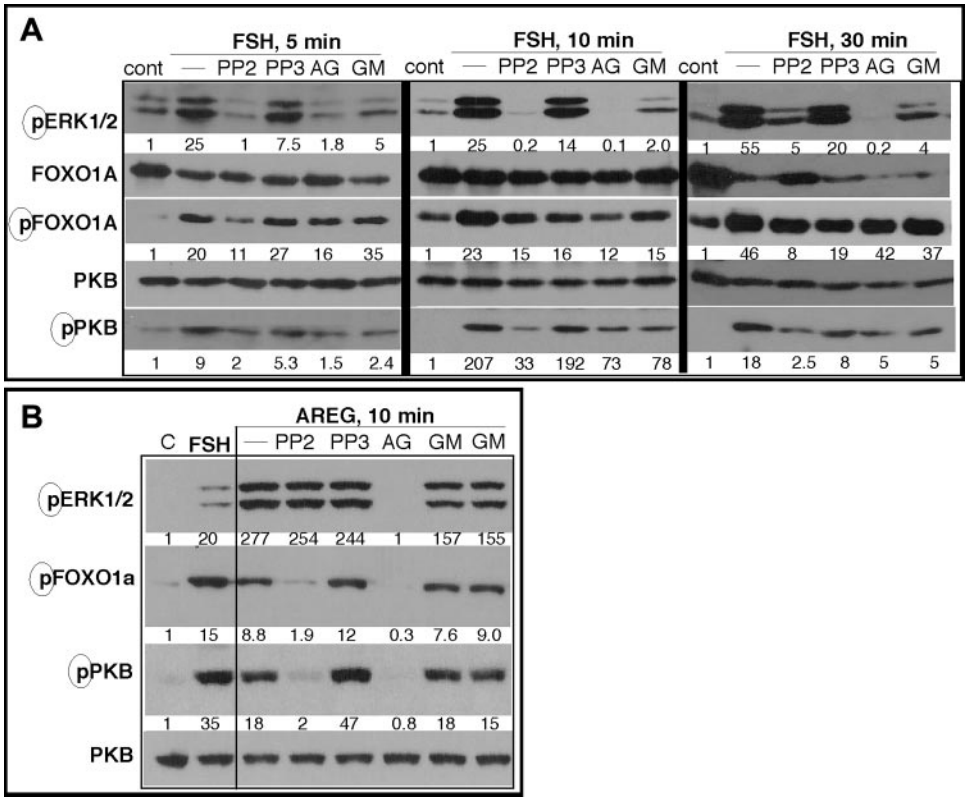


Fig. 3. FSH-Mediated Phosphorylation of ERK1/2, PKB, and FOXO1a Are Dependent on SFK Activity as Well as EGFR Activation

A, Primary granulosa cells were cultured overnight in defined media. At that time, the media were removed and replaced with fresh media containing selective kinase inhibitors PP2 (20 μM or its inactive isoform PP3; 20 μM) or AG1478 (AG, 10 μM) or the general metalloprotease inhibitor (galardin, GM6001; 10 μM) for 1 h and then stimulated with FSH (100 ng/ml) for 5, 10, and 30 min. Cell lysates were prepared in boiling SDS extraction buffer and analyzed by Western blotting using specific antibodies as indicated. B, AREG-mediated phosphorylation of ERK1/2, PKB, and FOXO1a was compared with that of FSH. Granulosa cells were cultured overnight and treated for 1 h without or with inhibitors as described above (panel A). FSH or AREG (100 ng/ml) were added for 10 min as indicated. Cell lysates were prepared as above. Each experiment was repeated twice with similar results. Quantitative changes in the intensity of immunoreactive bands were obtained as in Fig. 2. GM, GM6001; cont, control.

inhibitor galardin (GM6001), implicated in the cleavage and shedding of growth factor ligands from the cell surface (31). 4-amino-7-phenylpyrazol(3,4-d)pyrimidine (PP3), an inactive compound related to PP2, had lesser effects on FSH-mediated ERK1/2 phosphorylation compared with PP2. FSH-mediated phosphorylation of PKB and FOXO1a was also reduced selectively by PP2 compared with PP3. Inhibiting EGFR activity (AG1478) or protease activity (GM6001) reduced phosphorylation of PKB to a greater extent than FOXO1a. However, determining the precise levels of phosphor-FOXO1a is somewhat difficult because the levels of FOXO1a protein were reduced progressively in cells exposed to FSH, most notably at 30 min. This FSH-induced decrease in FOXO1a protein was also partially reversed by PP2 but not by the other inhibitors, indicating that FSH not only increases FOXO1a phosphorylation but also acts to reduce FOXO1a protein by a SFK-dependent mechanism. As in previous studies (Fig. 2), AREG more potently stimulated ERK1/2 phosphorylation than did FSH (Fig. 3B). AREG also stimulated PKB and FOXO1a phosphorylation in a manner that was sensitive to AG1478 as well as PP2 but not to PP3 or GM6001. These results provide additional evidence that SFKs mediate FSH and AREG activation of PKB and FOXO1a, that SFKs *and* EGFR activation are critical for FSH phosphorylation of ERK1/2, and only EGFR activation is required for AREG-mediated phosphorylation of ERK1/2.

c-SRC, FYN, and the c-ABL Are Expressed in Granulosa Cells and Phosphorylated in Response to FSH

To determine which SFK members might be expressed in granulosa cells, we reviewed our cumulus oocyte complex (COC) microarray database (44) and determined that mRNAs encoding *cSrc, Fyn*, and the cSRC-related factor *Abl1* were expressed at relatively high levels. RT-PCR analyses confirmed that mRNA encoding each was present in ovarian cells (data not shown). Based on these observations, Western blot analyses were done using a specific antiphosphoty-

rosine antibody that recognizes specific tyrosine phosphorylation sites in c-SRC and FYN as well as c-ABL (29). As indicated in Fig. 4A, treatment of cells with FSH for 10 min increased immunoreactive bands at 145 kDa corresponding to c-ABL and at 60/59 kDa corresponding to c-SRC and FYN, respectively. Little or no change in the levels of nonphosphorylated c-SRC and FYN were observed in these same samples (Fig. 4B). Similar results were observed in cells exposed to FSH for 30 min (Fig. 4C). At each time point, FSH-induced phosphorylation of c-ABL and c-SRC/FYN was reduced to a greater extent by PP2 than by PP3, AG1478, or GM6001, indicating that FSH-induced phosphorylation of SFKs, especially c-ABL, was dependent on SFK phosphorylation, hence activation. However, a role for EGFR/growth factor receptor activation may also be involved because AG1478 and GM6001 also reduced c-ABL phosphorylation but not to the same extent as PP2. Importantly, FSH-mediated phosphorylation of c-ABL at 10 min was approximately 3.5-fold greater than that observed in response to AREG (5.8 compared with 1.7-fold; 15 compared with 5.2-fold; Fig. 4, D and E), indicating that FSH is more potent than AREG in mediating this response. PP2 reduced the levels of phospho-c-ABL in cells exposed to AREG more potently than did PP3. AG1478 also reduced AREG-induced phosphorylation of c-ABl. Neither FSH nor AREG markedly altered levels of non-phosphorylation c-ABL in these cells (Fig. 4E). Thus, FSH activation of c-ABL and c-SRC/FYN appears to occur rapidly and is dependent, in part, on a PP2-sensitive step.

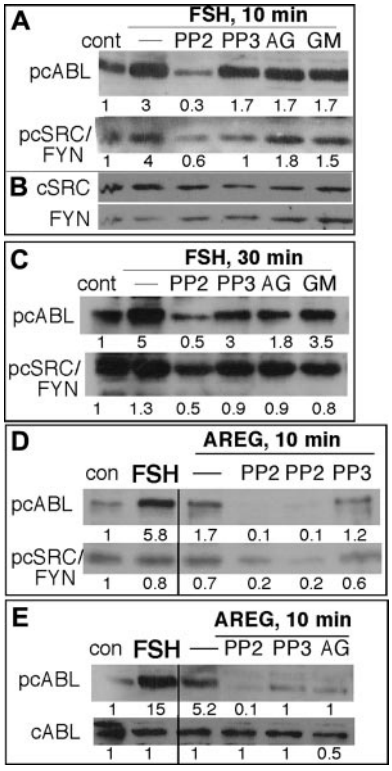


Fig. 4. FSH-Increased Phosphorylation of c-ABL Occurs in a PP2 Sensitive, SFK-Dependent Manner

Primary granulosa cells were cultured overnight in defined media, pretreated with kinase inhibitors PP2 (20 μM or its inactive isoform PP3, 20 μM) or AG1478 (AG, 10 μM) or the general protease inhibitor (galardin, GM6001; 10 μM) for 1 h and then stimulated with FSH (100 ng/ml) for 10 min (panel A) or 30 min (panel B). AREG-mediated phosphorylation of c-ABL and c-SRC/FYN was compared with that of FSH. Granulosa cells were cultured with the same inhibitors as above for 1 h before addition of FSH (100 ng/ml) or AREG (100 ng/ml) for 10 min (D and E). Cell lysates were prepared as above. Each experiment was repeated twice with similar results. Quantitative changes in immunoreactive band intensities were obtained as in Fig. 2. GM, GM6001; con or Cont, control.

FSH and AREG Mediate PKB, p38MAPK, and ERK1/2 Phosphorylation by PKA-Independent Mechanisms that Involve Receptor Tyrosine Kinase Activity and MEK1

Because FSH and AREG activated RAS, we next compared the ability of FSH and AREG to activate not only ERK1/2 and PKB but also p38MAPK. For this we analyzed a detailed time course and the effect of specific inhibitors of FSH- and AREG-signaling cascades on the phosphorylation responses. Granulosa cells were cultured overnight in defined, serum-free medium and then exposed to FSH or AREG for selected time intervals (0–60 min). As shown in Fig. 5A, the response to each agonist exhibited a distinct pattern. FSH stimulated demonstrable increases in the levels of phospho-ERK1/2, phospho-p38MAPK, and phospho-PKB within 10 min. Whereas levels of phospho-EKR1/2 were highest 60min after FSH, levels of phospho-p38MAPK and PKB were maximal at 20 min. In contrast, AREG stimulated a dramatic increase in phospho-ERK1/2 within 5 min that decreased by 20 min and then rebounded slightly at 30–60 min, a pattern that has been observed in three separate experiments. Although AREG also increased the phosphorylation of p38MAPK and PKB at 5 min, the response was much less than that observed for ERK1/2. Levels of phospho-p38MAPK and phospho-PKB also exhibited a biphasic pattern with a secondary increase at 20 min followed by reduced levels at 60 min.

These distinct temporal patterns of FSH- and AREG-induced phosphorylation suggest that each ligand acts via distinct receptor signaling cascades that converge on downstream targets. To determine which signaling cascades were activated by FSH and AREG, inhibitors of two tyrosine kinases (EGFR, AG1478 and SFKs, PP2), p38MAPK (SB203580), nonactive and activated MEK1 (PD98059 and U1026, respectively), and protein kinase A inhibitor (PKI) were tested. Granulosa cells were cultured overnight and exposed to each inhibitor for 1 h before the addition of FSH or AREG (Fig. 5, B and C, respectively). As shown, FSH increased the phosphorylation of ERK1/2, p38MAPK, and PKB at 30 min. With the exception of PKI, which

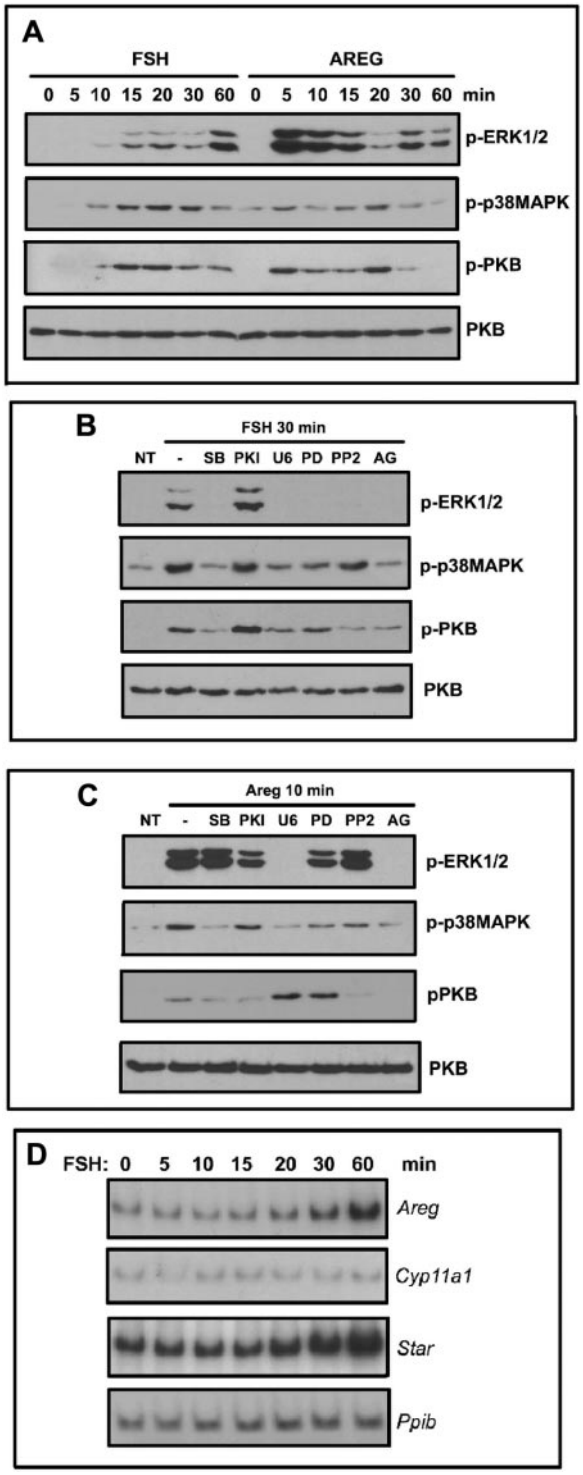


Fig. 5. FSH- and AREG-Stimulated Phosphorylation of ERK1/2, PKB, and p38MAPK Is Independent of PKA

A, Time course. Primary granulosa cells were cultured overnight in defined media and then stimulated with FSH (100 ng/ml) or AREG (100 ng/ml) for 0–60 min. Cell lysates were prepared in boiling SDS extraction buffer and analyzed by Western blotting. B and C, Kinase Inhibitors. Additional granulosa cells were cultured overnight and then incubated in the presence or absence of specific kinase inhibitors for 1 h before treatment with agonist FSH for 30 min (panel B) or AREG for 10 min (panel C) or FSH 5–10 min and AREG 10 min (panel D). Inhibitors used include: SB (SB203580, 20 μM), protein kinase inhibitor (PKI, 50 μM), U1026 (U6, 10 μM), PD98059 (PD, 20 μM), PP2 (20 μM), and AG1478 (AG, 10 μM). D, Granulosa cells were cultured with FSH as in panel A. At selected time intervals total RNA was extracted, and the levels of *Areg*, *Cyp11a1*, *Star*, and cyclophilin (*Ppib*; internal standard) mRNA were analyzed by semiquantiative RT-PCR. Each experiment was repeated twice with similar results. Quantitative changes in immunoreactive band intensities and RT-PCR products were obtained as in Fig. 2. NT, No treatment.

increased the levels of pERK1/2, p38MAPK, and pPKB, all other inhibitors reduced or blocked the FSH-mediated phosphorylation of the kinases analyzed, indicating that the SFKs, p38MAPK, EGFR, and MEK1 are all required. Most dramatic was the complete absence of phosphor-ERK1/2 in FSH-treated cells exposed to inhibitors of the EGFR/MEK pathway (AG1478, PD98059, U1026) as well as the p38MAPK blocker, SB203580, and the SFK blocker, PP2. Notably, SB203580 blocked FSH-mediated phosphorylation of PKB but PP2 did not block FSH-mediated phosphorylation of p38MAPK, indicating that FSH-mediated phosphorylation of p38MAPK does not involve SFKs. These results indicated that FSH activated numerous pathways, all of which were stimulated independently of PKA at the time interval analyzed.

In sharp contrast to FSH, AREG potently increased the phosphorylation of ERK1/2 at 10 min (Fig. 5C). Moreover, this response was most completely blocked by either the EGFR tyrosine kinase inhibitor (AG1478) or the activated MEK1 inhibitor (U1026). The inhibitor of nonactivated MEK1 (PD98059) and the SFK inhibitor PP2 were less effective. Unexpectedly, PKI reduced AREG-mediated phosphorylation of ERK1/2. AREG mediated only modest increases in the phosphorylation of p38MAPK and PKB at 10 min. Whereas the AREG-mediated phosphorylation of p38MAPK was reduced most effectively by SB203580, U1026, and AG1478, the low levels of phospho-PKB were increased by the MEK1 inhibitors (PD98059 and U1026). Collectively, these results indicate that the effects of FSH on ERK1/2, p38MAPK, and PKB phosphorylation are complex and mediated, at least in part, by SFKs and the EGFR tyrosine kinase as well as by p38MAPK itself.

FSH Induces Expression of *Areg* mRNA

The apparent convergence of FSH signaling with the EGFR cascade on ERK phosphorylation indicated that FSH activation of the FSH receptor might impact this growth factor cascade directly via activation of SFKs. However, recent studies in mice have shown that FSH can induce expression of the EGF-like factors including AREG in cumulus cells and granulosa cells of ovulating follicles (37, 38), thereby providing specific

ligands to activate the EGFR-signaling cascade. Therefore, we next sought to determine whether FSH could mediate rapid induction of AREG in these immature granulosa cells obtained from E-primed rats. As shown in Fig. 5D, low levels of *Areg* mRNA were present in cells cultured overnight in serum-free, defined medium. FSH induced a detectable increase in the expression of *Areg* mRNA within 20 min, a response that was increased further at 30 and 60 min (Fig. 5D). In these same samples, expression of *Star* mRNA also increased within 20–60 min. Thus, the rapid phosphorylation of ERK1/2 by FSH observed at 5–10 min (Figs. 3 and 4) occurs before demonstrable increases in *Areg* mRNA. However, the heightened levels of ERK1/2 phosphorylation at 60 min (Fig. 5A) occur in concert with increased *Areg* mRNA at 30–60 min (Fig. 5D). These results indicate that there may be two mechanisms by which FSH receptor activation leads to ERK1/2 phosphorylation, the first involving SFK activation and EGF-like factor shedding (GM6001-sensitive) and the second involving induction of AREG, each of which can impact RAS (29) (Figs. 2B and 3, A–D).

RAS Is an Important Regulator of Granulosa Cell Function

Because RAS is a downstream target of many tyrosine kinases, including the EGFR and SFKs and because FSH activated RAS via SFK and EGFR-sensitive mechanisms (Fig. 2B), we next examined whether FSH-dependent phosphorylation of PKB, p38MAPK, and/or ERK1/2 required RAS. For these studies, granulosa cells were infected with an adenovirus expressing a dominant-negative form of RAS (DN-RAS) or an empty vector for 16 h (45). At that time granulosa cells were stimulated with FSH or AREG for 30 min (Fig. 6A). In cells exposed to the empty vector, FSH induced phosphorylation of PKB, p38MAPK, and ERK1/2 whereas AREG increased the levels of phospho-ERK1/2 and phospho-p38MAPK but not phospho-PKB at the same 30-min time interval. In cells expressing DN-RAS, the phosphorylation of ERK1/2 and p38MAPK by either FSH or AREG was completely abolished whereas phosphorylation of PKB was increased in a manner similar to that observed in cells with empty vector treated with FSH or AREG (Fig. 6A). Thus, DN-RAS has a potent and selective negative impact on ERK1/2 and p38MAPK phosphorylation induced by AREG and FSH in granulosa cells. To determine whether this was specific to RAS or might also be mediated by RAP1, additional cells were infected with an adenovirus expressing an empty vector or RAP1-GAPII and treated with the same agonists. In contrast to DN-RAS, RAP1-GAPII had little or no effect on the levels of phospho-PKB and phospho-p38MAPK and only marginally reduced levels of phospho-ERK1/2 (data not shown). Thus, RAS is a potent mediator of AREG- and FSH-induced ERK1/2 and p38MAPK

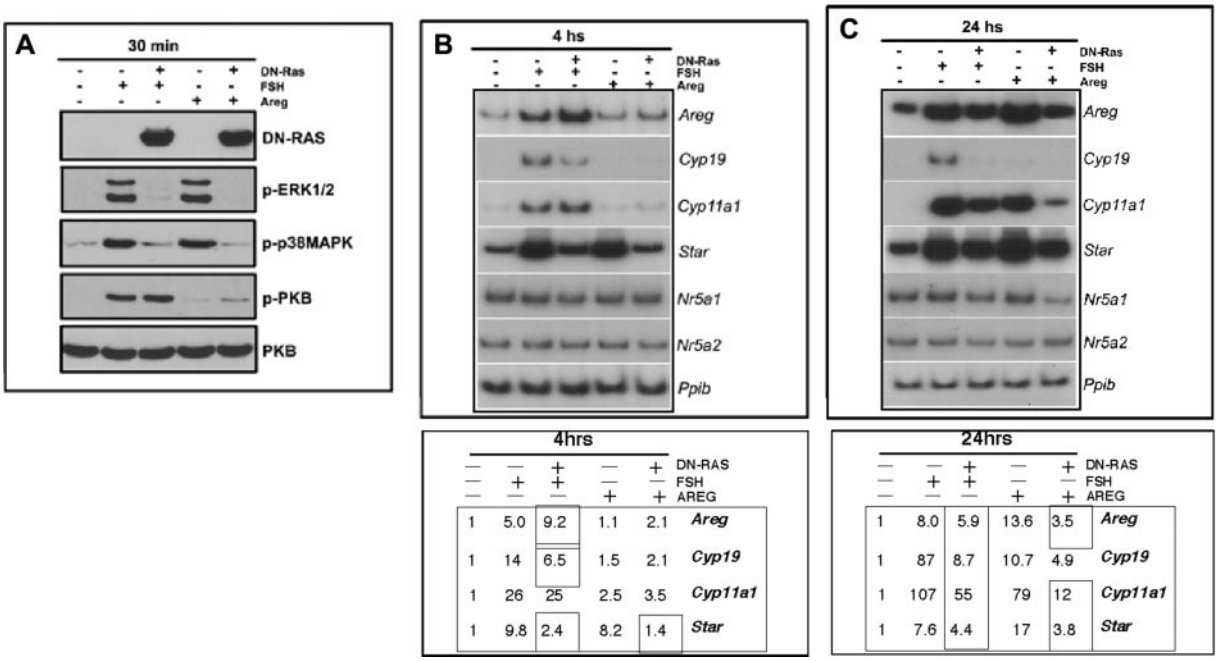


Fig. 6. DN-RAS Impacts FSH- and AREG-Mediated Signaling Cascades and Gene Expression

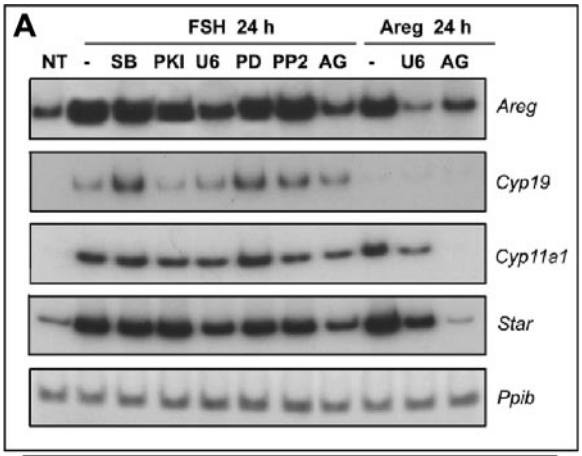
Granulosa cells were cultured for 8 h in defined medium and then infected with an adenoviral vector expressing DN-RAS for 16 h. At that time the cells were stimulated with FSH or AREG for 30 min (A), 4 h (B), or 24 h (C). Whole-cell extracts were prepared to analyze changes in protein phosphorylation (panel A) or RNA was extracted for selective RT-PCR analyses (panels B and C). Each experiment was repeated twice with similar results and quantitated to document relative changes in RNA levels.

phosphorylation but does not impact ligand-induced PKB phosphorylation in this context.

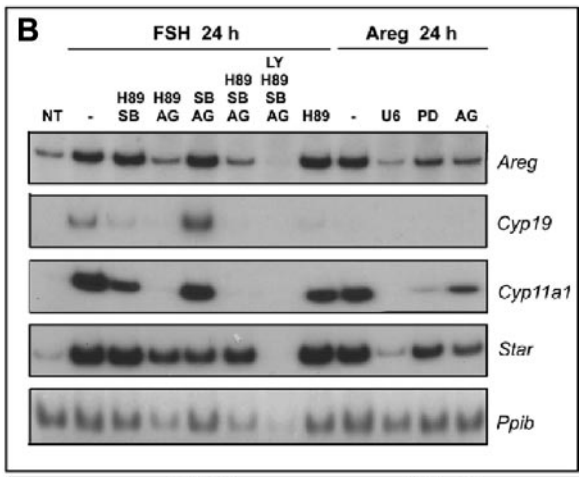
RAS Is Important for the Expression of Selected FSH- and AREG-Induced Genes

To determine the relative impact of FSH and AREG on the expression of specific genes related to differentiation in this granulosa cell model system, additional granulosa cells were infected with the same adenovirus expressing a DN form of RAS or an empty vector for 16 h. At that time granulosa cells were stimulated with FSH or AREG for 4 h or 24 h (Fig. 6, B and C, respectively). At 4 h, DN-RAS enhanced FSH induction of *Areg* mRNA but reduced FSH-dependent induction of *Cyp19* and *Star* mRNAs without affecting expression of P450scc (*Cyp11a1*), the transcriptional regulators steroidogenic factor 1 (*Nr5a1*) and liver receptor homolog 1 (*Nr5a2*), or cyclophilin B (*Ppib*; the latter used as an internal control). At this same 4-h time interval, AREG alone had little or no effect on the expression of the selected genes with the exception of *Star*. AREG induction of *Star* mRNA was similar to that of FSH and also reduced by the presence of DN-RAS. At 24 h, expression of FSH-induced expression of *Areg*, *Cyp19a*, *Cyp11a1*, and *Star* and *Nr5a1*mRNAs was also reduced in the DN-RAS-expressing cells. Likewise, AREG induced expression of *Areg*, *Cyp11a* , and *Star* mRNAs was also reduced by DN-RAS. However, AREG did not impact *Cyp19* expression. Collectively, these results indicate that agonist-induced expression of *Areg*, *Cyp19*, *Cyp11a, Star*, and, to a lesser extent, *Nr5a1* mRNAs is modulated by RAS most dramatically at later stages of differentiation.

To further analyze the potential role of EGFR signaling on genes presumed to be regulated by FSH, granulosa cells were cultured with FSH in the absence or presence of specific inhibitors of PKA (PKI or H89), p38MAPK (SB203580), MEK1 (U1026 or PD98059), SFKs (PP2), or EGFR tyrosine kinase (AG1478) alone or in combinations for 24 h (Fig. 7, A and B, respectively). As shown, *Cyp19a* mRNA was induced by FSH but not by AREG, confirming results in Fig. 6. Treatment of the cells with inhibitors of p38MAPK (SB20380) inactive MEK1 (PD98059), or SFKs (PP2) enhanced FSH-induced expression of aromatase (*Cyp19a*) between 3- to 4-fold whereas inhibitors of activated MEK1 and EGFR tyrosine kinase (AG) enhanced the FSH-mediated effects on *Cyp19* mRNA approximately 1.4- to 2-fold. In contrast, the PKA inhibitor PKI had little or no effect on FSH induction of *Cyp19a* mRNA. These data suggest that FSH induction of *Cyp19a* mRNA is regulated by pathways in addition to PKA. FSH-induced expression of *Areg*, *Cyp11a1*, and *Star* was selectively reduced by the inhibitors of activated MEK1 (U1026), SFKs (PP2), and EGFR (AG1478) but not by PKI. AREG-induced expression of *Areg*, *Cyp11a1*, and *Star* was reduced markedly by blockers of MEK1 and the EGFR tyrosine kinase (Fig. 7A).



	FSH, 24h							AREG, 24h			
NT	—	SB	PKI	U6	PD	PP2	AG	—	U6	AG	
1	3.4	3.3	3.3	1.1	3.2	4.8	2.1	7.9	1.1	0.56	*Areg*
1	24	98	18	34	88	75	44	6.2	3.4	4.1	*Cyp19*
1	20	17.6	16.3	12.7	28.6	12.0	9.9	22.6	6.5	0.12	*Cyp11a1*
1	14	15.1	17.7	9.2	12.8	13.3	5.8	26.5	9.9	0.80	*Star*



	FSH, 24h							AREG, 24h				
NT	—	SB H89	AG H89	SB AG	SB AG H89	SB/AG H89/LY	H89	—	U6	PD	AG	
1	4.6	7.9	1.1	6.8	2.1	0.2	8.9	7.4	0.9	4.7	3.4	*Areg*
1	96	21	103	275	1.5	0.4	16.4	5.9	0.3	0.7	0.3	*Cyp19*
1	87	31	2.0	73	0.9	0.6	38	47	0.5	1.6	10.6	*Cyp11a1*
1	39	35	18	27	14.8	0.8	24.5	24.8	0.3	5.9	9.7	*Star*

Fig. 7. FSH-Mediated Differentiation of Granulosa Cells Is Mediated by PKA and EGFR Signaling Pathways

Granulosa cells were cultured overnight in defined serum-free medium, pretreated for 1 h with various kinase inhibitors alone (A) or in combination (B) and then cultured with FSH or AREG for an additional 24 h. Total RNA was isolated and RT-PCR analyses were done to determine the expression of selected genes. Cyclophilin B was used as an internal control. Each experiment was repeated twice with similar results and quantified as in Fig. 2 to document relative changes in RNA levels. NT, No treatment; AG, AG1478.

More dramatic changes in the expression of *Areg*, *Cyp19a*, *Cyp11a1*, and *Star* mRNAs were observed when granulosa cells were cultured with FSH in the presence of combinations of kinase inhibitors for 24 h. Specifically, FSH-induced expression of *Areg* and *Star* mRNAs was not altered by the PKA inhibitor H89 alone or when H89 was combined with the p38MAPK inhibitor (SB) but was dramatically reduced when H89 was combined with the EGFR inhibitor (AG). In contrast, FSH induced expression of *Cyp19* and *Cyp11a1* mRNAs was effectively reduced by all inhibitor combinations except when SB and AG. These results suggest that inhibition of EGFR tyrosine kinase and p38MAPK together removes negative signals to restore FSH induction of *Cyp11a1* and to enhance FSH transactivation of the *Cyp19* gene 3-fold. (Fig. 7B; data not shown). The combination of AG1478 and SB203580 did not markedly affect FSH-induced gene expression and adding SB203580 with H89 and AG1478 did not further reduce FSH-induced gene expression compared with the effects of H89 and AG1478. When cells were cultured with FSH and all four inhibitors (H89, SB203580, AG1478 as well as the PI3K inhibitor LY) for 24 h, the effect appeared to be was toxic because the expression of all genes including cyclophilin B, *Ppib*, the internal control, were reduced.

At 24 h, AREG alone induced expression of *Areg*, *Cyp11a1*, and *Star* mRNAs but did not alter expression of *Cyp19a* mRNAs in granulosa cells (Fig. 7B). The inductive effects of AREG on *Cyp11a1* and *Star* mRNAs were reduced by the presence of inhibitors of the EGFR (AG1478) as well as activated and nonactivated MEK1 (U1026 and PD98059, respectively). Collectively, these results indicate that FSH induction of *Areg*, *Cyp11a1*, and *Star* is most potently blocked by inhibitors of PKA and EGFR tyrosine kinase. Moreover, AREG alone impacts the expression of *Areg*, *Cyp11a1*, and *Star* mRNAs, suggesting that AREG mediates some of the long-term effects of FSH.

Based on these results, we hypothesized that both FSH and AREG regulate the expression of *Cyp11a1* and *Star* mRNAs in granulosa cells. To test this further, granulosa cells were cultured for 24 h in defined medium without or with FSH, a specific PKA agonist N^6-Benzoladenosine-3′,5′-cyclic monophosphate (6-Bnz-cAMP), an EPAC-specific agonist 8-pMeOPT-cAMP, AREG alone, or AREG in combination with either 6-Bnz-cAMP or 8-pMeOPT-cAMP. FSH alone induced expression of *Cyp19a*, *Cyp11a1*, *Star*, and *Areg* mRNA, 38-, 101-, 17-, and 7.4-fold, respectively ($n = 2$; Fig. 8). The PKA and EPAC agonists alone were comparatively ineffective. In contrast, AREG alone also induced expression of these genes (2.3-, 42-, 19.6-, and 7.5-fold, respectively). When cells were treated with AREG and the PKA agonist 6-Bnz-cAMP, expression levels of *Cyp11a1* and *Star* mRNAs were consistently increased 1.3-fold. In contrast, the EPAC agonist did not enhance the effects of AREG. These results indicate that a PKA-specific agonist alone is ineffective in inducing FSH target genes and only marginally promotes the effect of AREG. Moreover, FSH and AREG exerted additive effects because *Cyp11a1* and *Star* mRNAs increased 1.5- to 2.5-fold, respectively, above that observed with FSH alone whereas Cyp19 levels were reduced 10-fold compared with FSH alone. Thus, FSH induction of AREG appears to impact the expression of specific genes that are associated with FSH-mediated differentiation to a luteal phenotype, such as *Star* and *Cyp11a1* and *Sgk* (data not shown).

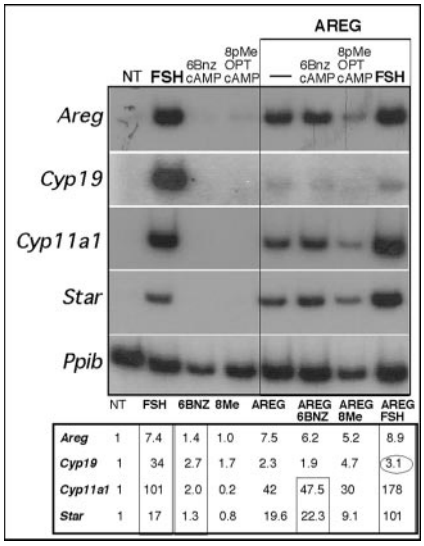


Fig. 8. PKA Activation Alone Is Not Sufficient to Induce Granulosa Cell Differentiation

Granulosa cells were cultured overnight in defined serum-free medium and then cultured an additional 24 h in the presence of testosterone without or with FSH, a PKA-selective agonist 6-Bnz-cAMP, an EPAC-selective agonist 8-pMeOPT-cAMP, AREG alone, or AREG with 6-Bnz-cAMP, 8-pMeOPT-cAMP, or FSH. Total RNA was isolated for RT-PCR analyses. Each experiment was repeated twice with similar results. Quantitative changes in RT-PCR products were quantified as in Fig. 2. NT, No treatment.

FSH and AREG Act Synergistically to Induce Granulosa Cell Differentiation and the Expression of Steroidogenic Genes

Because FSH and AREG increased *Cyp11a1* and *Star* mRNA in these naïve granulosa cells, we hypothesized that changes in CYP11a1 protein levels would also be observed. For these studies granulosa cells were cultured in medium alone or with either FSH, AREG, or FSH and AREG for 24 h. For immunofluorescent analyses the cells were cultured on coverslips, fixed and labeled with a CYP11a1 antibody (Fig. 9A). For Western blots, cell lysates were prepared in boiling sodium dodecyl sulfate (SDS) buffer, resolved and probed with CYP11a1, phospho-ERK1/2-, and phospho-PKB-specific antibodies (Fig. 9B). Both the immunostaining and Western blots show that FSH increased mito-

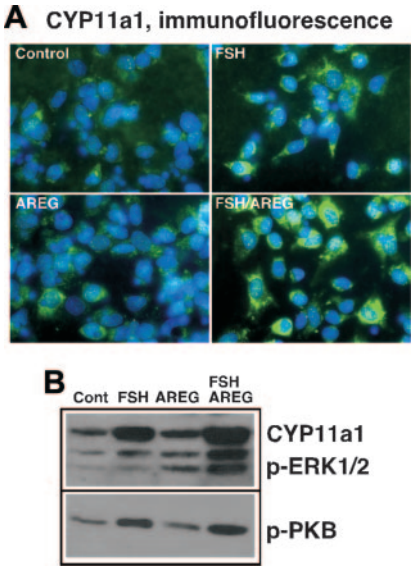


Fig. 9. Expression of CYP11a1 Protein Is Regulated by FSH and AREG

Granulosa cells were cultured in defined medium overnight and then exposed to FSH (100 ng/ml), AREG (100 ng/ml), or FSH and AREG for 24 h. For cells cultured on coverslips, the medium was removed at 24 h, and the cells were fixed in 4% paraformaldehyde and processed as described in *Materials and Methods*. CYP11a1 protein was visualized using a specific polyclonal anti-CYP11a1 antibody and fluorescent secondary antibody (Alexis 588, *green*). For whole-cell extracts, cells were lysed in boiling SDS medium and then analyzed by Western blotting using the same CYP11a1 antibody as well as phospho-ERK1/2- and phospho-PKB-specific antibodies. Cont, Control.

chondrial CYP11a1 protein levels in many cells whereas AREG alone increased CYP11a1 protein in fewer cells. However, FSH and AREG stimulated marked increases in the intensity of immunostaining for CYP11a1 in most cells and increased the level of protein visualized on the Western blot. The increase in CYP11a1 protein correlated with increased levels of phospho-ERK1/2 and phospho-PKB in these cells, indicating that both FSH and AREG act to increase CYP11a1 protein, and this involves, in part, enhanced ERK1/2 phosphorylation as well as PKB.

DISCUSSION

The binding of FSH to its cognate FSH receptors expressed exclusively on the surface of granulosa cells initiates not only the production of cAMP via activation of adenylyl cyclase but other signaling cascades as well (2, 14)(summarized in Fig. 10). Using primary granulosa cells expressing the endogenous FSH receptor as a model system, we document, for the first time, that FSH binding to its receptor rapidly (within 5–15 min) activates both RAP1 and RAS by mechanisms that are independent of either PKA or PI3K activation (Fig. 10). Furthermore, our results indicate that cAMP binding to EPAC is the primary pathway by which FSH activates RAP1 and is at least one pathway by which FSH activates p38MAPK. Although our observations that FSH activates RAS in primary cultures of granulosa cells contrast with those published previously (26), the activation of RAP1 and RAS by FSH that we observed in immature granulosa cells is not completely unexpected. There is increasing evidence that GPCRs, including the LH receptor, exhibit complex cross talk with tyrosine kinase-regulated pathways that lead to RAS activation and ERK1/2 phosphorylation (35, 43, 46). The divergent results that we have obtained compared with those of Cottom *et al.* (26) are due most likely to the different and short time intervals being analyzed in each study. This is compounded by the difficulty in capturing at a single time point the rapid and sequential events that occur after FSH binding to its receptor. The complexity of FSH signaling is highlighted by the fact the mechanisms by which FSH activates RAS likely involve more than one pathway. Specifically, FSH-mediated phosphorylation of ERK1/2 was blocked by both SFK inhibition as well as EGFR tyrosine kinase inhibition. Because the initial, rapid phosphorylation of ERK1/2 by FSH is sensitive to SFK inhibitors, it is likely that the SRC kinases (c-SRC and FYN) and the SRC-related kinase c-ABL, which we show are present in, and are also phosphorylated rapidly in response to, FSH mediate some of the rapid responses of the liganded FSH receptor (Fig. 10).

An important role of SRC kinases in transducing FSH and LH receptor action appears to be emerging from several different studies. Notably, SRC family kinase inhibitors block FSH- (26) (Figs. 2 and 3) and LH- (43) dependent phosphorylation of ERK1/2 by mechanisms that in the case of LH (35) appear to be independent of the EGFR activation. However, in the case of FSH, both SFK and EGFR pathways appear to be involved and possibly linked. Of physiological relevance, recent studies have shown that mice in which the *c-Src* gene has been disrupted are infertile and exhibit impaired follicular development and luteinization (47, 48). In support of these studies, a key role for SRC kinase activation downstream of GPCR activation has also been observed for arginine vasopressin-stimulated phosphorylation of ERK1/2 via the V2 vasopressin receptor (28) and α2-agonist activation via the α2-adrenergic receptor (29). Although the precise mechanisms by which the gonadotropins, arginine vasopressin, and α2 agonists activate SFKs remain to be determined, several possible candidates include the G protein receptor kinases and β-arrestins that can recruit SFKs to liganded GPCRs via scaffolding events (28, 29, 32, 49). β-Arrestins have also been shown recently to impact β-adrenergic receptor-mediated phosphorylation of ERK1/2 (46). However, phosphorylation of ERK1/2 by GPCR has also been postulated to occur by β-arrestin-independent mechanisms that may be mediated by G proteins (29) or a phosphotyrosine phosphatase (26). Our studies clearly show that

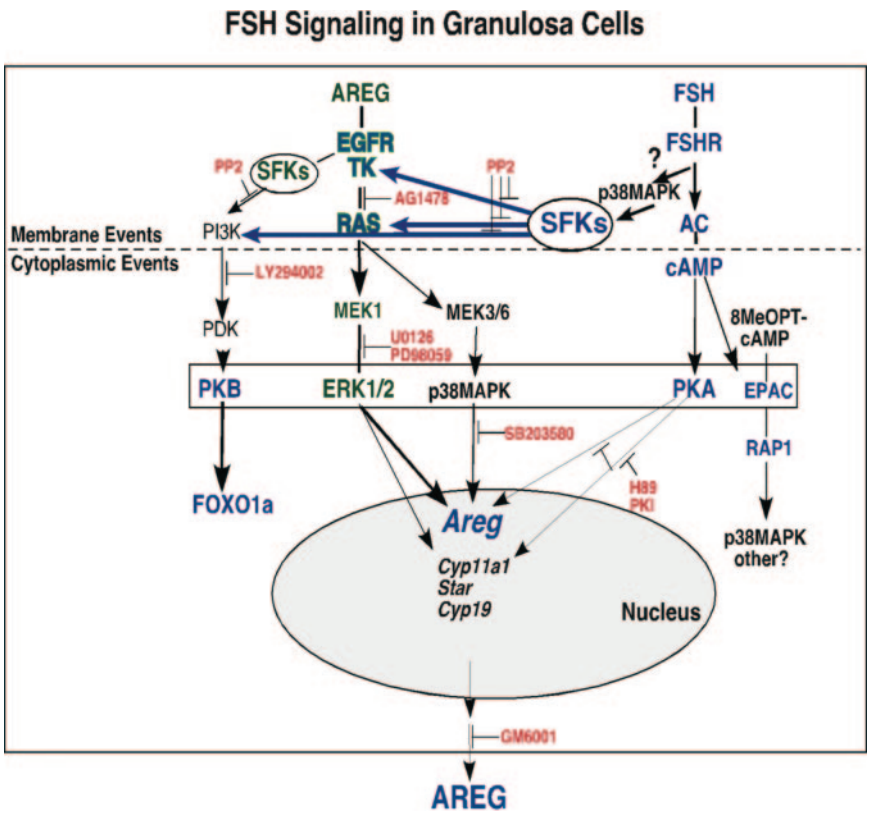


Fig. 10. Schematic of Signaling Cascades Activated by FSH in Immature Rat Granulosa Cells

FSH binding to its cognate receptor on the surface of granulosa cells is known to activate adenylyl cyclase, leading to the production of cAMP and activation of PKA. Shown herein, FSH via cAMP also rapidly activates EPAC that is selectively expressed in granulosa cells of immature ovaries and its downstream target, the small GTP-binding protein RAP1. Activation of this pathway leads to a modest increase in p38MAPK phosphorylaton in these immature granulosa cells. During the same rapid time interval, FSH activated other membrane-related factors such as SFKs and RAS leading to increased levels of phospho-ERK1/2, p38MAPK, PKB, and FOXO1a. FSH activation of RAS and phosphorylation of ERK1/2 in granulosa cells occurred by mechanisms that appear to require SFKs (inhibited by PP2), EGFR tyrosine kinase activity (inhibited by AG1478), and MEK1 (inhibited by U1026, PD98059), respectively. The specific factors leading to the phosphorylation of p38MAPK are less well defined but appear to involve RAS as well as a weaker input from EPAC/RAP1. SFKs (but not EGFR tyrosine kinase activity or RAS) mediated FSH- and AREG-stimulated phosphorylation of PKB and its target FOXO1a. The induction of *Areg* mRNA by FSH in these immature granulosa cells appears to occur primarily via ERK1/2 and PKA, leading to the production of ligand that further activates the EGFR pathway and the differentiation of granulosa cells observed at 24 h. FSH-mediated proteinase activity also appears to enhance EGFR activity. Whereas the FSH-induced expression of *Cyp19a* mRNA is critically dependent on PKA (inhibited by H89 and PKI), the expression of *Areg*, *Cyp11a1*, and *Star* mRNAs also required activation of ERK1/2 (inhibited by AG1478). Importantly, FSH and AREG act in an additive manner to enhance ERK1/2 phosphorylation and CYP11a1 expression at 24 h. Thus, FSH binding to its receptor rapidly orchestrates the coordinate activation of at least three diverse membrane-associated signaling cascades (AC, RAS, and SFK) that converge downstream to activate specific kinases (PKA, ERK1/2, and PKB/FOXO1a) that impact the expression of selected genes during granulosa cell differentiation. *Bold lines* indicate major pathways analyzed. AC, Adenylyl cyclase; TK, tyrosine kinase.

FSH activates both RAS and ERK1/2 phosphorylation by mechanisms that are sensitive to PP2 inhibition and that are temporally related to FSH-mediated phosphorylation of c-ABL that is also remarkably sensitive to PP2. Of potential relevance is the recent observation that c-ABL phosphorylates the EGFR at Y1173 and that this blocks EGFR endocytosis, thereby prolonging EGFR activation (50).

An additional mechanism by which FSH activates RAS and leads to ERK1/2 phosphorylation depends on the activation of the EGFR directly by SFKs and/or by induction/activation of specific EGF-like factors, including AREG, which bind the EGFR (Fig. 10). The ligand-activated EGFR is known to activate RAS and the MAPK pathway (24, 39, 42). That ERK1/2 phosphorylation is downstream of FSH and EGFR pathways is supported by the observations that FSH-mediated phosphorylation of ERK1/2 in immature granulosa cells obtained from preantral and small antral follicles is highly sensitive to inhibition by the EGFR tyrosine kinase inhibitor AG1478, the MEK1 inhibitors (U1026 and PD98590), and DN-RAS as well as to the protease inhibitor GM6001. Taken together, these results indicate that the EGFR system is a target of FSH

action in granulosa cells at this early stage of differentiation as well as in granulosa cells of preovulatory follicles (24, 38). Importantly, FSH-mediated phosphorylation of ERK1/2 was not sensitive to an IGF receptor inhibitor, indicating that FSH is not acting via IGF-I (data not shown). Thus, cross talk between the FSH receptor and the EGFR is highly specific, time dependent, and likely involves more than one process. In marked contrast, AREG-mediated phosphorylation of ERK1/2 in these immature granulosa cells was relatively insensitive to SFK inhibition or p38MAPK inhibitors. Similarly, activation of the EGFR and phosphorylation of ERK1/2 in MA-10 cells is insensitive to SFK inhibition (35). These results provide further evidence that activation of the EGFR-mediated phosphorylation of ERK1/2 is direct whereas the effects of FSH on ERK1/2 are multifaceted.

The signaling cascades by which FSH induces expression *Areg* mRNA and other EGF-like factors in granulosa cells are not yet entirely clear. However, they likely involve a combination of several signaling cascades including RAP1 activation and p38MAPK phosphorylation (24) as well as PKA and, based on the results presented herein, involve activation of the EGFR itself. The promoter of the *Areg* gene has a functional CREB binding site that is sensitive to PKA regulation in other cells (51, 52). However, in granulosa cells no one has conclusively shown that *Areg* is exclusively a PKA-regulated gene and in these immature granulosa cells PKA alone does not appear to be effective. Moreover, epiregulin and betacellin are also targets of LH and FSH in granulosa cells and are presumed targets of PKA and/or ERK (24, 37, 38). Other kinases including p38MAPK and ERK1/2 are known to activate CREB (30), but this may be cell type and time dependent (2). That FSH induction of *Areg* mRNA at 4 h was increased in cells exposed to DN-RAS but reduced in cells exposed to FSH for 24 h indicates further that the ERK1/2 pathway may exert negative and positive regulatory effects on transcriptional regulation of this gene that are time dependent.

A novel role for EGFR signaling in immature granulosa cells was revealed by the ability of AREG alone to modulate the expression of genes presumed to be specific targets of cAMP and PKA. These genes encode the steroidogenic enzymes involved in progesterone biosynthesis, *Cyp11a1* and *Star*, as well as other luteal cell markers. Not only did FSH and AREG induce expression of these genes but the induction of these genes at 24 h post agonist treatment was reduced by DN-RAS, inhibitors of the EGFR (AG1478), and activated MEK1 but not by the presence of the PKA inhibitor H89 alone. These results indicate that more than one pathway is critical for the regulation of these genes because combinations of inhibitors were far more effective than any one inhibitor alone in blocking induced gene expression. Notably, combining H89 and AG1478 potently reduced expression of *Cyp11a1* and *Star* mRNAs whereas combining H89 with the p38MAPK inhibitor SB203580 did not differ from H89 alone. Thus, expression of *Cyp11a1* and *Star* appear to be regulated coordinantly by PKA and ERK1/2 pathways. These extend recent studies showing that the ERK1/2 pathway impacts expression of steroidogenic genes in more mature granulosa cells and cumulus oocyte complexes obtained from preovulatory follicles (24, 53–55). The EGFR pathway and ERK1/2 also impact the expression of other genes involved in granulosa cell differentiation to a luteal cell phenotype and include *Sgk,* a PKB-related kinase (13), and *Sfrp4,* a regulator of WNT/FRIZZLED signaling cascades (data not shown) (56). These results provide a mechanism to explain previous results showing that the tyrphostin AG18 blocked gonadotropin induction of steroidogenic enzymes and luteinization (57, 58). The results presented herein also support the recent studies by Hsieh *et al.* (59) who have shown that mice null for *Areg* and that also harbor a hypomorphic allele for the EGFR are subfertile due to altered ovarian responses to gonadotropin treatment. Specifically, ovaries of these mutant mice contain many nonovulated, nonluteinized follicles with reduced expression of *Cyp11a1* mRNA after equine CG and human CG treatments. Thus, it is tempting to speculate that the induction of the EGF-like factors by LH in preovulatory follicles and their activation of the EGFR impact genes controlling the luteinization process.

In contrast, the PKA inhibitor H89 alone reduced FSH-induced expression of *Cyp19a* mRNA, confirming that this kinase has a preeminent role in regulating transcription of this gene. However, the PKA-selective agonist 6-Bnz-cAMP alone did not induce expression of the *Cyp19a* mRNA in these cells. These results combined with the evidence that DN-RAS reduced FSH induction of *Cyp19a* mRNA indicate that PKA is unlikely to be the sole regulator of this gene in immature granulosa cells and may explain the delayed induction of this gene 24 h post FSH stimulation. Because AREG alone did not induce aromatase, it is likely that RAS or SFKs act on other signaling factors to impact expression of this gene. Potential targets of RAS or SRC include NR5a1 and/or GATA4 that are known to impact transactivation of the *Cyp19a* gene (10, 60). Thus, FSH-mediated transcription of the aromatase/*Cyp19* gene also appears to require multiple signaling events.

Although the cAMP-regulated guanine nucleotide exchange factor EPAC is highly expressed in granulosa cells and although FSH can activate RAP1 via EPAC, the downstream targets of this cell signaling system in these cells remain elusive. However, recent studies indicate that EPAC may play a role in cell migration and activation of specific genes via the activation of specific RAS-related factors, namely R-RAS (61) or by regulating ERK5 and phosphodiesterase4 (20). Although we had originally proposed that EPAC might be the key factor by which FSH mediates phosphorylation of PKB, the results obtained herein indicate that EPAC exerts a supportive but not primary role in this regard. Clearly, PKA, p38MAPK, and

ERK1/2 are not involved in FSH-mediated phosphorylation of PKB. Rather, FSH- and AREG-induced phosphorylation of PKB is mediated, in part, by SFK activities because the SFK inhibitor PP2, but not its inactive analog PP3, blocked both FSH- and AREG-induced phosphorylation of PKB and FOXO1a (Fig. 10). Moreover, PP2 partially blocked the FSH-mediated decrease in FOXO1a protein. Because FSH rapidly reduces FOXO1a mRNA in granulosa cells (62), this process may involve FSH activation of SFKs, leading to decreased FOXO1a protein. Alternatively, SFKs may enhance FOXO1a degradation via other pathways. Collectively, these data provide further evidence that SFKs are activated by FSH-liganded receptors and impact key steps in granulosa cell differentiation. Importantly, FSH- and AREG-mediated phosphorylation of PKB occurs independently of RAS activation (*i.e*. was not affected by DN-RAS) and was negatively regulated by EGFR tyrosine kinase activation. Thus, the liganded EGFR and liganded FSH receptor can both activate SFKs by mechanisms that are independent of RAS. These results provide supporting evidence that there are specific proteins that interact with these receptors at the cell surface. c-ABL may be one of the critical factors (50, 63).

In summary (Fig. 10), the results presented herein provide the novel evidence that FSH rapidly activates the small GTP-binding proteins RAP1 and RAS in immature, relatively undifferentiated granulosa cells. Whereas activation of RAP1 involves cAMP-mediated activation of EPAC, the mechanisms by which FSH activates RAS appear to involve multiple factors that are activated in a time-dependent manner. Most convincingly, we show that FSH rapidly increases ERK1/2 phosphorylation by mechanisms that include activation of SFKs, EGFR tyrosine kinase, and MEK1. Activation of the EGFR appears to be dependent in part on SFKs as well as on FSH-mediated induction/activation of the EGF-like factors, including AREG. Moreover, FSH activation of PKB and FOXO1a is also dependent on SFKs but is clearly independent of RAS. Lastly, we provide the evidence that inductions of AREG and EGFR activation are key events that enhance granulosa cell differentiation and the expression of some genes characteristic of the luteal cell phenotype. Thus, activation of the ERK pathway combined with activation of PKA and PKB appear to dictate terminal differentation of granulosa cells to a nondividing, highly steroidogenic state.

MATERIALS AND METHODS

Reagents

Media and cell culture reagents and materials were purchased from Invitrogen, Inc. (Carlsbad, CA), Sigma (St. Louis, MO), Research Organics (Cleveland, OH), Fisher Scientific (Fairlawn, NJ), Corning Inc. (Corning, NY), and HyClone Laboratories, Inc. (Logan, UT). Trypsin, soybean trypsin inhibitor, DNase, 17β-estradiol, 8-bromo-cAMP, and propylene glycol were all purchased from Sigma. Ovine FSH (oFSH-16) was a gift of the National Hormone and Pituitary Program (Rockville, MD). AREG (mouse recombinant, carrier free) was obtained from R&D Systems (Minneapolis, MN). Control and adenoviral vectors expressing DN-RAS were gifts from Dr. Luis Parada, University of Southwestern Medical School (Dallas, TX). An adenoviral vector expressing RAP1-GAPII was a gift from Dr. Naoki Mochizuki (Osaka, Japan). The EPAC-specific cAMP analog 8-pMeOPT-2′-O-Me-cAMP, 6-Bnz-cAMP, and 8-Br-cAMP were purchased from Biolog Life Science Institute (Bremen, Germany). Antibodies for phospho-MAPK/ERK1/2 (no. 9101), PKB (no. 9272), phospho-PKB (Ser 473) (no. 9271), FKHR(FOXO1a) (no. 9462) phospho-FKHR(FOXO1a) (no. 9461), phospho-p38-MAPK (Thr180/Tyr182) (no. 9211), c-ABL (no. 2862), and FYN (no. 4023) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-SRC antibody was from R&D Systems and antiphospho-SRC (Tyr418) was from Stressen Bioreagents (Ann Arbor, MI). Antibodies for RAS (no. 05-516) and RAP1 (no. 07-331) as well as the RAS and RAP pull-down kits were purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). Affinity-purified EPAC antibody was used as previously described. The CYP11a1 (P450scc) antibody was kindly provided Dr. Dale B. Hales (University of Illinois at Chicago). Inhibitors of selected kinases and receptors LY294002 (no. 440204), H89 (no. 371962), PKI (no. 476485), SB203580 (no. 559398), PD98059 (no. 513001), U0126 (no. 662005), AG1478 (no. 658548), PPP (no. 307247), picropodophyllin 2 (no. 407247), and 3 (no. 529573), and GM6001 (no. 364205) were purchased from Calbiochem (San Diego, CA). Qiagen RNAeasy minikits were used for RNA isolation (QIAGEN, Chatsworth, CA). Electrophoresis and molecular biology grade reagents were purchased from Sigma and Bio-Rad Laboratories, Inc. (Hercules, CA) and enhanced chemiluminescence (ECL) from Pierce Chemical Co. (Rockford, IL). Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). RT-PCR reagents were purchased from Promega Corp. (Madison, WI). α-^{32}P[dCTP] was obtained from ICN Radiochemicals (Costa Mesa, CA).

Animals

Immature (23 d of age) Sprague Dawley Holtzman female rats were obtained from Harlan, Inc., (Indianapolis, IN). Rats were injected sc with 0.2 ml estradiol (7.5 mg 17β-estradiol/1 ml propylene glycol) once daily for 3 d to stimulate the growth of large preantral follicles (9). Animals were housed under a 14-h light/10-h dark schedule in the Center for Comparative Medicine at Baylor College of Medicine and provided food and water *ad libitum*. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Baylor College of Medicine.

Primary Granulosa Cell Cultures

Granulosa cells were harvested from estrogen-primed immature rats as previously described (7, 64). Briefly, cells were cultured at a density of 1×10^6 cells in 2 ml serum-free medium (DMEM-F12 containing penicillin and streptomycin) in multiwell (35 mm) dishes that were serum coated (1% fetal calf serum). Cells were cultured in defined medium overnight (0 h) followed by the addition of FSH (100 ng/ml), forskolin (10 μM), and/or other agonists/inhibitors as indicated in the figures and figure legends. FSH was used to stimulate differentiation of the granulosa cells. 8-pCPT-2′-O-Me-cAMP, 8-pMeOPT-2′-*O*-Me-cAMP, 6-Bnz-cAMP, and 8Br-cAMP (all at 100 μM) were used to determine the relative effects of cAMP on specific cell functions. Cell cultures were pretreated with the inhibitors LY294002 (25 μM), H89 (10 μM), PKI (50 μM), SB203580 (20 μM), PD98059 (20 μM), U0126 (10 μM), AG1478 (10 μM), PPP (10 μM), PP2 and PP3 (10 μM), and

GM6001 (10 μM) for 1 h before agonist exposure unless otherwise noted.

RNA Isolation and RT-PCR Assay

Total RNA was isolated from granulosa cells using Qiagen RNAeasy Minikits according to specifications provided by the manufacturer. Specific primer pairs for each gene analyzed are listed in Table 1. Specific primer pairs for the cyclophilin B were used to determine relative levels of expression. For RT-PCRs, the reverse transcriptase step contained 500 ng of input RNA and AMV-RTase (Promega) was performed using the manufacturer's instructions. PCR amplification of each gene was performed as previously described (56, 65) (Table 1) using 2 μl (one tenth total volume) of the preceding reverse transcriptase step as a template for amplification reactions. The amplified cDNA products were resolved by acrylamide gel electrophoresis, and radiolabeled PCR products were quantified by phosphoimage analysis (Amersham Biosciences, Piscataway, NJ).

Cell Extracts

Total cell extracts were prepared according to the method of Ginty *et al*. (66) by adding 80 μl hot (100 C) Tris buffer containing 10% SDS and β-mercaptoethanol to each 35-mm well. The cells were rapidly scraped with a rubber policeman and the extract transferred to a microcentrifuge tube and heated at 100 C for 5 min. Extracts were stored at 4 C until analyzed by SDS-PAGE. Protein isolated from whole tissue was prepared by homogenization of tissues in whole cell extract buffer (10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 400 mM KCl, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, 1 mM vanadate, 1 mM diethyl dithiocarbamic acid, 0.1 mg/ml aprotinin) followed by centrifugation (1 min in microfuge) to isolate soluble protein. Soluble protein concentrations in each sample were determined by Bradford assay (Bio-Rad Laboratories, Inc.).

Table 1. Oligonucleotides and Protocols for RT-PCRs

Oligonucleotides, cycles of amplification, and annealing temperatures
Areg: SE: 5′-CTGGCAGTGAACTCTCCACA-3′ AS: 5′-CTTACGGCGGAGACAAAGAC-3′ – 28 cycles, 59 C
Cyp11a1: SE.: 5′-GTACTTGGGCTTTGGCTGGG-3′ AS: 5′-CAGGTCCTGCTTGAGAGGCT-3′ – 25 cycles, 60 C
Cyp19: SE: 5′-TGCACAGGCTCGAGTATTTCC-3′ AS: 5′-ATTTCCACAATGGGGCTGTCC-3′ – 26 cycles, 63 C
Pp1b: SE: 5′-AGAGCACCAAGACAGACAGC-3′ – 28 cycles, 59 C AS: 5′-TCTACTCCTTGGCAATGGC-3′
Star: SE: 5′- AGTGGAACCCAAATGTCA-3′ AS: 5′-CGAACTCTATCTGGGTCTG-3′ – 29 cycles, 55 C
Nr5a1: SE: 5′ CGAAGGTGCATGGTATTTAAGG-3′ AS: 5′-GTGTGGGTAGTGACACAAGGTG-3′ – 28 cycles, 59 C
Nr5a2: SE: 5′-TTGAAGTGTGAACCCGATGA-3′ AS: 5′-TGAAGGGAACGGAGTCTCAC-3′ – 28 cycles, 59 C

SE, Sense; AS, antisense; PCRs: 94 C for 2 min, 94 C for 30 sec, followed by a specific temperature (as indicated above), then 72 C for 30 sec repeated for the number of amplification cycles as indicated above, and lastly 15 C for at least 5 min.

Western Blotting

Total cell extracts or lysates were resolved using one-dimensional SDS-PAGE with 4.5% stacking and 10% separating acrylamide gels. Western blots were run with either 30 μg of whole cell extract protein for *in vivo* analysis, or 20 μl of cell extracts prepared by the Ginty method for *in vitro* experiments. Proteins were electrophoretically transferred to 0.45-mm Immobilon membranes, washed briefly in Tris-buffered saline (TBS), and then blocked for 1 h at room temperature in TBS and 0.1% Tween 20 (TBS-T) containing 5% milk. Membranes were incubated overnight at 4 C with 1:1000 diluted primary antibodies (unless otherwise noted in figure legends) in TBS-T containing 2.5% milk with constant agitation. The membranes were then washed three times (7 min) with TBS-T before treatment with a secondary antibody (1:10000 antirabbit-horseradish peroxidase unless otherwise noted in figure legends). After washing as described above, immunoreactive bands were visualized with ECL according to the manufacturer's instructions. Immunoreactive bands were quantified by image analysis using ImageQuant (Amersham Biosciences). Equal loading was determined by Ponceau-S staining after protein transfer.

Immunohistochemistry

Ovaries were collected and fixed in 4% paraformaldehyde (EMD Chemicals Inc., Gibbstown, NJ), dehydrated, embedded in paraffin, and sectioned following standard histological procedures. Sections (7 μm) were mounted on siliconized slides and were stained using a polyclonal antibody specific to EPAC (19) using a 1:50 dilution in 1% normal goat serum (NGS) in PBS. Nonspecific signals were blocked with 3% NGS in PBS. Signal detection was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with a goat antirabbit horseradish peroxidase-conjugated (1:200 dilution in 1% NGS in PBS) according to manufacturer's instruction.

Immunocytochemistry

Primary granulosa cells were plated on serum-coated coverslips in 24-mm wells and cultured overnight in defined medium. After hormone treatment for 24 h, the coverslips were washed once in PBS and the cells fixed in 4% formaldehyde in PBS for 30 min. After washing three times in PBS, the cells were permeabilized with 0.5% NP-40 in PBS for 20 min, washed, and blocked for 1 h with 5% BSA. Coverslips were then incubated overnight at 4 C with an anti-CYP11a1 polyclonal antibody (diluted 1:1500 in 5% BSA containing PBS). After PBS washes, goat antirabbit conjugate (Alexa Fluor 488; Molecular Probes, Inc., Eugene, OR) diluted 1:200 was used to visualize Cyp11a1. Coverslips were mounted on siliconized slides using Vectastain mounting solution with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, CA). Fluorescent images were captured using an Axioplan fluorescent microscope.

RAP1 and RAS Pulldown Assays

RAP1 and RAS pulldown assays were performed using RAP1 and RAS Activation Assay Kits (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, cultured cells were stimulated as described above and in the figure legends. After stimulation, cells were washed with ice-cold PBS and lysed, and the lysates were subjected to centrifu-

gation to clear insoluble cellular debris. Supernatants were incubated for 45 min with agarose slurries containing either a RAL GDS RAP1-binding domain (for RAP1) or RAF1 RAS-binding domain (for RAS) to bind activated RAP1 and RAS, respectively. The bound agarose beads were washed and then resuspended with 2× Laemmli reducing sample buffer. Samples were boiled for 5 min to release bound RAP1 or RAS before Western blot analysis.

Statistical Analysis

The data were represented as the arithmetic mean plus or minus SEM. Student's *t* test was performed using Prism 5 for Macintosh to analyze the significance of difference between the two groups being compared. $P < 0.05$ was considered significant.

Acknowledgments

We thank Dr. Luis Parada (University of Texas Southwestern Medical School) for the DN-RAS adenoviral vector and Dr. Dale B. Hales (University of Illinois at Chicago). We also thank Dr. Zhilin Lui for critical evaluation on the manuscript and Yuet Lo for her expert technical contributions to the studies.

Received January 12, 2007. Accepted May 21, 2007.

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This work was supported in part by the following: National Institutes of Health (NIH) Grant HD-16272 (to J.S.R.); National Research Service Award 5F32 HD045060 (to C.M.W.); and NIH Grant GM66170 (to X.C.).

Author Disclosure Summary: C.M.W., H.-Y.F., and X.C. have nothing to declare. J.S.R. is a consultant with Bayer Schering Pharma (Berlin, DE).

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