In this report we sought to elucidate the mechanism by which the follicle-stimulating hormone (FSH) receptor signals to promote activation of the p42/p44 extracellular signal-regulated protein kinases (ERKs) in granulosa cells. Results show that the ERK kinase MEK and upstream intermediates Raf-1, Ras, Src, and L-type Ca\textsuperscript{2+} channels are already partially activated in vehicle-treated cells and that FSH does not further activate them. This tonic stimulatory pathway appears to be restrained at the level of ERK by a 100-kDa phosphotyrosine phosphatase that associates with ERK in vehicle-treated cells and promotes dephosphorylation of its regulatory Tyr residue, resulting in ERK inactivation. FSH promotes the phosphorylation of this phosphotyrosine phosphatase and its dissociation from ERK, relieving ERK from inhibition and resulting in its activation by the tonic stimulatory pathway and consequent translocation to the nucleus. Consistent with this premise, FSH-stimulated ERK activation is inhibited by the cell-permeable protein kinase A-specific inhibitor peptide Myr-PKI as well as by inhibitors of MEK, Src, a Ca\textsuperscript{2+} channel blocker, and chelation of extracellular Ca\textsuperscript{2+}. These results suggest that FSH stimulates ERK activity in immature granulosa cells by relieving an inhibition imposed by a 100-kDa phosphotyrosine phosphatase.

The cytoplasmic p42/p44 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERKs) comprise a critical convergence point in the signaling pathways initiated by a variety of receptor agonists that promote cellular differentiation or proliferation. For the classic receptor tyrosine kinase-initiated pathway, growth factors like epidermal growth factor (EGF) induce the autophosphorylation of their receptors and create specific binding sites for Src homology 2-containing proteins such as Grb2 (1). Grb2 complexed to Sos associates with the receptor tyrosine kinase, and Sos stimulates GDP release from Ras, leading to Ras activation. Active Ras then binds to Raf-1, leading to its activation, and Raf-1 in turn catalyzes the serine phosphorylation and activation of the MAPK/ERK kinase MEK. MEK then catalyzes the phosphorylation of ERK on regulatory Thr and Tyr residues, resulting in ERK activation.

Guanine nucleotide-binding protein-coupled receptors (GPCRs) are also well known activators of ERK; however, there are a variety of pathways by which GPCRs promote ERK activation. Often, GPCRs such as those activated by lysophosphatic acid or angiotensin II promote the transactivation of a receptor tyrosine kinase as evidenced by its increased tyrosine phosphorylation (2). Receptor tyrosine kinase transactivation directs the tyrosine phosphorylation of adaptor proteins such as Shc, recruitment of the Grb2-Sos complex, and subsequent Ras activation. It is less clear how GPCRs promote the tyrosine phosphorylation of the receptor tyrosine kinase, although Src activation downstream of the G\textsubscript{6y} has been implicated in some cells (3, 4). For those GPCRs whose activated G\textsubscript{a} subunits promote increased intracellular Ca\textsuperscript{2+} and consequent activation of Pyk and Src leading to EGF receptor (EGFR) transactivation, Src appears to catalyze the tyrosine phosphorylation.

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of this receptor tyrosine kinase (5). GPCRs can also stimulate EGFR activation by stimulating the proteolytic cleavage and resulting release of the soluble EGFR ligand, heparin binding EGF (6).

The G protein-regulated second messenger cAMP has also been shown to both inhibit and activate ERKs, depending on the cell type. In cells where cAMP inhibits growth factor-stimulated cell proliferation and ERK activation, cAMP via PKA inhibits Raf-1 activity, although the relevant PKA substrate has been controversial (7, 8). A recent report shows that the selective PKA substrate in this pathway appears to be Src (9). In fibroblasts, PKA-catalyzed Src phosphorylation directs the activation of Rap1, which binds and sequesters Raf-1, thereby preventing Ras activation of Raf-1 (9). Conversely, in PC12 cells, cAMP stimulates differentiation, and in HEK293 cells transfected with the β2-adrenergic receptor, cAMP via PKA promotes Rap1 phosphorylation and activation of B-Raf, leading to MEK and ERK activation (10, 11). cAMP can also bind to and directly activate the Rap1 guanine nucleotide exchange factor EPAC independent of PKA (12, 13), leading to B-Raf and ERK activation (14). In melanocytes, where cAMP leads to cell differentiation, cAMP independent of PKA promotes Ras and B-Raf activation, leading to ERK activation independent of Rap1 and EPAC (15). Thus, depending on the cell type, cAMP appears to utilize a variety of pathways to modulate ERK activity.

Ovarian granulosa cells comprise a unique cellular model in which the majority of both the differentiation and proliferation responses to the agonist follicle-stimulating hormone (FSH) are mediated by cAMP (16). The FSH receptor is a seven-transmembrane GPCR coupled to adenyl cyclase (17) and is expressed exclusively on ovarian granulosa cells in female mammals (18). FSH stimulates both granulosa cell proliferation as well as differentiation to a preovulatory phenotype (16). Although the induction of cyclin D2 can be stimulated in primary granulosa cell cultures by cAMP (19), the proliferative response to FSH is poorly understood and likely includes a paracrine component from surrounding thecal cells since rat granulosa cells do not proliferate in serum-free media in the presence of FSH alone (16, 20). The differentiation response is readily induced in serum-free granulosa cells by FSH and is characterized by the induction of enzymes required for estrogen and progesterone biosynthesis, the luteinizing hormone receptor, the type II regulatory (RII) β subunit of PKA (16, 21), inhibin-α (22), and an A kinase-anchor protein AKAP20 (23). We have shown that FSH-stimulated activation of the immediate early genes c-Fos and serum glucose-corticoid kinase as well as inhibin-α are mediated in part via the apparently direct phosphorylation of histone H3 on Ser-10 by PKA (24). Additionally, FSH leads to ERK activation in target ovarian granulosa cells in an apparently PKA-dependent manner, based on the ability of the PKA inhibitor H89 to inhibit FSH-stimulated ERK activation (24, 25). Recent results using a transformed granulosa cell line support a role for PKA in FSH-stimulated ERK activation (26). However, the mechanism by which PKA leads to ERK activation in granulosa cells is not known.

In this investigation we sought to identify the cellular pathway by which FSH promotes ERK activation in primary granulosa cells. Results show that MEK, Raf-1, Ras, and L-type Ca2+ channels are already partially activated in vehicle-treated granulosa cells. This pathway appears to be restrained at the level of ERK by a 100-kDa phosphotyrosine phosphatase (PTP) that associates with ERK in vehicle-treated cells. FSH promotes the PKA-dependent phosphorylation of this PTP and its dissociation from ERK, leading to ERK activation and translocation to the nucleus. Consistent with this premise, FSH-stimulated ERK activation is inhibited by the cell-permeable PKA-specific inhibitor peptide PKI as well as by inhibitors of MEK, Src, EGFR tyrosine kinase activity, a Ca2+ channel blocker, and chelation of extracellular Ca2+ (26). These results suggest that FSH enhances ERK activity in immature granulosa cells by relieving an inhibition imposed by a 100-kDa PTP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ovine FSH (oFSH-19) was kindly provided by Dr. A. F. Parlow of the National Hormone and Pituitary Agency of the National Institute of Diabetes and Digestive and Kidney Diseases (Torrence, CA). The following were purchased. H89, AG1478, GF109203X, 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), nifedipine, PP1 (4-amino-5-[4-methylphenyl]-7-[3-(t-butyl)pyrazolo][3,4-d]pyrimidine), A23187, ionomycin, okadaic acid, and phorbol myristic acid (PMA) were from LC Laboratories (San Diego, CA); BayK8644, myristoylated PKA inhibitor (PKI) 14–22 amide, farnesyltransferase inhibitor 1, and calcium blocker, and chelation of extracellular Ca2+ were from Upstate Biotechnology (Lake Placid, NY); anti-MAPK/ERK phosphorylated on Thr-202 and Tyr-204 was from Promega (Madison, WI); anti-MAPK/ERK antibody was from Zymed Laboratories Inc. (San Francisco, CA); anti-protein kinase C (PKC) (α, anti-Raf1 (C-12), anti-B-Raf (C-19), anti-ERK2-agarose conjugate, anti-AKT, and anti-Rap1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-MEK phosphorylated on Ser-17 and Ser-21, total MEK, anti-AKT phosphorylated on Thr-389, and anti-histone H3 phosphorylated on Ser-10, anti-CREB phosphorylated on Ser-133, anti-phospho-Tyr, Raf-1 Ras binding domain (BD) and Raf GDS-Rap BD glutathione-agarose conjugate were from Transduction Laboratories (Lexington, KY); recombinant human EGF was from Intergen Co. (Purchase, NY); ADP-agarose was from Sigma; Trizol™ was from Invitrogen. All other chemicals were from sources previously described (28–30). The preimmune serum and polyclonal anti-PTP-RL antibody, directed to GST-mPTP-RL 147–549 (31), were kindly provided by Dr. Rafael Pulido. An affinity-purified monoclonal anti-PTPRβ7 was generated by the Ogata laboratory by immunizing mice with a maltose binding fusion protein containing residues 298–656 in the cytoplasmic portion of murine PTPRβ7. An antibody that recognizes active Src unphosphorylated on Tyr-532 was previously described (32).

**Granulosa Cell Culture, Immunofluorescence, and Western Blotting**—Granulosa cells were isolated from ovaries of 28-day-old Sprague-Dawley rats primed with subcutaneous injections of 1.5 mg of estradiol-17β on days 22–25 to promote growth of preantral follicles (23, 29). Cells were either plated on fibronectin-coated 33-mm plastic dishes (Falcon) at a density of ~5–10 x 10⁶ cells/dish in serum-free medium (29), as indicated, or on coverslips (for immunofluorescence) and treated with indicated additions ~20 h after plating. Treatments were terminated by aspirating medium and rinsing cells once with PBS. Total cell extracts were collected by scraping cells in 0.5 ml of SDS sample buffer (33) followed by heat denaturation. Protein concentrations were controlled by plating identical cell numbers per plate in each experiment then loading equal volumes of total cell extract per gel lane. Equal protein loading was confirmed by total ERK, PKCα, phosphatidylinositol 3-kinase, or Ponceau S staining as indicated. Collection of soluble cell extracts is described below. Granulosa cell proteins were separated by SDS-PAGE on 12% or 12% acrylamide in running gel (34) and transferred to Hybond C-extra nitrocellulose (Amersham Biosciences). Blots were incubated with primary antibody overnight at 4°C, and antigen-antibody complexes were detected by enhanced chemiluminescence (Amer sham Biosciences). For immunofluorescence, cells were treated as indicated, fixed with 3.7% formaldehyde, permeabilized with 1% Triton X-100 in PBS, washed, blocked for 1 h in 1% bovine serum albumin in PBS, incubated overnight at 4°C with anti-histone H3 phosphorylated on Ser-10 (1:100 dilution, monoclonal antibody from New England Biolabs/Cell Signaling) and anti-ERK phosphorylated on Thr-202 and Tyr-204 was from Promega (Madison, WI); anti-MAPK/ERK antibody was from Zymed Laboratories Inc. (San Francisco, MA); anti-StAR antibody was kindly provided by Dr. Dale Buchanan Hales (27); anti-Ras and -PKCδ antibodies were from Transduction Laboratories (Lexington, KY); recombinant human EGF was from Intergen Co. (Purchase, NY); ADP-agarose was from Sigma; Trizol™ was from Invitrogen. All other chemicals were from sources previously described (28–30). The preimmune serum and polyclonal anti-PTP-RL antibody, directed to GST-mPTP-RL 147–549 (31), were kindly provided by Dr. Rafael Pulido. An affinity-purified monoclonal anti-PTPRβ7 was generated by the Ogata laboratory by immunizing mice with a maltose binding fusion protein containing residues 298–656 in the cytoplasmic portion of murine PTPRβ7. An antibody that recognizes active Src unphosphorylated on Tyr-532 was previously described (32).
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shields™ mounting medium (Vector Laboratories, Burlingame, CA). Slides were analyzed by a Zeiss LFM 510 confocal microscope.

Rap Phosphorylation, Ras, Rap, and Raf Activation Assays, and Immunoprecipitations—Raf activity was measured by an immunocomplex kinase assay using purified His6-MEK1 as substrate (35). After treatment of cells (6 × 106 cells/dish) with vehicle, FSH, or EGF for 2 min, cells were lysed in buffer A (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 2 mM dithiothreitol, 0.23 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% Nonidet P-40, and 0.1% deoxycholate), and insoluble cell debris was removed by centrifugation at 15,000 × g for 2 min. Raf-1 and B-Raf were immunoprecipitated overnight from the soluble cell extract as protein A + Gagarose complexes. Immunoprecipitates were washed, bated for 20 min at 37°C in a 50-μl reaction mix containing 0.5 μg of His8-MEK1, 0.1 μg [γ−32P]ATP (10 μCi/tube), 15 mM MgCl2, 1 mM MnCl2, and 25 mM Tris-HCl, pH 7.4, followed by the addition of SDS sample buffer (33), heat denaturation, and SDS-PAGE.

To detect Rap1 phosphorylation, cells were incubated for 1 h in phosphate-free medium then overnight with 0.5 μCi of [32P]P/6 × 106 cells and treated as indicated. Cells were lysed in buffer B (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.23 mM PMSF, 5 μg/ml aprotinin, 1% Nonidet P-40, and 0.5% deoxycholate, 0.1% SDS). Insoluble cell debris was removed by centrifugation, and Rap1 was immunoprecipitated using anti-Rap1 antibody (Santa Cruz Biotechnology). Immunoprecipitates were washed, and immunoprecipitated Rap1 was mixed with SDS sample buffer and heat-denatured. After separation of proteins in the immunoprecipitated complex by SDS-PAGE, proteins were transferred to nitrocellulose and subjected to autoradiography and, after decay of radioactivity, to a Rap1 immunoblot.

To detect active Ras or Rap1, cells were treated as indicated, rinsed with phosphate-buffered saline, lysed in buffer C (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 10% glycerol, 25 mM NaF, 10 mM MgCl2, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) or buffer B containing protease inhibitors, respectively, and soluble extract was collected. The soluble extract was incubated with control GST plus glutathione-agarose, GST-tagged Raf-1 Ras BD, or Ras GDS-Rap BD glutathione-agarose complexes. Immunoprecipitates were washed, and immunoprecipitated Rap1 was mixed with SDS sample buffer and heat-denatured. After separation of proteins in the immunoprecipitated complex by SDS-PAGE, proteins were transferred to nitrocellulose and subjected to autoradiography and, after decay of radioactivity, to a Rap1 immunoblot.

For anti-ERK-agarose pull-downs from total ovarian extracts, soluble extracts enriched in PTP-SL, ovaries of 13 estrogen-treated rats (see Methods) were subjected to ultrasonic disruption, cell debris was removed by centrifugation, and the supernatant was mixed with 0.06 ml of ADP-agarose or anti-ERK-agarose complexes. Immunoprecipitates were washed, bated for 20 min at 37°C in a 50-μl reaction mix containing 0.5 μg of His8-MEK1, 0.1 μg [γ−32P]ATP (10 μCi/tube), 15 mM MgCl2, 1 mM MnCl2, and 25 mM Tris-HCl, pH 7.4, followed by the addition of SDS sample buffer (33), heat denaturation, and SDS-PAGE.

For Ca2+ channel immunoprecipitations, 24 × 106 cells were treated as indicated, rinsed with phosphate-buffered saline, and frozen at −70°C. Using an antibody (anti-FP1) that recognizes the 210-kDa α subunit of class C L-type Ca2+ channel independent of its phosphorylation state (12), the αc subunit was immunoprecipitated from cell extracts solubilized in buffer H (1% Triton X-100, 10 mM EDTA, 10 mM MgCl2, 25 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM p-nitrophenyl channel

Results were analyzed using Student’s t test (p ≤ 0.05) (42) and are presented as the means ± S.E. (n > 3) or as the means ± range (n = 2).

RESULTS

FSH-stimulated ERK Activation Is PKA-dependent—We first investigated the time course of FSH-dependent ERK activation in serum-free cultures of primary rat granulosa cells.
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**Fig. 1. Time course of FSH-stimulated phosphorylation of ERK and CREB and expression of StAR.** Granulosa cells were treated for the indicated times with 50 ng/ml FSH followed by preparation of total cell extracts, as described under "Experimental Procedures." After SDS-PAGE and transfer of proteins to nitrocellulose, blots were probed with the indicated antibodies to phosphorylated (PH) and/or total p42/p44 MAPK/ERK (ERK), PH-CREB, and StAR. Results are representative of two independent experiments. Results for PH-ERK and -CREB at times 0, 10 min and 1 and 4 h were previously reported (24).

ERK activation, identified by the phospho-specific ERK antibodies, was readily detected by 10 min and was reduced by 1 h after FSH addition (Fig. 1). ERK activity remained low at 48 and 72 h after FSH addition, when granulosa cells had differentiated to the mature phenotype, as evidenced by the induction of StAR (Fig. 1), the protein responsible for mobilizing cholesterol substrate for steroidogenesis (43). Increased phosphorylation of CREB on Ser-133, an established PKA target (44), was detected by 10 min and undetectable by 4 h post-FSH. For subsequent studies, cells were treated for 10 min to detect maximal ERK activation unless otherwise indicated.

To determine whether or not FSH-stimulated ERK activation was cAMP- and PKA-dependent, cells were treated with FSH and forskolin (Fig. 2A) or the cell-permeable cAMP analog CPT-cAMP (Fig. 2B) or with FSH in the absence and presence of the cell-permeable-selective PKA inhibitor peptide, Myr-PKI (Fig. 2C). FSH stimulated ERK phosphorylation 4.1 ± 0.7-fold (n = 6) (Fig. 2A). Forskolin and CPT-cAMP mimicked FSH and activated ERK 3.1 ± 0.7 (n = 5) and 5.7-fold, respectively. Myr-PKI reduced FSH-stimulated ERK activation by 53 ± 8% (n = 3). CREB phosphorylation in response to forskolin, CPT-cAMP, and the PKA inhibitor mirrored that of ERK. These results support our earlier studies (25) with the PKA inhibitor H89 (45, 46) and confirm that the majority of FSH-stimulated ERK activation in this granulosa cell model is PKA-dependent.

Treatment of granulosa cells with FSH for 30 min resulted in localization of the majority of phosphorylated/active ERK in the nucleus (Fig. 3e) in conjunction with phosphorylated histone H3 (Fig. 3f). Both ERK and histone H3 phosphorylations were abrogated by pretreatment of cells with Myr-PKI (Fig. 3, m and n). These results are consistent with the hypothesis that FSH stimulates ERK activation to promote changes in gene expression leading to granulosa cell differentiation.

Cell-permeable cAMP analogs are also known to mimic the long term responses to FSH, leading to granulosa cell differentiation (16). That PKA is also required for the differentiation of granulosa cells to a preovulatory phenotype is evidenced by the ability of the PKA inhibitor H89 (Fig. 4) and Myr-PKI (not shown) to block FSH-stimulated induction of AKAP80 (by 98 ± 1.5%, n = 2), H89 or Myr-PKI also inhibited the induction by FSH of StAR expression (not shown) and progesterone production (by 97.7 ± 2.3%, n = 2).

FSH-stimulated ERK phosphorylation as well as CREB phosphorylation was independent of the inhibitory G protein $G_i$, based on the inability of pertussis toxin, which inhibits receptor-stimulated $G_i$ activation, to modulate FSH-stimulated ERK activation (not shown). These results are consistent with a direct action of PKA activated downstream of the FSH receptor to modulate ERK activity rather than the pathway reported for the $\beta_2$-adrenergic receptor in which the PKA-phosphorylated $\beta_2$-adrenergic receptor preferentially promotes activation of $G_i$ and consequent ERK activation via Gi6 (47).

FSH-stimulated ERK Activation Is Dependent on MEK Activity, but FSH Does Not Stimulate MEK Activation—Because ERK activation requires the upstream kinase MEK, we utilized the MEK inhibitor PD98059 (48) to confirm that FSH-stimulated ERK activation also required MEK. Results showed that PD98059 fully blocked (by 100%, n = 2) acute FSH-stimulated ERK activation (Fig. 5A) but did not affect CREB phosphorylation (Fig. 5B), consistent with an earlier report (24). PD98059 also reduced the FSH-stimulated induction of AKAP80 with granulosa cell differentiation to a mature phenotype (Fig. 5C) by 90 ± 9% (n = 3) but did not consistently modulate progesterone (22 ± 12% inhibition, n = 5) or inhibit (3 ± 3% inhibition, n = 2) secretion or StAR expression (not shown) by these cells. However, when we evaluated the ability of FSH to stimulate MEK phosphorylation, results showed that MEK exhibited a detectable level of phosphorylation that was not enhanced by FSH or forskolin (10-min treatments) but was strongly increased by EGF (Fig. 5D) and the PKC activator PMA (Fig. 5E). Consistent with this result, a detailed time course of FSH treatment of granulosa cells confirmed equivalent MEK phosphorylation at time 0 (i.e., in the absence of FSH) and at 10–20 min post-FSH addition when FSH stimulated maximal ERK phosphorylation (Fig. 5F). These results show that FSH-stimulated ERK activation is dependent on MEK activation but that FSH does not stimulate MEK activation. However, mitogens like EGF or PMA that activate ERK through mechanisms independent of PKA promote strong MEK activation.

FSH Does Not Enhance B-Raf or Raf-1 Activities—The presence in granulosa cells of detectable MEK phosphorylation that was unaffected by FSH suggested that upstream enzymes in this pathway might also exhibit detectable activity in the absence of FSH. The activities of B-Raf and Raf-1 were evaluated in an immunocomplex kinase assay using recombinant Hs-$\alpha$-MEK1 as substrate. Results in Fig. 6A showed that neither B-Raf nor Raf-1 activities was increased by FSH. Moreover, although the commonly high basal level of B-Raf activity (49) was readily detected and not affected by EGF (compare lanes 1 and 3), Raf-1 activity was detected in vehicle-treated cells and was strongly enhanced by EGF (compare lanes 4 and 6). These results indicate that FSH does not stimulate B-Raf or Raf-1 activation. Rather, a basal level of activity for both kinases was detected.

FSH actually stimulated the phosphorylation of Raf-1 on Ser-259 (Fig. 5F), an established inhibitory phosphorylation site (14, 50). Coincident with the inhibition of Raf-1 activity upon its phosphorylation on Ser-259, both MEK and ERK phosphorylations decreased beginning ~30 min post-FSH addition (see Fig. 5F). Based on evidence (a) that Raf-1 is phosphorylated on Ser-259 by AKT (50), (b) that FSH stimulates AKT phosphorylation/activation (Ref. 53 and Fig. 5G), and (c) that FSH-stimulated Raf-1 phosphorylation is inhibited by the phosphatidylinositol 3-kinase inhibitor wortmannin (not shown), we can conclude that FSH-stimulated Raf-1 phosphorylation is most likely downstream of FSH-stimulated AKT.

FSH Does Not Activate Ras or Rap1—We also evaluated the activation state of the upstream activators Ras and Rap1 in vehicle- and FSH-treated cells. Active Ras, indicated by Ras-GTP binding to the Ras BD on Raf-1 (14), was readily detected in vehicle-treated granulosa cells (Fig. 6B, lane 1). FSH did not increase the amount of Ras bound to the Ras BD of Raf-1 (Fig. 6B) (the reduced binding of Ras to the Ras BD of Raf-1 in...
response to FSH was not a consistent observation). Inhibition of the farnesylation of Ras by the cell-permeable farnesyltransferase inhibitor 1, resulting in reduced Ras but not Rap-1 function (54), also reduced FSH-dependent ERK activation by 66% (n = 2) (Fig. 6C), consistent with an obligatory role for Ras in FSH-stimulated ERK activation. Although direct activation by cAMP of the guanine nucleotide exchange factors for Rap1 cannot explain PKA-dependent ERK activation in granulosa cells especially in the absence of detectable FSH-stimulated B-Raf activation (12, 13, 55), PKA is reported to phosphorylate Rap1 to promote its activation (57). To determine whether Rap1 was phosphorylated in response to FSH treatment granulosa cell ATP pools were labeled with 32P, and cells were pretreated with or without the PKA inhibitor H89 and then treated with vehicle or FSH for 10 min followed by a Rap1 immunoprecipitation. Results showed that although Rap1 was readily immunoprecipitated (Fig. 6D, lower panel), phosphorylated Rap1 was not detected in vehicle or FSH-treated granulosa cells (Fig. 6D, upper panel). Moreover, FSH did not stimulate the GTP loading (activation) of Rap1, as evidenced (Fig.
by the equivalent binding of GTP-Rap1 to the Ral GDS-Rap BD in vehicle and FSH-treated granulosa cells. Taken together, these results suggest the existence of a tonic stimulatory pathway leading to modest activation of Rap1, Ras, Raf-1, and MEK in vehicle-treated granulosa cells. In the following experiments, we seek to identify upstream components of this tonic pathway.

**Upstream Components of the Tonic Pathway Leading to MEK Activation Include Src, the EGFR, and Extracellular Ca2**

We initially determined whether Src activity contributed to FSH-stimulated ERK activation. Pretreatment of cells with the Src inhibitor PP1 completely blocked FSH-stimulated ERK activation but did not affect CREB phosphorylation (Fig. 7A). Blots probed with an antibody that detects active Src by recognizing unphosphorylated Tyr-532 (designated "dephospho-Src antibody") (32) showed that Src activity was readily detected in untreated granulosa cells (Fig. 7B, lane 1) and not further activated at 10-min post-FSH (Fig. 7B, lane 2) or at earlier or later FSH treatment times (Fig. 5F). Consistent with this result, the general tyrosine kinase inhibitor genistein completely blocked FSH-stimulated ERK phosphorylation (Fig. 8A). The EGFR-selective tyrosine kinase inhibitor AG1478 (58) also completely prevented FSH-, forskolin-, and EGF-stimulated ERK phosphorylation, whereas only EGF-stimulated CREB phosphorylation was blocked by AG1478 (Fig. 8B). The effectiveness of AG1478 to inhibit acute EGF-stimulated tyrosine phosphorylation of the EGFR is shown in Fig. 8C (compare lanes 4 and 8). Inhibition of the tyrosine kinase activity of nerve growth factor receptor by AG879 did not affect FSH-stimulated ERK activation (not shown). However, although a basal level of EGFR tyrosine phosphorylation was detected in vehicle-treated cells and EGF stimulated the tyrosine phosphorylation of the EGFR (Fig. 8C), as detected in a membrane pellet fraction obtained from 106 cells, we could not detect any transactivation of the EGFR by FSH or forskolin (Fig. 8C, compare lanes 1–3). Equivalent results were obtained when the EGFR was immunoprecipitated and probed with phospho-Tyr antibody and when tyrosine phosphorylated proteins were immunoprecipitated with phospho-Tyr antibody and probed with EGFR antibody (not shown). The EGFR inhibitor AG1478 also reduced the FSH-stimulated induction of AKAP80 by 90% (n = 2) (not shown) but did not affect FSH-stimulated progesterone secretion (8 ± 8% inhibition, n = 2). These results suggest that EGFR activation is obligatory for FSH-stimulated ERK activation.
To ascertain whether Src was upstream or downstream of EGFR activity, we determined whether the EGFR inhibitor AG1478 inhibited Src activity using the dephospho-Src antibody (which detects active Src) to probe blots of cell extracts treated with vehicle or FSH (Fig. 8D). Results showed that the dephospho-Src signal was not reduced by AG1478, in contrast to FSH-stimulated ERK phosphorylation, which was reduced by 85 ± 12% (n = 3). Rather, levels of active Src were elevated in cells pretreated with AG1478. The basis for this elevation in the active Src signal is not known. This result suggests that Src activity lies upstream rather than downstream of the EGFR, leading to FSH-stimulated ERK activation.

We also determined whether extracellular Ca\(^{2+}\) contributed to the ability of FSH to stimulate ERK phosphorylation. Depletion of extracellular Ca\(^{2+}\) by EGTA strongly reduced FSH-stimulated ERK phosphorylation (by 86 ± 5%, n = 4), whereas the effect on CREB phosphorylation was variable (30 ± 20%, n = 2) (Fig. 9A). The Ca\(^{2+}\) ionophores A23187 (Fig. 8D) and ionomycin (Fig. 9B) also promoted ERK phosphorylation. To determine whether Ca\(^{2+}\) entry was mediated via Ca\(^{2+}\) channels, cells were pretreated with the Ca\(^{2+}\) channel inhibitor nifedipine. Results (Fig. 9C) showed that nifedipine reduced both basal and FSH-stimulated ERK phosphorylation (by 97 ± 2.5%, n = 2) but not affect CREB phosphorylation. Consistent with a role for Ca\(^{2+}\) entry into granulosa cells via a Ca\(^{2+}\) channel, we determined whether FSH stimulated the phosphorylation on Ser-1928 and the resulting activation of the α subunit of class C L-type Ca\(^{2+}\) channel (39). To this end, cells were treated for 10 min with vehicle or FSH, and the α\(_{1C}\) subunit of the L-type Ca\(^{2+}\) channel was immunoprecipitated from cell extracts using an antibody (anti-FP1) that recognizes total α\(_{1C}\) subunit L-type Ca\(^{2+}\) channel independent of its phos-
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Fig. 8. FSH-stimulated ERK activation is dependent on EGFR activity. In panel A, granulosa cells were preincubated for 30 min with vehicle (Veh) or 30 μg/ml genistein then treated for 10 min with vehicle or 50 ng/ml FSH. Total cell extracts were probed with the indicated antibodies. Results are representative of two experiments. PH, phosphorlated. In panel B, granulosa cells were preincubated for 15 min with vehicle or 250 nM AG1478 then treated for 10 min with vehicle, 50 ng/ml FSH, 10 μM forskolin, or 25 nM EGFR. Total cell extracts were probed with the indicated antibodies. Results are representative of more than five experiments. In panel C, granulosa cells were preincubated 15 min without or with 250 nM AG1478 and then treated for 5 min with 50 ng/ml FSH, 10 μM forskolin, or 25 nM EGFR. Cells were separated in (detergent-free) buffer E, and a membrane pellet was collected, as detailed under “Experimental Procedures.” Proteins in the total pellet fraction were separated by SDS-PAGE, blotted to nitrocellulose, and probed with phospho-Tyr (PH-Y) antibody. Results are representative of three experiments. In panel D, granulosa cells were pretreated 15 min with vehicle or 250 nM AG1478 then treated for 10 min with vehicle, 50 ng/ml FSH, or 125 μM A23187 (A23). Blots were probed with the indicated antibodies. Results are representative of three experiments.

Phosphorylation state. The resulting blots were probed first with an affinity-purified antibody that detects α1C subunit phosphorylated at Ser-1928 (anti-CH3P), stripped, and reprobed with an antibody that detects total α1C subunit protein at 210 kDa (anti-FP1) (39). Results showed that the α1C subunit L-type Ca2+ channel was already phosphorylated in vehicle-treated granulosa cells and that FSH did not enhance its phosphorylation (Fig. 9D). Consistent with results that suggest that L-type Ca2+ channels are phosphorylated and open in vehicle-treated cells, the L-type Ca2+ channel agonist BayK8644 did not enhance ERK phosphorylation over levels in vehicle-treated cells (Fig. 9E).

The requirement for Ca2+ in the pathway by which FSH stimulates ERK phosphorylation was shown to be independent of PKC based on the inability of the PKC inhibitor GF109203X to inhibit FSH-stimulated ERK phosphorylation (Fig. 9F). The effectiveness of this inhibitor is evidenced by its ability to prevent PMA-stimulated ERK phosphorylation.

We next determined whether the effect of Ca2+ on FSH-stimulated ERK activation was upstream or downstream of EGFR activity. As shown in Fig. 8D, pretreatment of cells with the EGFR inhibitor AG1478 blocked the ability of the Ca2+ ionophore A23187 to stimulate ERK phosphorylation (by 86 ± 6%, n = 4), suggesting that Ca2+ is upstream of the EGFR. We also determined whether the Ca2+ effect was upstream or downstream of Src. Pretreatment of cells with the Src inhibitor PP1 reduced the ability of the Ca2+ ionophore A23187 to stimulate ERK phosphorylation (by 75%, Fig. 9G), suggesting that the Ca2+ signal is also upstream of Src. CREB phosphorylation, however, was not affected by PP1.

Taken together, these results suggest the existence of a tonic stimulatory pathway leading to MEK activation in (serum-free) vehicle-treated granulosa cells. That FSH can stimulate ERK phosphorylation suggests that MEK-stimulated ERK activation must be suppressed in granulosa cells and that FSH must in some manner overcome this inhibition. In the following experiments, we investigated the phosho tyrosine phosphatase PTP-SL (60). This phosphatase has been shown to bind to and inactivate ERK by stimulating the dephosphorylation of the regulatory Tyr residue (Tyr-204 in ERK1; Tyr-185 in ERK2) (31, 61). Phosphorylation of PTP-SL by PKA on Ser-231 in the kinase interaction motif inhibits its ability to bind to and consequently catalyze the dephosphorylation of ERK (61, 62).

A 100-kDa PTPs Associated with ERK and FSH Stimulates Its Phosphorylation—We first determined whether or not PTP-SL was expressed in rat ovaries. Proteins reactive with anti-PTP-SL antibody were readily detected at ~220, 180, 100, and 66 kDa in total granulosa cell extracts (Fig. 10A, lane 1). Detergent-soluble rat brain (Fig. 10A, lane 2) and ovarian (Fig. 10B, lane 2) extracts exhibited prominent anti-PTP-SL-reactive bands at 100 and 66 kDa that were absent when the blot was probed with preimmune serum (Fig. 10B, lane 1). The 100-kDa anti-PTP-SL-reactive band was retained in a soluble ovarian extract eluted from DEAE-cellulose with ~0.1–0.15 M salt (Fig. 10C, lane 1) but was undetectable in the 0.18–0.25 M salt DEAE eluate (lane 2). This result suggests that the 100-kDa anti-PTP-SL-reactive band is localized to the cytosolic fraction of granulosa cells.

We determined whether the soluble 100-kDa anti-PTP-SL-reactive band exhibited PTP activity. Using the same DEAE
extracts as those blotted in Fig. 10C, lanes 1 and 2, we performed an in-gel PTP assay in which a $^{32}$P-labeled tyrosine-phosphorylated substrate was incorporated into the gel. Results confirmed the presence of PTP activity specifically at 100 kDa only in the samples containing anti-PTP-SL reactivity (Fig. 10C, lanes 3 and 4).

PTP-SL along with striatal-enriched protein-tyrosine phosphatase and hematopoietic-PTP belong to a subfamily of PTPs grouped on the basis of sequence conservation and regulation of ERK activity (31, 60, 63–65). PTP-SL and striatal-enriched protein-tyrosine phosphatase exist as both membrane and cytosolic forms that are produced by alternative splicing of the two precursor genes (60, 65). PTP-SL, brain-enriched PTPBR7, and the PC12-enriched PC12-PTP are all isoforms of the PTP-SL gene produced by alternative splicing (60, 64, 66). However, the molecular masses for all the identified family members are <80 kDa. For example, reported sizes for these proteins are ~39 kDa for cytosolic PC12-PTP (66), ~45 kDa for cytosolic PTP-SL, and ~65–80 kDa for membrane-bound PTP-SL and PTPBR7, respectively (31, 67). Although the ~66-kDa band detected by anti-PTP-SL antibody (Fig. 10, A and B) might correspond to the membrane-bound isoform of PTP-SL, the abundant signal at 100 kDa in rat ovarian and brain extracts is either a new PTP-SL isoform or the product of a closely related but distinct gene.³ We therefore determined by Northern blot whether a larger PTP-SL transcript was detected in rat ovarian and brain extracts. The largest mRNA reported for PTP-SL is ~4 kilobases (60). Using a cDNA corresponding to amino acid residues 147–288 of PTP-SL, we detected a weak signal of ~8.6 kilobases in rat brain and ovarian extracts under low stringency washing conditions (Fig. 10D). This result is consistent with the likely presence of a larger PTP-SL-like transcript in rat ovaries and brain. Taken together, these results show that a 100-kDa PTP-SL-like PTP is present in soluble ovarian extracts.

In the following experiments we determined whether one of the anti-PTP-SL-reactive proteins in ovarian extracts was complexed with ERK. We first evaluated whether ovarian PTP-SL-reactive proteins were pulled down by anti-ERK-agarose. Using a detergent-solubilized ovarian extract (with anti-PTP-SL reactivity at 66 and 100 kDa), the 100-kDa PTP was selectively immunoprecipitated by anti-ERK agarose and did not bind to control ADP-agarose conjugates (Fig. 11A). Most of the 100-

³ Neither the 66- nor the 100-kDa proteins in total granulosa cell extracts is recognized by anti-PTPBR7, whereas this antibody detected a predominant ~70-kDa band in rat brain likely corresponding to PTPBR7.
kDa PTP contained in soluble ovarian extracts (which exhibited anti-PTP-SL reactivity only at 100 kDa) bound to anti-ERK-agarose (Fig. 11B, lane 5). The 100-kDa PTP was only minimally detected in the anti-ERK-agarose flow-through fraction (lane 3). In contrast, all of the 100-kDa PTP was present in the ADP-agarose flow-through (Fig. 11B, lane 2), and none was detected in the ADP-agarose eluate (lane 4). Consistent with these results, total ERK protein was readily detected in the flow-through fraction from ADP-agarose (lane 2), and total ERK protein was only detected in anti-ERK-agarose eluate (lane 5) and not in ADP-agarose eluate (lane 4). Thus, the majority of the ovarian 100-kDa PTP appears to be complexed with ERK.

We next determined whether the 100-kDa PTP was phosphorylated in response to FSH. Granulosa cells in which the cellular ATP pools with labeled 32P were treated with vehicle or FSH for 15 min then subjected to immunoprecipitation with anti-PTP-SL or control antibody. Results in Fig. 11C demonstrated increased phosphorylation of a band at 100 kDa in PTP-SL immunoprecipitates from FSH-treated cells. Increased phosphorylation of the 100-kDa band was not detected in control anti-PTP-SL immunoprecipitations (Fig. 11C). Phosphorylation of the 100-kDa band in PTP-SL immunoprecipitates was stimulated by forskolin and blocked by pretreatment of cells with the PKA inhibitor H89 (Fig. 11D). These results suggest that FSH via PKA promotes the phosphorylation of the 100-kDa PTP in granulosa cells.

Finally, we determined whether FSH stimulated the dissociation of the 100-kDa PTP from ERK. Granulosa cells were treated for 10 min with vehicle or FSH. Detergent-enriched cell extracts were then subjected to anti-ERK-agarose pull-downs followed by a PTP-SL Western blot. Results showed that the PTP signal at 100 kDa was reduced by 57% in FSH compared with vehicle-treated cells (Fig. 11E). These results show that FSH promotes the release of a portion of the ERK from the 100-kDa PTP.

**DISCUSSION**

FSH is obligatory for follicular development beyond the preantral stage (68). It is well established that cAMP mediates FSH-dependent induction of granulosa cell differentiation to a preovulatory phenotype (16). Among the genes induced include those for increased steroidogenesis including P450(O aromatas and P450(B chain cleavage, membrane receptors including those for luteinizing hormone and prolactin (16), signaling and anchoring proteins such as RIIβ and AKAP80 (23), and hormones such as inhibin-α (22). As depicted in Fig. 12, FSH via cAMP promotes activation of PKA (29), leading to the translocation of the PKA catalytic subunit to the nucleus (29, 69), and phosphorylation of CREB (24, 70). FSH via PKA also promotes phosphorylation of histone H3 (24, 29) and induction of the immediate early genes serum glucocorticoid kinase (71) and c-Fos (72). Because the majority of the actions of cAMP are mediated by PKA, it has been assumed that the actions of FSH to induce genes leading to the preovulatory phenotype are dependent on PKA. In support of this hypothesis, we previously reported that the association of phosphorylated histone H3 with promoters of serum glucocorticoid kinase, inhibin-α, and c-Fos was blocked by the PKA inhibitor H89 (24). Induction of AKAP80 as well as StAR expression and inhibin-α and progesterone secretion are also inhibited by the PKA inhibitor H89 (see “Results”).

FSH also promotes the activation of ERK (24–26) and down-
stream RSK2 (24, 25). The PKA dependence of ERK activation in response to FSH in primary granulosa cells was conclusively established in this report. The goal of these studies was to identify the cellular mechanism by which PKA, activated downstream of the FSH receptor, promoted ERK activation. For these studies we used a serum-free primary granulosa cell culture model to identify the signaling pathway by which FSH activates ERK. Utilizing this model, our results show that ERK activation appears to be obligatory for the induction of AKAP80, based on the ability of the MEK inhibitor PD98059 to block this response to FSH. ERK activation, however, does not appear to be necessary for other PKA-mediated responses of granulosa cells to FSH, such as progesterone and inhibin secretion. PKA-dependent histone H3 phosphorylation is also independent of ERK activation (24).

Our results support the existence of a tonic stimulatory pathway leading to partial MEK but not ERK activation in (serum-free) vehicle-treated granulosa cells. This conclusion is based on evidence that FSH does not promote the activation of MEK, yet MEK inhibition with PD98059 blocks the ability of FSH to activate ERK (shown herein) as well as downstream RSK2 (24). We ruled out the well described pathways by which cAMP/PKA stimulates Rap1 activation leading to activation of B-Raf, MEK, and ERK (10, 11, 55) based on the inability of FSH to activate Rap1, B-Raf, or MEK (Fig. 12). Rather, MEK as well as the upstream activator Raf-1 exhibit detectable activity in the absence of FSH, and inhibition of Ras actions inhibits FSH-stimulated ERK activation. This tonic pathway leading to Ras activation appears to include Ca²⁺ entry, at least in part, through phosphorylated and activated L-type Ca²⁺ channels, leading to the activation of Src, the EGFR, and Ras followed by activation of Raf-1 and MEK (Fig. 12). However, we do not know what stimulates phosphorylation of the Ca²⁺ channel and consequent Ca²⁺ entry into granulosa cells. Notably, mitogens like EGF and PMA that activate ERK independent of PKA promote strong activation of MEK.

We have shown that granulosa cell extracts express a soluble 100-kDa PTP that is recognized by an antibody developed against PTP-SE. Moreover, a portion of the ovarian ERK is complexed with the 100-kDa PTP, based on the ability of anti-ERK-agarose to selectively pull-down ERK complexed to this PTP. PTP-SE has been shown to complex with ERK in other cell types, resulting in ERK dephosphorylation on the regulatory Tyr residue (Tyr-204 in ERK1) and consequent ERK inactivation (31, 61). It has also been established that PTP-SE is a PKA substrate, phosphorylated on Ser-231 (62). Similarly, striatal-enriched protein-tyrosine phosphatase and hematopoietic PTP, two other members of this subfamily of PTPs, are both

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**PKA-dependent ERK Activation**

Fig. 11. FSH stimulates the phosphorylation of the 100-kDa PTP in granulosa cells. In panel A, ovarian extracts (1.36 mg protein) in (detergent-enriched) buffer A were incubated with control ADP-agarose or anti-ERK agarose. Agarose pellets were washed, heat-denatured, and subjected to SDS-PAGE, blotted to nitrocellulose, and probed with anti-PTP-SL antibody. Agarose pellets were washed, heat-denatured, and subjected to SDS-PAGE, blotted to nitrocellulose, and probed with anti-PTP-SL antibody. Results are representative of two experiments. IB, immunoblot. In panel B, soluble ovarian extracts were prepared as described in the legend to Fig. 10C in detergent-free buffer D, loaded onto a DEAE-cellulose column, and batch-eluted with 0.15 M potassium phosphate; the eluate was concentrated to 1.15 ml. After removing an aliquot (Input), equal volumes of concentrated eluate were mixed overnight with control ADP-agarose and anti-ERK agarose. The flow-through (FT) that did not bind to agarose was collected, agarose was washed, and bound proteins eluted with SDS-sample buffer, as detailed under “Experimental Procedures.” After SDS-PAGE and blotting to nitrocellulose, blots were probed with the indicated antibodies. The percentage of original input volume mixed with agarose conjugates that was loaded onto SDS-PAGE is indicated. Results are representative of two experiments. In panels C and D, cellular ATP pools were prelabeled with 32Pi, and granulosa cells were pretreated (panel D) for 60 min with vehicle (Veh) or 10 μM H89 and then treated for 15 min with vehicle or 50 ng/ml FSH. Cell extracts were prepared in detergent-enriched buffer F and then subjected to immunoprecipitation (IP) with the indicated antibodies (Ab), as detailed under “Experimental Procedures.” After SDS-PAGE, gels were dried and exposed to film. Results in each panel are representative of at least two separate experiments. In panel E, granulosa cells were pretreated for 15 min with 1 μM okadaic acid, a Ser/Thr protein phosphatase 2A preferential inhibitor (56), then treated for 10 min with vehicle or FSH, sonicated for 1 min in (detergent-enriched) buffer F, and sonicate was centrifuged at 15,000 × g for 5 min, and the supernatant was mixed with 0.06 ml of anti-ERK-agarose for 4 h at 4°C. IB, immunoblot. For the rest of details see the panel A legend. Results are representative of two separate experiments.
PKA-dependent ERK Activation

...pathway leading to MEK activation appears to be initiated by the entry of extracellular Ca²⁺. Because FSH has been shown to increase the entry of Ca²⁺ into granulosa cells (52), one would predict that ERK would remain active as a result of the positive feedback actions of Ca²⁺ coupled with actions of PKA to maintain the 100-kDa PTP in a phosphorylated conformation. However, FSH-stimulated ERK activation is transient (see Fig. 5F). This is in part attributable to the phosphorylation of Raf-1 on Ser-259 most likely by AKT (50) activated in response to FSH, resulting in the inactivation of Raf-1 (14, 50) and consequent reduction in the phosphorylation of MEK.

The ability of EGTA, the L-type Ca²⁺ channel blocker nifedipine, the Src inhibitor PP1, the EGFR tyrosine kinase inhibitor AG1478, and the Ras inhibitor to completely prevent FSH-stimulated ERK activation indicates that the tonic pathway involving extracellular Ca²⁺, Src, the EGFR, and Ras is the predominant route to the ERK regulated by FSH in granulosa cells rather than an alternative pathway such as one involving Rap1. This conclusion is substantiated by our inability to detect enhanced MEK phosphorylation in response to FSH.

This is the first report demonstrating agonist-stimulated modulation of ERK activity in conjuction with agonist-stimulated PKA-dependent phosphorylation of a PTP in a physiological cell model. Thus, these studies support a physiological role for this PTP in regulating ERK activity in an intact cellular model in response to an extracellular ligand.

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