Insulin Receptor Substrate 1, the Hub Linking Follicle-stimulating Hormone to Phosphatidylinositol 3-Kinase Activation*

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The ubiquitous phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates many cellular functions. However, the mechanism by which G protein-coupled receptors (GPCRs) signal to activate PI3K is poorly understood. We have used ovarian granulosa cells as a model to investigate this pathway, based on evidence that the GPCR agonist follicle-stimulating hormone (FSH) promotes the protein kinase A (PKA)-dependent phosphorylation of insulin receptor substrate 1 (IRS1) on tyrosine residues that activate PI3K. We report that in the absence of FSH, granulosa cells secrete a subthreshold concentration of insulin-like growth factor-1 (IGF-1) that primes the IGF-1 receptor (IGF-1R) but fails to promote tyrosine phosphorylation of IRS1. FSH via PKA acts to sensitize IRS1 to the tyrosine kinase activity of the IGF-1R by activating protein phosphatase 1 (PP1) to promote dephosphorylation of inhibitory Ser/Thr residues on IRS1, including Ser*789. Knockdown of PP1β blocks the ability of FSH to activate PI3K in the presence of endogenous IGF-1. Activation of PI3K thus requires both PKA-mediated relief of IRS1 inhibition and IGF-1R-dependent tyrosine phosphorylation of IRS1. Treatment with FSH and increasing concentrations of exogenous IGF-1 triggers synergistic IRS1 tyrosine phosphorylation at PI3K-activating residues that persists downstream through protein kinase B (AKT) and FOXO1 (forkhead box protein O1) to drive synergistic expression of genes that underlies follicle maturation. Based on the ability of GPCR agonists to synergize with IGFs to enhance gene expression in other cell types, PP1 activation to relieve IRS1 inhibition may be a more general mechanism by which GPCRs act with the IGF-1R to activate PI3K/AKT.

The phosphatidylinositol-3 kinase (PI3K) signaling pathway regulates transcription, translation, proliferation, and apoptosis (1, 2). Whereas PI3K is classically activated by receptor tyrosine kinases, such as the insulin-like growth factor-1 receptor (IGF-1R), PI3K is also activated in many cells by G-protein-coupled receptors (GPCRs). However, the mechanisms by which GPCRs signal to activate PI3K are much less understood compared with classical activation by receptor tyrosine kinases (1). We have used rat ovarian granulosa cells (GCs) as a model to elucidate the mechanism by which the GPCR agonist follicle-stimulating hormone (FSH) activates PI3K, based on prior evidence that FSH activates PI3K in a protein kinase A (PKA)-dependent manner (3).

FSH is an obligatory regulator of ovarian follicle maturation. During the menstrual cycle, preantral follicles that encompass developing oocytes mature to a preovulatory stage in response to elevated levels of FSH (4). Administration of exogenous FSH also restores follicle development in anovulatory women (5). Finally, mice harboring null alleles for either the FSH receptor or FSHβ lack preovulatory follicles and are infertile (6, 7). Traditionally, FSH has thus been considered both necessary and sufficient to promote follicle maturation.

FSH acts exclusively on GCs within follicles to regulate the expression of ~500 genes (8) through both relief of repression and activation via numerous transcription factors (9–17). FSH binds its GPCR, leading to the activation of PKA via adenylyl cyclase-mediated cAMP production (18). Transcriptional responses to FSH are dependent upon activation of PKA, based on the ability of the catalytic inhibitor PKI or H89 to inhibit target gene expression in cultured GCs (9, 13, 19, 20). Likewise, lentiviral expression of a constitutively active PKA catalytic subunit mutant (PKA-CQR) closely mimics gene expression patterns of FSH (21), suggesting that PKA is necessary and sufficient for most transcriptional actions of FSH.

It has been known for some time that Igf1-null mice, like FSH knock-out mice, lack preovulatory follicles and are infertile despite a normal complement of primordial follicles (22). Recent studies have shown that IGF-1 (in rodents) (23) or IGF-2 (in humans) (24) is produced by cultured GCs and is necessary for FSH-dependent target gene expression. IGF-1 and IGF-2 share commonality in transducing intracellular signals through the IGF-1R in GCs (23). Classically, the IGF-1R phosphorylates adapter proteins, such as insulin receptor substrate 1 (IRS1), to facilitate activation of downstream targets (25). IRS1 is tyrosine-phosphorylated by the IGF-1R within Tyr*-Met-X-

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2 The abbreviations used are: IGF-1R, insulin-like growth factor-1 receptor; IGF-1 and -2, insulin-like growth factor-1 and -2, respectively; IRS1, insulin receptor substrate 1; GC, granulosa cell; AKT, protein kinase B; GPCR, G protein-coupled receptor; PP1 and PP2, protein phosphatase 1 and 2, respectively; LH, luteinizing hormone; FOXO1, forkhead box protein O1; CREB, cAMP-response element-binding protein; PKI, PKA-inhibitory peptide; Myr-PKI, myristoylated PKI; qPCR, quantitative PCR; ANOVA, analysis of variance; p-PKA, phospho-PKA; MLC, myosin light chain.
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Met (Y*XM) motifs (where an asterisk denotes a phosphorylation residue) that are then bound by PI3K, leading to its allosteric activation (26, 27). Downstream of PI3K, the kinase AKT plays a central role in many cell processes, including regulation of transcription and translation (2).

PI3K inhibitors or expression of a dominant negative AKT block the induction of FSH target genes in GCs (10, 12, 13, 28–31), indicating the requirement of PI3K/AKT in FSH-dependent gene expression responses. Exogenous IGF-1 alone (in the absence of FSH) also activates PI3K/AKT in GCs (3) but does not promote gene expression (23, 32, 33). However, numerous studies have reported that exogenous IGF-1 potentiates gene expression responses to FSH (23, 31–38). IGF-1 does not alter the affinity of FSH for its receptor (37), suggesting an intracellular intersection of the two hormonal pathways downstream of the FSH receptor. Consistent with this conclusion, the expression of a constitutively active AKT mutant in GCs amplifies gene expression responses to FSH (29). Together, these results suggest that FSH and IGF-1 signaling pathways can intersect upstream of AKT in the PI3K pathway to regulate gene expression. We utilized this information to elucidate the mechanism by which FSH via PKA activates PI3K in GCs.

We report that activation of PI3K/AKT in rat GCs stems from the phosphorylation of IRS1 Y*XM motifs by the IGF-1R. GCs constitutively secrete a subthreshold concentration of IGF-1 in the absence of FSH that partially activates the IGF-1R but is not sufficient to promote IRS1 Y*XM phosphorylation. In response to FSH, PKA activates protein phosphatase 1 (PP1) to dephosphorylate inhibitory Ser/Thr residues on IRS1, rendering IRS1 responsive to the tyrosine kinase activity of the IGF-1R. Through the same mechanism, as concentrations of IGF-1 exceed those secreted by GCs and become sufficient to stimulate IRS1 Y*XM phosphorylation, FSH synergistically enhances IRS1 Y*XM and downstream AKT phosphorylations in a PKA- and PP1-dependent manner. The synergistic Y*XM phosphorylation of IRS1 in response to FSH and exogenous IGF-1 contributes to the synergistic gene expression seen in the presence of both hormones. These results provide a mechanistic explanation both for the FSH/PKA-dependent tyrosine phosphorylation of IRS1 to drive PI3K activation and for the dual requirement of FSH and IGF-1 in follicular maturation.

Experimental Procedures

Materials—The following were purchased: ovine FSH-19 (National Hormone and Pituitary Agency of the NIDDK, National Institutes of Health); recombinant human IGF-1 (Atlanta Biologicals); myristoylated PKA-inhibitory peptide (Myr-PKI), GM6001, H89 dihydrochloride, okadaic acid, tautomycin, and lactacystin (EMD Millipore Corp.); NVP-AEW451 (Cayman Biochemical Co.); and sodium orthovanadate (Na3VO4) (Sigma-Aldrich).

Animals and GC Culture—Sprague-Dawley, CD-outbred rats were from a breeder colony (originally from Charles River Laboratories) maintained in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals by protocols approved by the Washington State University Animal Care and Use Committee. Immature female rats were primed with subcutaneous injections of 1.5 mg of estradiol from postnatal day 21 to 23 to promote preantral follicle growth, followed by euthanasia and dissection of ovaries. Ovaries were punctured with a 27-gaue needle, and GCs were isolated by centrifugation. Isolated GCs were seeded on fibronectin-coated (Corning, Inc.) plates in serum-free DMEM/F-12 (Invitrogen) with 1 nM estradiol, 100 units/ml penicillin, and 100 μg/ml streptomycin at a density of ~1 × 10⁶/ml unless otherwise described. Treatments began ~18 h postplating. For media washout experiments, 3 × 10⁶ GCs were plated in 5 ml of medium and incubated for ~18 h. For washout, medium over GCs was aspirated, and GCs were rinsed twice with PBS with gentle agitation, followed by medium replacement and incubation for the times indicated. For adenoviral transductions, GCs were incubated for 4 h following plating and transduced with adenoviruses for the indicated time intervals.

Western Blotting—Following treatments, total cell lysates were collected in SDS lysis buffer as described previously (3) and heat-denatured. GCs do not proliferate under the above culture conditions; therefore, to control protein concentrations, identical cell numbers were plated for each sample treatment, and equal volumes were loaded in gel lanes. Equal protein concentration was confirmed by loading controls, as indicated. Samples were subjected to Western blotting analysis using standard procedures with the following conditions. Electrophoresed samples were transferred to Amersham Biosciences Protran™ nitrocellulose (GE Healthcare Life Sciences), blots were blocked in 5% dry milk solution for ≥1 h at room temperature, blots incubated with primary antibody solution containing 5% dry milk (unless otherwise indicated below) overnight at 4 °C, and antigen-antibody complexes were detected using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

The following antibodies were purchased from Cell Signaling Technology, Inc. (primary concentration, catalogue, lot, species of origin): phospho-AKT(Ser473) (1:500, 9271, 12, rabbit), phospho-AKT(Thr308) (1:500, 9275, 19, rabbit), AKT (1:1000, 9272, 25, rabbit), phospho-CREB(Ser133) (1:1000, 9191, 9, rabbit), phospho-ERK1/2 (1:1000, 9107, 7, mouse), phospho-FOXO1(Ser256) (1:500, 9461, 5, rabbit), phospho-GAB2(Tyr452) (1:2000 in 5% BSA + 0.1% Tween, 3881, 3, rabbit), phospho-GAB2(Ser159) (1:1000, 3884, 1, rabbit), phospho-glycogen synthase kinase 3β(Ser7) (1:1000, 9336, 12, rabbit), phospho-IGF-1R( Tyr1135/1136) (1:500 in 5% BSA + 0.1% Tween, 3024, 11, rabbit), IGF-1R (1:1000, 9750, 1, rabbit), IRS1 (1:1000, 2382, 4, rabbit), phospho-IRS1(Ser381) (1:500 in 5% BSA + 0.1% Tween, 2389, 2, rabbit), phospho-MLC(Ser19) (1:500, 3671, 3, rabbit), phospho-AKT substrate (RRX(S*/T*)) (1:3000 in 5% BSA + 0.1% Tween, 9624, 16, rabbit), S6 (1:500, 2317, 4, mouse), and phospho-S6(Ser235/236) (1:1000, 2211, 22, rabbit). The following antibodies were purchased from Santa Cruz Biotechnology, Inc.: phospho-IRS1(Tyr929) (1:500 + 0.1% Tween, 17200-R, L2010, mouse), IGF-1R (rabbit, used for immunoprecipitation), IRS1 (rabbit, used for immunoprecipitation), PP1α (1:500, 443, D1713, rabbit), PP1β (1:500, 373782, H2312, mouse), and SHP2 (1:1000, 7384, L2010, mouse). Secondary antibody concentra-
tions were adjusted for each primary antibody used for signal intensity within the linear range. Band intensity was quantified by densitometry analysis via Quantity One software (Bio-Rad). Signal intensity was normalized to loading controls. If treatments altered target protein expression levels, phosphorylated protein band intensity was normalized to total target protein, as indicated.

\[ \text{cDNA Synthesis and qPCR—} \]

GCs treated as indicated were collected in 5-prime RNA-Iso lysis reagent (Thermo Fisher Scientific), and RNA was isolated by isopropyl alcohol/ethanol precipitation. cDNA was synthesized from 500 ng of isolated RNA using qScript cDNA SuperMix (Quanta Biosciences). cDNA was diluted 1:5, and 5 \( \mu l \) was used in each subsequent real-time reaction with Fast SYBR Green Master Mix (Applied Biosystems) on an ABI Fast 7500 real-time machine (Applied Biosystems). Primers were optimized for concentration, and efficiency was determined. Only primers with efficiencies between 95 and 105% were used. \( \text{Rpl19} \) was used as the endogenous control, and the \( \Delta \Delta CT \) method was used to analyze relative mRNA changes between treatment groups. The following primers were used to detect mRNA: \( \text{rInha} \), 5’-TG GAG CGC CGC TGG ATC GTA-3’ (forward) and 5’-GCA TCC CGC AGC TAC CAT-3’ (reverse); \( \text{rLhcr} \), 5’-TCC AGA ACA CCA AAA ACC TGC-3’ (forward) and AAG GGT TCG GAT GCC TGT G-3’ (reverse); \( \text{rCyp11a1} \), 5’-GGG TGG ACA CGA CCT CCA T-3’ (forward) and 5’-ACC TTC AAG TTG TGT GCC ATT TC-3’ (reverse); \( \text{rL19} \), 5’-GTC ACC TGG ATG CGA AGG A-3’ (forward) and 5’-GCC TTG TCT GCC TTC AGT-3’ (reverse).

\[ \text{Immunoprecipitation—} \]

Post-treatment, \( ~10^7 \) GCs were collected in 750 \( \mu l \) of lysis buffer containing 50 \( \mu M \) Hepes, pH 7.0, 150 \( \mu M \) NaCl, 1% IGEPA L, 20 \( \mu M \) NaF, 2 \( \mu M \) Na3V04, 2 \( \mu M \) Na2P2O7, 5 \( \mu M \) EGTA, 5 \( \mu M \) EDTA, 20 \( \mu M \) benzamidine, 10 \( \mu M \) isobutylmethylxanthine, 10 \( \mu M \) soybean trypsin inhibitor (Sigma-Aldrich), 10 \( \mu g/ml \) calpain inhibitor III, 10 \( \mu g/ml \) antipain (EMD Millipore Corp.), and 1 \( \times \) Halt™ protease inhibitor mixture (Thermo Scientific). Lysates were sonicated twice for 15 s on ice and then cleared by centrifugation (14,000 \( \times g \) at 4 °C for 10 min). After 30 \( \mu l \) was taken for input samples, soluble extracts were precleared with Protein A/G beads (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C on a rotator and then incubated with 20 \( \mu g \) of antibody-agarose conjugate or IgG-agarose conjugate control overnight at 4 °C on a rotator. Agarose beads were washed three times in wash buffer containing 50 \( \mu M \) Hepes, pH 7.0, 150 \( \mu M \) NaCl, 10% glycerol, 0.1% Triton X-100 (Sigma-Aldrich). Subsequent samples were analyzed by Western blotting. Inputs represent ~4%, and bound samples represent ~96% of total sample volume.

\[ \text{Lentivirus-mediated Expression of a Constitutively Active Mutant of the Catalytic Subunit of PKA (PKA-CQR)—} \]

Construction of the PKA-CQR lentiviral vector and reverse transduction of immature rat GCs were as described previously (21). Following reverse transduction of GCs with PKA-CQR or control EGFP, a GC suspension was seeded on calf serum-coated plates in serum-free M199 medium containing 30 ng/ml testosterone. Plated GCs were then treated without or with FSH, as indicated, and collected for Western blotting analysis.

\[ \text{In Vitro Phosphorylation Assays—} \]

Washed immunoprecipitates were incubated for 1 h at room temperature in a final volume of 100 \( \mu l \) containing 50 \( \mu M \) Tris-HCl, pH 7.5, 10 \( \mu M \) ATP, 10 \( \mu M \) MgCl2, and ~6 \( \mu g \) of recombinant bovine heart PKA catalytic subunit (Sigma-Aldrich) or water. Pellets were washed two times, and bound proteins were subjected to Western blotting analysis.

\[ \text{IGF-1 ELISA—} \]

Medium IGF-1 concentrations were determined using a Quantikine™ ELISA kit (R&D Systems).

\[ \text{Statistical Analysis—} \]

One-way ANOVA followed by Tukey’s multiple-comparison test \( (p < 0.05) \) was performed on Western, qPCR, and ELISA data using Prism software (GraphPad Software, Inc.). Two-way ANOVA was used to test for interaction between FSH and IGF-1. Any data not significant by two-way ANOVA was still significant by one-way ANOVA \( (p < 0.05) \).

\[ \text{Results} \]

\[ \text{FSH and IGF-1 Synergistically Regulate Gene Expression in a PKA-dependent Manner—} \]

To elucidate the mechanism by which PKA activates P13K, we exploited the apparent intersection of FSH and IGF-1 in the P13K signaling pathway. Statistical synergy identifies a physical intersection between two signaling pathways, producing a greater than additive response (40). To this end, we initially sought to confirm the synergistic regulation of gene expression by FSH and exogenous IGF-1, focusing on three FSH- and P13K-dependent genes required for follicle maturation and female fertility: \( \text{Inha} \) (inhibin-\( \alpha \)) (41), \( \text{Lhcr} \) (luteinizing hormone (LH) receptor) (42), and \( \text{Cyp11a1} \) (P450sc c (P450 side chain cleavage)) (reviewed in Ref. 32) (Fig. 1, A–C). Primary, serum-free cultures of GCs from immature female rats were treated with vehicle or FSH in the presence of increasing concentrations of exogenous IGF-1 for 48 h. To evaluate the influence of PKA on the FSH-dependent responses, GCs were pretreated with H2O or the PKA-selective inhibitor \( \text{Myr-PKI} \) (43) for 1 h. Accumulation of mRNA for select genes was measured by qPCR relative to \( \text{Rpl19} \) (ribosomal protein L19). Results show that exogenous IGF-1 alone was not sufficient to induce significant gene expression; however, IGF-1 at 1 and 5 ng/ml potentiated the gene expression responses to FSH for all three targets over control FSH samples lacking exogenous IGF-1. The induction of \( \text{Inha}, \text{Lhcr}, \) and \( \text{Cyp11a1} \) by FSH was abrogated by Myr-PKI, indicating the requirement for PKA in the synergistic gene expression responses to FSH and IGF-1.

\[ \text{P13K/AKT Is Activated in Response to FSH and IGF-1 via IGF-1R-dependent IRS1 Y*MXM Phosphorylation—} \]

Based on the ability of IGF-1 to enhance FSH-stimulated gene expression, we sought to identify the molecular basis for this synergistic response. We focused on the P13K/AKT pathway because both FSH and IGF-1 activate this pathway in GCs (3), and activation of this pathway is necessary for the expression of many FSH target genes, including \( \text{Inha}, \text{Lhcr}, \) and \( \text{Cyp11a1} \) (10, 12, 13, 28–31).

P13K activation is most accurately monitored by the phosphorylation of AKT on Thr308 rather than Ser473, based on evidence that Ser473 phosphorylation is retained in cells lacking...
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In response to IGF-1, PI3K is activated upon binding to dual IGF-1R-dependent Tyr-phosphorylated Y*MXM motifs on IRS1 (26, 27). Treatment of GCs with the IGF-1R antagonist NVP-AEW541 inhibited the phosphorylation of IRS1 (Tyr989), a canonical Y*MXM motif (Y989MXXM), in response to FSH and IGF-1 (Fig. 2A). IGF-1R(Y1135/1136M) phosphorylation, an indicator of IGF-1R activity (48), was detectable in the absence of FSH, and in marked contrast to IGF-1, FSH did not regulate the activity of the IGF-1R (Fig. 2A). CREB(Ser133) phosphorylation by PKA is independent of PI3K and IGF-1R (3) and serves as a negative control. These results confirm that the IGF-1R is necessary for FSH-stimulated PI3K activation that promotes phosphorylation of AKT (Thr308) and strongly suggest that the IGF-1R is the tyrosine kinase that phosphorylates IRS1 (Tyr989).

If the IGF-1R is the tyrosine kinase that directly phosphorylates IRS1 (Tyr989), then the IGF-1R should co-immunoprecipitate with IRS1. Representative results (Fig. 2, B and C) of co-immunoprecipitations from GC lysates show an association of IGF-1R and IRS1 that is independent of FSH. AKT (Ser473) phosphorylation acts as a positive control for FSH stimulation. These results are consistent with previous results identifying an FSH-independent interaction between IRS1 and PI3K in GCs (3). We thus conclude that activation of PI3K in response to FSH and IGF-1 is triggered by IGF-1R-dependent tyrosine phosphorylation of IRS1 within canonical Y*MXM motifs, leading to phosphorylation of AKT on Thr308.

FSH Activates PI3K through IGF-1R Primed by Subthreshold Levels of Secreted IGF-1—GCs constitutively secrete a relatively low concentration of IGF-1, reaching 1 ng/ml over 48 h in culture (23), which is probably sufficient to partially activate the IGF-1R in the absence of FSH (see Fig. 2A). If the IGF-1R is already partially active in the absence of FSH, why is FSH or elevated concentrations of exogenous IGF-1 required to promote IRS1 (Tyr989) phosphorylation and consequent activation of PI3K/AKT? To address this question, we utilized a washout model to remove endogenous secreted IGF-1. Briefly, GCs were incubated overnight after plating, and culture medium was aspirated the next day, followed by PBS rinse and medium replacement. GCs were allowed to incubate in fresh medium for the indicated time intervals, followed by treatment with FSH. Lysates were analyzed by Western blotting, and medium IGF-1 concentrations were measured by ELISA. After medium washout, a sharp decline of secreted IGF-1 (Fig. 3A, bottom, lane 4) was accompanied by abolished AKT (Thr308, Ser473) phosphorylations in response to FSH (Fig. 3A, top). Although washout rapidly reduced IGF-1 concentrations to ~5 pg/ml, delayed inhibition of AKT (Thr308; Ser473) phosphorylations after washout (compare lanes 3 and 4) probably occurred due to the relatively slow dissociation of bound IGF-1 from the IGF-1R. As IGF-1 concentrations slowly recovered over time (Fig. 3A, bottom inset), FSH-stimulated AKT phosphorylations were restored between 6 and 9 h, suggesting an autocrine/paracrine role of secreted IGF-1 in the responses to FSH.

Recovery of IRS1 (Tyr989) and AKT (Thr308; Ser473) phosphorylations 8 h post-washout in response to FSH was significantly inhibited by the addition of recombinant rat IGF-binding protein 3 (rIGFBP3) (Fig. 3B). IGF-binding proteins are expressed in most cell types, including GCs (21), and function to sequester free extracellular IGFs (49). IGF-binding proteins can also be proteolytically cleaved by metalloproteases to release local IGFs (49); however, pretreatment of cultured GCs with the metalloprotease inhibitor GM6001 did not significantly inhibit FSH-
stimulated IRS1(Tyr989) or AKT(Thr308; Ser73) phosphorylations (Fig. 3C). Notably, FSH did not acutely regulate extracellular IGF-1 concentrations (Fig. 3A, bottom, lanes 1 and 2). Together, these results indicate that IGF-1 is the relevant IGF in rat GCs and that FSH does not acutely free IGF-1 from sequestration by IGF-binding proteins.

To further evaluate the necessity of secreted IGF-1 in IRS1 Y*MXM phosphorylation and PI3K/AKT activation, the washout model (Fig. 3A) was utilized in a rescue experiment (Fig. 3D). Medium over GCs was removed, fresh medium was added, and GCs were incubated for 0.5 h. Samples were then treated with H2O or Myr-PKI for 1 h (1.5 h total fresh medium incubation), followed by treatment with vehicle, FSH, and/or exogenous IGF-1 (1 ng/ml) for 15 min. After washout, attenuated IRS1(Tyr989) and AKT(Thr308; Ser73) phosphorylations in response to FSH (lane 3) were rescued by the addition of 1 ng/ml exogenous IGF-1 (lane 4). Importantly, the presence of 1 ng/ml exogenous IGF-1 alone after washout was not sufficient to promote IRS1 and AKT phosphorylations in the absence of FSH (lane 6). Inhibition of PKA by Myr-PKI (lane 5), as indicated by blunted CREB phosphorylation, prevented FSH-stimulated IRS1 and AKT phosphorylations. IGF-1R(Thr1135/1136) phosphorylation results indicate that secreted endogenous IGF-1 is sufficient to prime the IGF-1R (lanes 1 and 2) but inadequate to promote IRS1(Tyr989) phosphorylation in the absence of FSH (lane 6) and is therefore subthreshold for IRS1/PI3K/AKT activation. These data also show that PKA is required to promote IRS1(Tyr989) phosphorylation and subsequent PI3K activation but does not regulate IGF-1R activity.

FSH Synergizes with IGF-1 at IRS1 Y*MXM Phosphorylations, Even at Saturating IGF-1 Concentrations—We asked whether the intersection between the FSH and IGF-1 signaling pathways at the level of IRS1(Tyr989) phosphorylation persisted with increasing concentrations of exogenous IGF-1 and whether the FSH component remains PKA-dependent. We utilized the washout and recovery model (see Fig. 3D) expanded to include higher concentrations of IGF-1 (seen in Fig. 1). Analysis by two-way ANOVA was necessary to test for the interaction between IGF-1 and FSH or FSH plus Myr-PKI. A synergistic relationship, or an intersection between two signaling pathways, is identified quantitatively by significance under a two-way ANOVA (p < 0.05) and qualitatively by departure of graphed treatment groups from parallel (40).

Results (Fig. 4) show that FSH statistically synergizes with increasing concentrations of exogenous IGF-1, initially at IRS1(Tyr989) phosphorylation and downstream through AKT(Thr308; Ser73) to ribosomal protein S6(Ser235/236) phosphorylation. FSH and IGF-1 also synergistically enhanced the phosphorylation of the transcriptional repressor forkhead box protein O1 (FOXO1), but only up to 1 ng/ml of IGF-1, at which point FOXO1 phosphorylation appears saturated. FOXO1 is a direct target of AKT (2) and represses expression of several genes in GCs (10, 12, 50). Myr-PKI prevented the statistical synergism between FSH and IGF-1 at the levels of IRS1, AKT, S6, and FOXO1 phosphorylations. FSH and IGF-1 did not synergistically regulate IGF-1R(Tyr1135/1136) or CREB(Ser33) phosphorylations. Overall, these data indicate that PKA intersects with the IGF-1 pathway at IRS1(Tyr989), apparently sensitizing IRS1 to phosphorylation by the IGF-1R and thereby providing the synergistic activation of AKT and downstream targets by FSH and IGF-1. In the following studies, we sought to identify the mechanism by which PKA sensitizes IRS1 to tyrosine phosphorylation by the IGF-1R.

Rationale for Use of the Nonselective Kinase Inhibitor H89 as a Tool to Identify Signaling Events Required for FSH-stimulated IRS1(Tyr989) Phosphorylation—The ability of PKA to mediate the actions of FSH to promote phosphorylation of IRS1(Tyr989) and consequent phosphorylation of AKT(Thr308; Ser73) in GCs was previously established using the specific PKA inhibitor PKI (3) and is reported herein (see Figs. 3D and 4). Additional evidence was provided by results in which GCs were transduced with a lentivirus expressing a constitutively active PKA catalytic subunit mutant, PKA-CQR. PKA-CQR mimicked FSH-stimulated phosphorylation of AKT(Thr308; Ser73) as well as IRS1(Tyr989) and CREB(Ser33) (Fig. 5A). The relatively weak FSH-stimulated IRS1(Tyr989) phosphorylation at 20 min is...
attributed to slight differences in GC culture conditions with PKA-CQR reverse transduction. Taken together, these results show that PKA is necessary and sufficient to trigger IRS1 phosphorylation and PI3K/AKT activation.

PKA regulation of PI3K in GCs was initially controversial based on evidence that H89 failed to block FSH-stimulated PI3K/AKT activation (28, 51, 52). In contrast to the specific PKA pseudosubstrate inhibitor PKI, H89 is a competitive ATP antagonist that lacks specificity toward PKA and is reported to inhibit numerous kinases more potently than PKA, including Rho-associated kinase, mitogen- and stress-activated protein kinase 1, and p70 S6 kinase (53, 54); H89 also inhibits non-kinase targets (54).

Consistent with results described above, pretreatment of GCs with 5 μM H89 did not significantly inhibit FSH-stimulated IRS1 phosphorylation (28) (100 ± 0.0%), and blunted GAB2 phosphorylation (77.0 ± 6.3%), a direct target of PKA in GCs (3). These results indicate that H89 at 5 μM exhibits a higher affinity toward S6 kinase than PKA, consistent with previous results (53, 54). Although the effects of H89 on the PI3K pathway are paradoxical, in subsequent studies we utilized H89 as a tool to parse out signaling events in GCs that are necessary for FSH to promote IRS1 phosphorylation.

FIGURE 3. GCs secrete IGF-1 that is necessary for FSH-stimulated IRS1 phosphorylation but not sufficient to elicit responses downstream the IGF-1R. A, for washout following overnight incubation, medium over GCs was aspirated and replaced with fresh medium followed by preincubation for the indicated time intervals (0–9 h), as detailed under “Experimental Procedures.” GCs were then treated with vehicle or FSH for 15 min. Whole-cell lysates were analyzed by Western blotting (top), and corresponding medium IGF-1 concentrations were measured by ELISA (bottom). The bottom inset is a magnified view of IGF-1 concentrations from fresh medium preincubations. ELISA concentrations are represented as mean ± S.E. (error bars) (n = 3); following washout, GCs were incubated with PBS or 50 ng/ml recombinant IGF-binding protein 3 for 8 h. GCs were then treated with vehicle or FSH for 15 min. As analyzed by Western blotting, recovery of FSH-stimulated phosphorylations of IRS1(Tyr989), AKT(Thr308), CREB(Ser133), and AKT(Ser473) was inhibited by recombinant IGF-binding protein 3 (IGFBP3) (91.8 ± 3.6, 60.2 ± 6.8, 35 ± 4.1, and 64.4 ± 6.8%, respectively). Results are represented as percentage inhibition ± S.E. (n = 3). C, GCs were pretreated for 1 h with DMSO or 10 μM GM6001, followed by vehicle (v), FSH (F), or IGF-1 (50 ng/ml) for 15 min. Representative images show that GM6001 did not affect FSH-stimulated IRS1, AKT, or CREB phosphorylations and did not affect IGF-1-stimulated IRS1 and AKT phosphorylations. Results represent n = 2. D, washout was performed, as in A, followed by incubation for 0.5 h. GCs were then pretreated with H2O or 25 μM Myr-PKI and incubated for 1 h (1.5 h of total incubation in fresh medium). GCs were then treated with vehicle or FSH and IGF-1 (1 ng/ml) for 15 min, and whole-cell lysates were analyzed by Western blotting. Results represent n = 3.
FIGURE 4. FSH synergistically enhances IGF-1 pathway responses, even at saturating IGF-1 concentrations. Washout and 1.5-h incubation was performed as described in the legend to Fig. 3D, expanding to include higher concentrations of IGF-1 (0–50 ng/ml) in the presence of vehicle (v), FSH (F), or FSH plus 30 μM Myr-PKI (PKI). Whole-cell lysates were collected and analyzed by Western blotting, with representative data shown (A) and densitometric quantification of probes for phosphorylated proteins shown in adjacent graphs (B–H). Data represent mean signal ± S.E. (error bars) (n = 3) analyzed by two-way ANOVA for interaction with FSH (IGF-1 alone) samples (*, p < 0.05; ****, p < 0.0001). All data sets not significant (n.s.) by two-way ANOVA are significant by one-way ANOVA (p < 0.05).
IRS1, the Hub Linking FSH to PI3K

FIGURE 5. Use of the nonselective inhibitor H89 to identify signaling events necessary for FSH-stimulated IRS1 Y*M phosphorylation. A, GCs were reverse transduced with lentiviruses expressing either EGFP or a constitutively active PKA catalytic subunit mutant (PKA-CQR) and treated with vehicle (v) or with FSH (F) for 20 min or 24 h. Whole cell lysates were collected and analyzed by Western blotting. Results are representative of three independent experiments. B, GCs were pretreated with DMSO or 5 μM H89 for 1 h and then treated with vehicle or FSH for 15 min or 1 h, and whole cell lysates were collected for Western blotting analysis. H89 inhibited FSH-stimulated IRS1 (Y989) phosphorylation. p-IRS1(Tyr989) (see Fig. 2B), 77.0 ± 6.3% (n = 3), and 8.7 ± 6.1% (n = 4), respectively. H89 did not significantly inhibit -fold FSH-stimulated dephosphorylation of MLC(Ser19) or H11006.

FIGURE 6. PKA-stimulated IRS1 Y*M phosphorylation is not a consequence of direct PKA phosphorylation of IRS1. A, IRS1 was immunoprecipitated from lysates of vehicle-treated GCs, as described under "Experimental Procedures." Immunoprecipitates of IRS1 or IgG controls were incubated with H2O or PKA catalytic subunit (C), and subsequent Western blots were probed using an antibody recognizing phosphorylated (p-) PKA substrates based on the PKA consensus motif or an antibody (Ab) against IRS1. Images represent two independent experiments. B, immunoprecipitations were performed from GCs treated for 15 min with vehicle or FSH using antibodies recognizing phosphorylated PKA substrates or IG1-1R versus IgG controls. Images represent two independent experiments. C, GCs were pretreated with DMSO or 5 μM H89 for 1 h, followed by treatment with vehicle or FSH for 15 min. Soluble lysates were subjected to immunoprecipitation (IP) using an antibody recognizing IRS1. Subsequent Western blots were probed using the phosphorylated PKA substrate antibody.

However, pretreatment of GCs with H89 abrogated the p-PKA substrate signal in IRS1 immunoprecipitates from FSH-treated GCs (Fig. 6C, lane 8). Based on evidence that H89 fails to inhibit the ability of FSH to promote IG1-1R-stimulated phosphorylation of IRS1(Tyr989) (see Fig. 5B), we interpret these results to suggest that the FSH-stimulated, PKA-dependent phosphorylation of IRS1 on Ser/Thr residues is not required for FSH-stimulated IRS1(Tyr989) phosphorylation.

FSH and IG1-1 Do Not Intersect through a Tyrosine Phosphatase—Previous studies have indicated the potential for PTEN (phosphatase and tensin homology) to act as a tyrosine phosphatase to regulate IRS1 Y*M phosphorylation (57). Therefore, we hypothesized that FSH could be inhibiting a tyrosine phosphatase such as PTEN to enhance the phosphorylation of IRS1 Y*M motifs. To test this, GCs were pretreated with H2O or 50 mM sodium orthovanadate (Na3VO4), a pan-tyrosine phosphatase inhibitor, followed by treatment with vehicle or FSH (Fig. 7A). -Fold stimulation of IRS1(Tyr989) phosphorylation between vehicle- and FSH-treated samples was not significantly inhibited by Na3VO4. Instead, IRS1(Tyr989) phosphorylation was enhanced in both vehicle- and FSH-treated
lanes, indicating a general sensitizing role by tyrosine phosphatases and not a regulatory role. AKT phosphorylations were concomitantly raised in vehicle- and FSH-treated samples. FSH-stimulated dephosphorylation of GAB2(Tyr452) served as a positive control (3); CREB(Ser133) phosphorylation served as a negative control.

FSH Activates PP1cβ to Dephosphorylate Inhibitory IRS1 Ser/Thr Residues—Extensive research has attributed the development of insulin resistance and type II diabetes to inhibitory Ser/Thr phosphatases on IRS1 that impair both tyrosine phosphorylation of IRS1 by the insulin receptor and activation of downstream targets like PI3K (44, 58). As a result, we asked whether FSH could be stimulating the dephosphorylation of inhibitory Ser/Thr residues within IRS1 to sensitize IRS1 to the tyrosine kinase activity of the IGF-1R. PP1- and -2 are two predominant members of the phosphoprotein phosphatase superfamily that account for over 90% of all eukaryotic protein phosphatase activity (59). We thus hypothesized that FSH could be activating PP1 or PP2 to stimulate IRS1 Y*M phosphorylation, as evidenced by dephosphorylation of IRS1(Y989), glycogen synthase kinase 3β(Ser21), and AKT(Thr308) phosphorylations by 98.6 ± 1.4% (n = 3) as compared with load control and 99.5 ± 0.4% (n = 3) as compared with total IRS1. C, GCs were transduced with adenosviruses expressing either scrambled shRNA or shRNA against the IRS1 isoform of PP1 for 72 h. GCs were then treated with vehicle or FSH, and lysates were collected for Western blotting. Knockdown of PP1β (76.7 ± 12.2% inhibited phosphorylation of IRS1(Y989), AKT(Thr308), AKT(Thr385), and CREB(Ser133) (72.7 ± 8.6, 73.7 ± 4.9, 68.6 ± 15.0, and 9.6 ± 37.8%, respectively) (n = 3). C. GCs were transduced for 18 h with adenosviruses expressing either GFP or the PKA-selective inhibitor PKI, followed by vehicle or FSH for 15 min. PKI inhibited FSH-stimulated dephosphorylation of IRS1(Ser789) and MLC(Ser19) (93.6 ± 6.2 and 89.7 ± 10.2%, respectively) and phosphorylation of CREB(Ser133) (88.3 ± 11.7%) (n = 3). All data from A–D are represented as mean percentage inhibition ± S.E.

We next utilized H89 to ascertain whether activation of PP1 by FSH was required for the ability of FSH to stimulate IRS1(Y989) phosphorylation. Results (Fig. 5B) show that pretreatment of GCs with H89 did not inhibit -fold FSH-stimulated activation of PP1, as evidenced by dephosphorylation of MLC(Ser19). This result supports the contribution of PP1 to FSH-dependent IRS1(Y989) phosphorylation.

To confirm the requirement for PP1 in FSH-stimulated IRS1 Y*M phosphorylation, targeted shRNA knockdown of PP1 isoforms via adenosviruses was performed (Fig. 8C). Knockdown of PP1β significantly inhibited FSH-dependent IRS1(Y989) and AKT(Thr385; Ser473) phosphorylations; CREB(Ser133) phosphorylation was unaffected. Based on our evidence that FSH activates PP1 and that PP1 is required for the ability of FSH to promote phosphorylation of IRS1(Y989), we sought to determine whether FSH via PP1 stimulates the dephosphorylation of inhibitory Ser/Thr resi-
dyes on IRS1. Additional probes of tautomycin-treated samples utilizing commercially available antibodies identified an inhibitory IRS1 Ser residue dephosphorylated in response to FSH in a PP1-dependent manner: Ser789 (Fig. 8B).

To test whether PP1 activation and IRS1 Ser dephosphorylation are PKA-dependent, GCs were transduced with an adenovirus expressing PKI (Fig. 8D). FSH-stimulated PP1 activation, as indicated by MLC(Ser19) dephosphorylation, and IRS1(Ser789) dephosphorylation were both abolished in the presence of PKI; CREB(Ser135) was a positive control. Furthermore, transduction of GCs with the constitutively active PKA catalytic subunit mutant PKA-CQR was sufficient to stimulate MLC(Ser19) dephosphorylation (Fig. 5A). Taken together, these results suggest that FSH in a PKA-dependent manner activates PP1 to dephosphorylate at least one inhibitory Ser residue on IRS1.

Finally, we determined whether the dephosphorylation of IRS1(Ser789) in response to FSH was inhibited by H89. Results (Fig. 5B) show that -fold FSH-stimulated IRS1(Ser789) dephosphorylation is retained in the presence of H89. This result supports our conclusion that PKA activation of PP1 to dephosphorylate IRS1(Ser789) contributes to the ability of FSH to promote IRS1(Tyr989) phosphorylation.

In summary, these data indicate that PKA is necessary and sufficient to stimulate the activation of PP1 that is required for the dephosphorylation of one or more inhibitory Ser/Thr residues on IRS1 and consequent Y*M phosphorylation of IRS1 by the IGF-1R to activate PI3K.

**PP1 Is Necessary for the Cooperative Regulation of PI3K by FSH and IGF-1**—Because PP1 is required for FSH-stimulated IRS1 and AKT phosphorylations with secreted endogenous IGF-1, we asked whether PP1 is also necessary for the synergistic response between FSH and exogenous IGF-1. GCs were pretreated with lactacystin and EtOH or tautomycin and subjected to medium washout with a 1.5-h incubation, followed by treatment with vehicle or FSH without or with exogenous IGF-1 (Fig. 9). IRS1 and AKT phosphorylations were statistically synergistic with FSH and IGF-1; IGF-1R(Tyr1135/1136) and CREB(Ser135) phosphorylations were not. Tautomycin blocked the synergistic phosphorylations of IRS1 and AKT. These data indicate that PP1 is necessary for the synergistic response of FSH and exogenous IGF-1.

**Discussion**

**Cross-talk between the IGF-1R and GPCRs to Activate PI3K**—The PI3K/AKT pathway is a central regulator of many cellular processes, including translation, differentiation, proliferation, and apoptosis (2). Whereas growth factor receptor tyrosine kinase agonists classically activate this pathway, a number of GPCRs also activate PI3K/AKT. For example, thyroid-stimulating hormone, LH, and β-adrenergic receptor agonists all activate the PI3K pathway. However, the mechanism by which GPCRs signal to activate PI3K/AKT is incompletely understood (1). It is interesting that cell responses to each of these GPCR agonists are reported to synergize with IGF-1: β-adrenergic receptor agonists synergize with IGF-1 to enhance L6 muscle cell proliferation (63, 64); thyroid-stimulating hormone activates the PI3K in the thyroid FRTL-5 cell in a PKA-dependent manner (65, 66), and its actions intersect with IGF-1/IGF-2 (67–69); and LH and IGF-1 synergize in preovulatory GCs at the level of gene expression (70, 71). Each of these GPCRs can activate PKA as well as other pathways (64 – 66, 72). Thus, the notion that IRS1 acts as the critical hub linking PKA with the IGF-1 pathway may not be unique to preantral GCs and the FSH receptor. Instead, we posit that IRS1 downstream of the IGF-1R may play a more universal role in GPCR-mediated activation of the PI3K/AKT pathway.

**Mechanism of PKA-enhanced IRS1 Y*M phosphorylation by the IGF-1R**—The well recognized synergy between FSH and IGF-1 at the level of gene expression (23, 31–38) spearheaded our identification of the site of intersection between these two signaling pathways and, consequently, the mechanism by which FSH via PKA activates PI3K/AKT. Our results place IRS1 Y*M phosphorylation as a pivotal step in the activation of PI3K/AKT in GCs in response to FSH. Although endogenous IGF-1 secreted from GCs partially activates the tyrosine kinase activity of the IGF-1R (see Fig. 2A), IRS1 Y*M phosphorylation fails to occur in the absence of FSH. Instead, phosphorylation of IRS1 Y*M and concomitant activation of PI3K/AKT require both endogenous IGF-1 and FSH, with the latter acting via PKA (see Figs. 3D and 5A). As IGF-1 concentration exceeds endogenous levels and becomes sufficient to stimulate IRS1/PI3K/AKT activation, FSH via PKA continues to enhance IRS1 Y*M phosphorylation with parallel activation of PI3K/AKT (see Fig. 4). Thus, the synergistic activation of AKT stems from the phosphorylation of IRS1 Y*M motifs. Further evidence of this linear pathway progression was observed upon treatment of GCs with the pan-tyrosine phosphatase inhibitor Na3VO4, which enhanced IRS1 Y*M and downstream AKT phosphorylations in both vehicle- and FSH-treated samples (see Fig. 7A). Our data are consistent with results utilizing Star promoter-reporter constructs in which the authors showed that IGF-1 enhances transcriptional activity only of FSH-responsive promoter regions (37), indicating the FSH/IGF-1 interaction in upstream signaling pathways. Hence, our data specify that IRS1 Y*M phosphorylation marks the point of intersection for FSH and IGF-1 signaling pathways, ultimately leading to a remodeled transcriptome required for proper follicle development.

Surprisingly, the ability of PKA to stimulate IRS1 Y*M phosphorylation occurs without enhanced activation of the IGF-1R (see Fig. 2), suggesting that PKA must regulate the accessibility of IRS1 to the tyrosine kinase activity of the IGF-1R. Extensive research has implicated inhibitory Ser/Thr phosphorylations on IRS1 in the development of type II diabetes, a disease characterized by impaired responses to insulin in target tissues (44, 58). Several Ser/Thr sites on IRS1 are phosphorylated in response to different stimuli, and mutation of select sites to Ala enhances insulin-mediated activation of targets downstream of IRS1, including PI3K (44, 73–77). IRS1(Ser789) is among the sites identified as inhibitory to PI3K activation (77, 78), and it is dephosphorylated in GCs with FSH in a PP1- and PKA-dependent manner (see Fig. 8). Finally, FSH-mediated IRS1 Y*M phosphorylation in the presence of endogenous IGF-1 (see Fig. 8, A and C) or exogenous IGF-1 (see Fig. 9) requires PP1. In sum and as depicted in Fig. 10, we propose that in the absence of FSH, IRS1 is restrained from tyrosine phos-
phorylation by the IGF-1R due to the presence of one or more inhibitory IRS1 Ser/Thr phosphorylations(s), including Ser\textsuperscript{789}. FSH via PKA activates PP1 to dephosphorylate the inhibitory Ser/Thr residues on IRS1, thereby permitting IGF-1R to phosphorylate IRS1 on Y\textsuperscript{M}X motifs to activate PI3K. The ability of Myr-PKI or tautomycin to inhibit IRS1 Y\textsuperscript{M}X phosphorylation in the presence of either endogenous secreted IGF-1 or increasing concentrations of exogenous IGF-1 further supports our model and defines PKA and PP1 as the predominant mediators of FSH and IGF-1 synergy. Future studies will analyze additional potentially inhibitory Ser/Thr phosphorylations on IRS1 (79) and determine the mechanism by which FSH activates PP1 in a PKA-dependent manner.

FSH-regulated Gene Expression in GCs Drives Follicle Maturation—A substantial shift in the GC transcriptome is necessary for the maturation of ovarian follicles to a stage that can mediate ovulation and oocyte maturation in response to the surge of LH. FSH signals primarily via PKA (21) not only to directly phosphorylate proteins, such as CREB (80, 81), histone H3 (9), and β-catenin (13), but also to activate signaling pathways, such as the MAPK/ERK (19) and PI3K/AKT (3) pathways. The regulation of target genes by FSH, such as Lhcgr, requires input from the PI3K/AKT pathway to relieve repression imposed by FOXO1 (13), PKA phosphorylation of β-catenin- (Ser\textsuperscript{552}; Ser\textsuperscript{675}) to relieve repression by T-cell factor 3 and to co-activate steroidogenic factor 1 (13), activation of specificity protein 1/3 (reviewed in Ref. 13), and input from PI3K/AKT and MAPK/ERK to activate GATA4 (15). Hence, activation of the PI3K pathway by IGF-1 in the absence of FSH is not sufficient to activate target genes required for follicle maturation (see Fig. 1). However, whereas FSH in the absence of IGF-1 activates CREB (see Fig. 3) and probably β-catenin and MAPK/
ERK (82), FSH in the absence of endogenous IGF-1 does not activate the PI3K/AKT pathway (see Fig. 3). Thus, gene expression that mediates follicle maturation requires both FSH and endogenously secreted IGF-1.

AKT Mediates Downstream Synergy of FSH and IGF-1—
Zeleznik et al. (29) showed that expression of a constitutively active AKT mutant in GCs amplified expression of FSH-regulated genes, thereby mimicking the effect of exogenous IGF-1. To date, FOXO1 is the predominant AKT-dependent target identified in GCs that modulates gene expression (10, 12, 83). AKT phosphorylation of FOXO1 relieves repression of an upstream target, namely IRS1. The requirement for PKA and PP1 to facilitate tyrosine phosphorylation of IRS1 by the IGF-1R forms the basis for the ability of FSH to both promote (with subthreshold endogenous IGF-1) and enhance (with exogenous IGF-1) phosphorylation of IRS1 Y*M motifs and subsequent PI3K/AKT activation. The ability of other GPCR agonists to synergize with IGF-1 to enhance gene expression, as reviewed above, suggests that the activation of PP1 may not be restricted to GCs and may by a more universal mechanism by which GPCRs activate PI3K.

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