G protein-coupled receptors (GPCRs) That Signal via Protein Kinase A (PKA) Cross-talk at Insulin Receptor Substrate 1 (IRS1) to Activate the phosphatidylinositol 3-kinase (PI3K)/AKT Pathway

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G protein-coupled receptors (GPCRs) activate PI3K/v-AKT thymoma viral oncoprotein (AKT) to regulate many cellular functions that promote cell survival, proliferation, and growth. However, the mechanism by which GPCRs activate PI3K/AKT remains poorly understood. We used ovarian preantral granulosa cells (GCs) to elucidate the mechanism by which the GPCR agonist FSH via PKA activates the PI3K/AKT cascade. Insulin-like growth factor 1 (IGF1) is secreted in an autocrine/paracrine manner by GCs and activates the IGF1 receptor (IGF1R) but, in the absence of FSH, fails to stimulate YXXM phosphorylation of IRS1 (insulin receptor substrate 1) required for PI3K/AKT activation. We show that PKA directly phosphorylates the protein phosphatase 1 (PP1) regulatory subunit myosin phosphatase targeting subunit 1 (MYPT1) to activate PP1 associated with the IGF1R-IRS1 complex. Activated PP1 is sufficient to dephosphorylate at least four IRS1 Ser residues, Ser\(^{318}\), Ser\(^{346}\), Ser\(^{612}\), and Ser\(^{789}\), and promotes IRS1 YXXM phosphorylation by the IGF1R to activate the PI3K/AKT cascade. Additional experiments indicate that this mechanism also occurs in breast cancer, thyroid, and preovulatory granulosa cells, suggesting that the PKA-dependent dephosphorylation of IRS1 Ser/Thr residues is a conserved mechanism by which GPCRs signal to activate the PI3K/AKT pathway downstream of the IGF1R.

Cells respond to an array of stimuli through both G protein-coupled receptors (GPCRs)\(^2\) and receptor tyrosine kinases (RTKs) under normal physiological conditions. Conversely, persistent activation of GPCRs or RTKs often drives mitogenic and metabolic responses that underlie tumorigenesis (1, 2). GPCRs and RTKs signal through the PI3K/v-AKT thymoma viral oncoprotein (AKT) pathway to orchestrate a broad spectrum of cellular functions (3–5). Because of its influential role in proliferation, apoptosis, migration, and metabolism, the PI3K/AKT pathway is actively pursued as a therapeutic target to treat dysregulated glucose homeostasis, autism, infertility, cancer, and the diverse pathologies of aging (6–9). However, the mechanism by which GPCRs activate the PI3K/AKT pathway, either alone or in conjunction with RTKs, is poorly understood (3, 10).

The RTKs for insulin and insulin-like growth factor 1 (IGF1) phosphorylate YXXM motifs on the adapter proteins insulin receptor substrate 1 (IRS1) and IRS2 that bind to and activate class I PI3K p85–p110 heterodimers (11, 12). PI3K generates the phosphoinositide 3,4,5-trisphosphate, which promotes phosphorylation of AKT on Thr\(^{308}\) by phosphoinositide-dependent kinase 1 (13). AKT is also phosphorylated on Ser\(^{473}\) by the mechanistic target of rapamycin complex 2 (14). Dual phosphorylation of AKT is necessary for full activation (15); however, Thr\(^{308}\) is generally considered a more reliable indicator of PI3K activity (7). AKT regulates many cellular functions through direct Ser/Thr phosphorylation of key signaling intermediates as well as transcription factors, such as forkhead box O1 (FOXO1) (4).

In contrast to the insulin and IGF1 receptors, GPCR-mediated activation of PI3K is poorly understood (10). A number of GPCR ligands are known to activate PI3K, including stromal cell-derived factor, sphingosine 1-phosphate, lysophosphatidic acid, carbachol, isoproterenol, prostaglandin E\(_2\), thyroid-stimulating hormone (TSH), FSH, luteinizing hormone (LH)/choriogonadotropin (CG), and others (10, 16–22). To date, the predominant mechanisms by which GPCRs activate PI3K are often tissue-specific (10) and involve either the binding of active Ras to the p110 catalytic subunit of PI3K (23), an event that can be enhanced upon phosphorylation of the p85 regulatory subunit.
unit by PKA (19, 24, 25) or by Gβγ (26), or direct binding of Gβγ to p110 (17, 26–29).

We used ovarian preantral granulosa cells (GCs) to model the mechanism by which the GPCR agonist FSH activates the PI3K/AKT cascade. Our previous work showed that FSH-stimulated PI3K/AKT activation is independent of Ras (30) but dependent on PKA, the IGF1R, and PP1 (protein phosphatase 1) (30, 31).

The requirement for PKA in FSH-stimulated PI3K/AKT activation was demonstrated by results showing that a constitutively active PKA catalytic mutant (PKA-CQR) mimicked FSH to promote phosphorylation of IRS1 on the YXXM motif Tyr^989 and AKT phosphorylation on Thr^308 and Ser^473 (31, 32). This result supports previous studies showing that pharmacological activators or an inhibitor of PKA (PKI) that functions as a catalytic subunit pseudosubstrate stimulated or blocked, respectively, the phosphorylation of these markers of PI3K/AKT pathway activation (30).

FSH-stimulated PI3K/AKT activation is also dependent on the IGF1R, based on the ability of the IGF1R antagonist NVP-AEW541 to block phosphorylation of IRS1(Tyr^989), AKT-(Thr^308), and AKT(Ser^473) in response to FSH (31). The IGF1R is constitutively activated by secreted IGF1 (~0.3 ng/ml), but the receptor fails to phosphorylate IRS1 YXXM motifs or activate PI3K/AKT in the absence of FSH (31).

PP1 is also required for FSH-stimulated PI3K/AKT activation, as evidenced by the ability of the PP1 inhibitor tautomycin as well as knockdown of the PP1 β catalytic subunit to significantly inhibit phosphorylation of IRS1(Tyr^989), AKT-(Thr^308), and AKT(Ser^473) in response to FSH (31). However, the mechanism by which FSH signals through PKA to stimulate IRS1 YXXM phosphorylation and PI3K/AKT activation in a PP1-dependent manner downstream of the IGF1R is not understood.

Blockade of the PI3K/AKT pathway by pharmacological inhibitors, a dominant-negative AKT, or inactivation of the AKT target FOXO1 abrogates the ability of FSH to promote GC proliferation as well to induce genes that define the preovulatory (PO) GC such as the LH/CG receptor and aromatase (33–35). Thus, this pathway and the genes it regulates are necessary for fertility (36–38).

In light of the physiological relevance of the PI3K/AKT pathway, in this report we sought to investigate the mechanism by which PKA stimulates IRS1 YXXM phosphorylation and PI3K/AKT activation in a PP1-dependent manner downstream of the IGF1R. We show that FSH, via PKA, activates PP1, present in a complex that includes IGF1R and IRS1, by directly phosphorylating one of its regulatory subunits to promote the dephosphorylation of at least four IRS1 Ser residues that facilitates IRS1 YXXM phosphorylation and PI3K/AKT activation. Moreover, we show in three additional cell types that the IGF1R-catalyzed phosphorylation of IRS1 and resulting PI3K activation are mediated by an apparently conserved PKA- and PP1-dependent mechanism that promotes the dephosphorylation of IRS1 Ser/Thr residues.

Results

**FSH Stimulates Multisite Dephosphorylation of Ser Residues on IRS1 via PKA and PP1—Insulin resistance is associated with Ser/Thr phosphorylation of IRS1 that impairs insulin-stimulated IRS1 YXXM phosphorylation and PI3K/AKT activation (7, 39). We used a panel of monoclonal antibodies (40) against IRS1 Ser/Thr phosphorylation sites (numbered according to the rat amino acid sequence) and immunoblot analysis to evaluate IRS1 Ser/Thr phosphorylation in response to FSH stimulation of preantral GCs isolated from the ovaries of immature rats (31). Although most IRS1 Ser/Thr phosphorylation was not changed by FSH, FSH significantly stimulated the phosphorylation of Ser^1216, Ser^1135, Ser^991, Ser^557, Ser^637, and Ser^332/336 1.5-fold or greater (Fig. 1). FSH also promoted the dephosphorylation of four Ser sites on IRS1 (Ser^789, Ser^612, Ser^446, and Ser^339) 1.5-fold or greater. Importantly, Ser^789, Ser^612, and Ser^339 have been reported previously as inhibitory sites on IRS1 (7, 41–43). Unexpectedly, IRS1(Ser^637) phosphorylation, which is usually detected during insulin resistance (7, 44–47), was undetectable in GCs.

To evaluate the PKA and PP1 dependence of IRS1 Ser/Thr dephosphorylation, cultured preantral GCs were pretreated with adenoviruses expressing either GFP or the PKA-selective inhibitor protein kinase inhibitor (PKI) (48) (Fig. 2, A and C–E). Alternatively, GCs were pretreated with lactacystin to block the degradation of IRS1 (31, 49) and incubated without or with the PKI inhibitor tautomycin (50) (Fig. 2, B and F–H). FSH-stimulated dephosphorylation of Ser^1216, Ser^1135, and Ser^991 was inhibited by both PKI and tautomycin. FSH-stimulated dephosphorylation of Ser^789 was also inhibited by PKI and tautomycin, as reported previously (31). FSH-stimulated dephosphorylation of Ser^612 in the absence of tautomycin was not significant by ANOVA because of the larger and more variable signal from tautomycin-treated samples; however, Ser^612 dephosphorylation was significant by Student’s t test (p < 0.05, Fig. 2H). cAMP response element binding protein (CREB) is a direct phosphorylation target of PKA in GCs (30) and served as a positive control for PKI treatments and a negative control for tautomycin treatments. Myosin light chain (MLC) is a direct target of PKP1 (51) and served as a positive control for tautomycin efficacy. Glycogen synthase kinase (GSK) 3β is a PP2 target (31) utilized to confirm the selectivity of tautomycin for PKP1 over PP2 (Fig. 2B).

**PKA and Its Regulatory Subunit MYPT1 Are Associated with IRS1—Co-immunoprecipitation experiments from GC lysates showed that PP1cβ (the β isofrom of the PK1 catalytic subunit) was constitutively associated with the complex of IRS1 and IGF1R (Fig. 3A). We found that the PKP1 regulatory subunit MYPT1 was also constitutively associated with the IRS1-IGF1R complex (Fig. 3A), consistent with its role to facilitate substrate specificity, catalytic regulation, and subcellular localization of PKP1 catalytic subunits (51). Additional co-immunoprecipitation studies showed that PP1cβ and MYPT1 were constitutively associated in GCs (Fig. 3, B and C). These results are consistent with our previous studies showing that shRNA knockdown of PP1cβ blocks FSH-stimulated IRS1 YXXM phosphorylation and PI3K/AKT activation (31).

**PKA Activates PP1 through Phosphorylation of MYPT1—PKA phosphorylates MYPT1 on several residues, although the regulatory effect and physiological significance of PKA-mediated MYPT1 phosphorylation remains unknown (reviewed in Ref. 53). We postulated that direct PKA phosphorylation of
MYPT1 activates PP1cβ. To test this hypothesis, the MYPT1-PP1cβ complex was isolated from GC lysates by immunoprecipitation and treated under cell-free conditions with either H2O or recombinant PKA catalytic protein (PKAc). Subsequent immunoblots probed for phospho-MYPT1(Ser668) (Fig. 4A) showed that PKAc phosphorylated MYPT1; MYPT1(Ser668) is contained within the sequence Arg-Arg-Arg-Ser668 that matches the PKA consensus phosphorylation motif (RR/KXXS/T) (13). Similarly, immunoblot analysis of whole GC lysates showed that FSH stimulated the phosphorylation of MYPT1(Ser668) in intact GCs, and this phosphorylation was inhibited by the PKA-selective inhibitor (48) myristoylated PKI (Myr-PKI) (Fig. 4, B and C).

Catalytic activity associated with the MYPT1-PP1cβ complex was measured using the protein phosphatase fluorogenic substrate 6,8-difluoro-4-methylumbelliferyl (DiFMUP) (54, 55). GCs were treated with vehicle or FSH for 15 min, and MYPT1 was isolated from lysates by immunoprecipitation. MYPT1 immunoprecipitates were incubated without or with PKAc, and phosphatase activity was measured via fluorescence emission from DiFMUP. PKAc activated MYPT1 isolated from vehicle-treated GCs (Fig. 4D). Interestingly, MYPT1 isolated from FSH-treated lysates had no significant phosphatase activity, which might be attributed to Ser668 dephosphorylation during immunoprecipitation isolation. However, the dephosphorylation of the PP1cβ direct target MLC (51) from whole-cell lysates indicated FSH-stimulated PP1cβ activity in primary cultures of GCs (Fig. 2B).

Finally, to assess phosphatase activity bound to the IRS1-IGF1R complex, the complex was isolated by immunoprecipitation using a panel of monoclonal antibodies that recognize phosphorylated Ser/Thr residues within IRS1. A, representative images. Arrows indicate bands of interest. B, densitometric quantification of phosphorylations was normalized to load controls, and -fold FSH-stimulated phosphorylation is represented by log10 scale. Black and green asterisks indicate IRS1 residues significantly (p < 0.05) phosphorylated or dephosphorylated 1.5-fold or greater with FSH, respectively. Results are represented as the mean ± S.E. (error bars) of -fold FSH-stimulated phosphorylation (n = 3).

FIGURE 1. FSH stimulates the phosphorylation and dephosphorylation of several Ser/Thr residues in IRS1. Primary cultures of rat preantral GCs were treated with vehicle (v) or FSH (F) for 15 min. Total cell lysates were collected for immunoblot analysis using a panel of monoclonal antibodies that recognize phosphorylated Ser/Thr residues within IRS1. A, representative images. Arrows indicate bands of interest. B, densitometric quantification of phosphorylations was normalized to load controls, and -fold FSH-stimulated phosphorylation is represented by log10 scale. Black and green asterisks indicate IRS1 residues significantly (p < 0.05) phosphorylated or dephosphorylated 1.5-fold or greater with FSH, respectively. Results are represented as the mean ± S.E. (error bars) of -fold FSH-stimulated phosphorylation (n = 3).
intact GCs, GCs were transfected with plasmids expressing GFP or a truncated (to residues 1–300) constitutively active (CA) form of MYPT1 (56). Transfection of GCs with CA MYPT1 stimulated dephosphorylation of IRS1(Ser318) in vehicle-treated cells (Fig. 5, B and E, compare lanes 1 and 2). Additionally, expression of CAMYPT1 in GCs stimulated phosphorylation of IRS1(Tyr989), a canonical YXXM motif (Tyr989–X–X–Met), and AKT(Thr308) equivalent to that of FSH (Fig. 5, B–D, compare lanes 2 and 3). Probes for total MYPT1 identified both endogenous MYPT1 (~140 kDa) and truncated CA MYPT1 (~30 kDa). Taken together, these results illustrated that PP1c–MYPT1 was necessary and sufficient to dephosphorylate Ser sites on IRS1 and promote IRS1 YXXM phosphorylation and PI3K/AKT activation.

**GPCR-mediated Activation of the PI3K/AKT Pathway Downstream of the IGF1R Is a Conserved Mechanism among Different Cell Types**—Based on the widespread expression of IGF1R in mammals (2, 57), we hypothesized that our model for GPCR-mediated activation of the PI3K/AKT pathway downstream of the IGF1R in preantral GCs might be conserved among different cell types. To test this hypothesis, we analyzed the PKA and IGF1R dependence of markers for PI3K/AKT activation in response to elevated cAMP in primary, serum-free cultures of rat PO GCs as well as serum-starved cultures of MCF7 human breast cancer and FRTL rat thyroid cell lines (for culture conditions refer to “Experimental Procedures”). In response to FSH, preantral GCs (used in the experiments above) differentiate to PO GCs that are phenotypically distinct from preantral GCs (20).

Cells were pretreated with the IGF1R-selective inhibitor NVP-AEW541 (58) or the PKA-selective inhibitor PKI (either Myr-PKI or adenoviral PKI). MCF7 and FRTL cells were then treated with the adenylyl cyclase activator forskolin to elevate intracellular levels of cAMP, whereas PO GCs were treated with human CG (hCG), a GPCR agonist for the LH/CG receptor that signals predominately via cAMP and PKA (reviewed in Ref. 20).
Human CG in PO GCs and forskolin in MCF7 and FRTL cells significantly stimulated IRS1(Tyr989) and AKT(Thr308) phosphorylation as well as the concomitant phosphorylation of MYPT1(Ser668) and dephosphorylation of IRS1(Ser318) over vehicle controls (Fig. 6 and *s*.01). Additionally, pretreatment with PKI blocked hCG- or forskolin-stimulated IRS1(Tyr989) and AKT(Thr308) phosphorylation (Fig. 6, A, B, G, H, M, and N). Furthermore, pretreatment with PKI blocked hCG- or forskolin-stimulated IRS1(Thr318) dephosphorylation as well as MYPT1(Thr668), IRS1(Tyr989), and AKT(Thr308) phosphorylation (Fig. 6, C–F, I–L, and O–R). It is worth noting that, in MCF7 cells treated with Myr-PKI, forskolin-stimulated MYPT1(Thr668) phosphorylation was statistically different from vehicle controls (p < 0.05) but significantly inhibited (63.1% ± 5.6% compared with H2O controls, p < 0.05; Fig. 6O). Also, basal phosphorylation of IRS1(Ser318) was decreased in the presence of Myr-PKI in MCF7 cells (Fig. 6P), a result that might reflect secondary effects on pathways that stimulate basal IRS1(Ser318) phosphorylation. Blockade of forskolin- and hCG-stimulated activation of the PI3K pathway by PKI established that forskolin and hCG promoted the cAMP-dependent activation of PKA in MCF7, FRTL, and PO GCs. Together, these data illustrate that cAMP-stimulated activation of the PI3K/AKT pathway required both the IGF1R and PKA in rat PO GCs, human MCF7 breast cancer cells, and rat FRTL thyroid cells.

**Discussion**

GPCRs constitute the largest family of cell surface proteins that transduce extracellular stimuli through complex signaling networks (1, 59). Among the signaling cascades regulated by GPCRs, the PI3K/AKT pathway intersects several vital cell functions, including apoptosis, proliferation, metabolism, growth, and glucose homeostasis (3–5). We utilized ovarian preantral GCs to elucidate how GPCRs can activate the PI3K/AKT pathway downstream of the IGF1R.

Several studies report that the GPCR agonist FSH stimulates activation of the PI3K/AKT pathway in preantral GCs (31–33,

**Figure 4.** PKA phosphorylation of MYPT1 stimulates MYPT1-associated phosphatase activity. A, MYPT1 was isolated by immunoprecipitation from untreated GC lysates. Immunoprecipitates were incubated under cell-free conditions with H2O or recombinant PKA catalytic subunit (C), and subsequent samples were analyzed by immunoblot. The image represents three independent experiments. B and C, GCs were pretreated with H2O or 50 μM Myr-PKI followed by vehicle or FSH for 15 min, and whole-cell lysates were analyzed by immunoblot. The asterisk indicates a significant (*, p < 0.05) or not significant (n.s.) FSH-stimulated MYPT1(Ser668) phosphorylation signal compared with vehicle controls of the same pretreatment. Data are represented as mean ± S.E. (error bars) analyzed by one-way ANOVA and Tukey’s multiple comparisons test (n = 3). **Figure 5.** MYPT1 is sufficient to stimulate IRS1 Ser dephosphorylation, IRS1 YXXM phosphorylation, and PI3K/AKT activation. A, the IRS1-IGF1R complex was isolated by immunoprecipitation (as in Fig. 3A) from lysates of GCs treated with vehicle (v) or FSH (F). Separately, MYPT1 was isolated by immunoprecipitation and treated with H2O (MYPT1 IP) or PKAc (activated MYPT1 IP) (as in Fig. 4, A and D). Isolated IRS1-IGF1R complexes from vehicle-treated lysates were combined with either MYPT1 IP (bound sample, lane 1) or activated MYPT1 IP (bound sample, lane 2) and incubated at room temperature for 3 h in phosphatase assay buffer. Results are representative of two independent experiments. Ab, antibody. B–E, GCs were transfected with plasmids expressing either GFP or a constitutively activate MYPT1 mutant (CA MYPT1) and treated with vehicle or FSH, and total cell lysates were collected for immunoblot analysis. B, representative images. Dotted lines indicate a cropped image from the same exposure. C–E, densitometric quantifications of target phosphorylations relative to load controls are represented as mean signal ± S.E. (error bars). Samples with a significantly different (*, p < 0.05; **, p < 0.01) or not significantly different (n.s.) signal from other treatment groups are indicated based on analysis by one-way ANOVA and Tukey’s multiple comparisons test (n = 3). veh, vehicle.
In a previous study, we reported that GCs secrete low levels of IGF1 that activate the IGF1R but fail to stimulate IRS1 YXXM phosphorylation in the absence of FSH (31). Furthermore, both PKA and PP1 are necessary for FSH-stimulated IRS1 YXXM phosphorylation and PI3K/AKT activation (31). Here we show that PKA activates PP1 via direct phosphorylation of the PP1 regulatory subunit MYPT1. PP1 and MYPT1 associate with the IRS1-IGF1R complex and mediate the dephosphorylation of four Ser residues on IRS1, three of which have been described previously as being inhibitory to PI3K activation (7, 41–43). Importantly, activated PP1c/H9252-MYPT1 mimics FSH-stimulated IRS1 Ser dephosphorylation, IRS1 YXXM phosphorylation, and PI3K/AKT activation. Therefore, our results support the hypothesis (depicted in Fig. 7) that FSH signals via PKA to activate PP1 and promote IGF1R signaling via dephosphorylation of IRS1 Ser residues to facilitate IRS1 YXXM phosphorylation, which activates the PI3K/AKT cascade.

Phosphoproteomic analysis of L6 skeletal muscle cells previously revealed the interaction of MYPT1 and IRS1 (62) as well as the potential role of MYPT1 in insulin responses (63); however, the role of MYPT1 was unknown. We show that CA MYPT1 (in the absence of FSH) stimulates IRS1 Ser dephosphorylation, IRS1 YXXM phosphorylation, and PI3K/AKT activation. Additionally, no significant additive affect was seen when CA MYPT1-expressing GCs were treated with FSH (Fig. 5, B–E, compare lanes 3 and 4), suggesting that the ability of FSH to stimulate IRS1 YXXM phosphorylation and PI3K activation is predominantly mediated by the activation of PP1c/H9252-MYPT1 and the dephosphorylation of IRS1 Ser residues. The inhibitory potential of IRS1 Ser/Thr phosphorylation is supported by decades of research on insulin signaling (reviewed in Refs. 7, 39, 64). Several studies report that mutation of select IRS1 Ser/Thr residues to Ala enhances insulin-stimulated IRS1 YXXM phosphorylation and PI3K/AKT activation (recently reviewed in Ref. 7). Additionally, Ser/Thr phosphorylation of IRS1 by various kinases is correlated with diminished insulin-stimulated PI3K activation (40). However, not all IRS1 Ser/Thr phosphorylation is inhibitory. For example, mutation of IRS1 (Ser1216) (human residue Ser1223) to Ala decreases insulin-stim-
phosphorylation of IRS1(Ser1216) in GCs, suggesting that activation of the insulin receptor (65). Interestingly, FSH stimulated the YXXM phosphorylation, which drives PI3K activation because of the presence of Ser/Thr phosphorylations on IRS1. PKA-activated downstream GPCRs phosphorylate the PP1 regulatory subunit MYPT1 to activate PP1. Activated PP1 then dephosphorylates Ser/Thr residues on IRS1, thereby stimulating IGF1R-dependent IRS1 YXXM phosphorylation to activate the PI3K/AKT pathway. The GPCR agonists FSH or hCG, the adenyllyl cyclase activator forskolin, the PKA inhibitor PKI, a CA MYPT1 mutant, the PP1 inhibitor tautomycin, and the IGF1R inhibitor NVP-AEW541 were utilized in the experiments outlined here.

ululated IRS1 YXXM phosphorylation in CHO cells expressing the insulin receptor (65). Interestingly, FSH stimulated the phosphorylation of IRS1(Ser1216) in GCs, suggesting that activating phosphorylation(s) might contribute to FSH-stimulated PI3K/ AKT activation. We previously reported that PKA promotes the direct phosphorylation of IRS1, although the contribution of this phosphorylation to PI3K/ AKT activation was not directly evaluated (31). Further studies are necessary to understand the integrated regulatory role of multisite IRS1 phosphorylation in various tissue backgrounds.

FSH regulates broad shifts in gene expression that drive the maturation of ovarian preantral follicles to a preovulatory phenotype capable of responding to the surge of LH that triggers ovulation and the resumption of meiosis in oocytes (Ref. 34 and reviewed in Ref. 20). The use of dominant negative or constitutively active AKT mutants or PI3K inhibitors illustrates that the PI3K/ AKT pathway plays a pivotal role in coordinating many vital gene expression responses to FSH in preantral GCs (33, 35, 66–70). FOXO family transcription factors are the predominant transcriptional regulators downstream of AKT that function as either activators or repressors (4, 71). In GCs, FOXO1 regulates ~60% of over 3700 genes up- or down-regulated by FSH (34). Additionally, inactivation of the genes encoding FOXO1 and FOXO3 in murine GCs impairs follicle maturation and fertility (72). Thus, transcriptional responses downstream of the PI3K/ AKT pathway are critical for normal maturation of ovarian follicles and, hence, fertility.

GPCRs activate the PI3K/ AKT pathway in a number of tissues across many mammals, including FSH receptors in preantral GCs, LH/CG receptors in PO GCs, β-adrenergic receptors in human MCF7 breast cancer cells, and TSH receptors in rat thyroid cells (19, 20, 25). Each of these ligand-activated GPCRs can signal via PKA (19, 25, 32). Our results support a conserved mechanism beyond preantral ovarian GCs by which GPCRs/ PKA cross-talk downstream of the IGF1R at IRS1 to activate the PI3K/ AKT pathway. Based on the near-ubiquitous expression of the IGF1R and PKA (73, 74), understanding the mechanism by which PKA can intersect downstream of the IGF1R will likely shed new light on how GPCRs impact normal and aberrant physiological responses. For example, activating mutations that induce tumorigenesis occur in a number of GPCRs that activate PKA, including the receptors for TSH, FSH, LH, neurenomed B, gastrin, and others (1). Therefore, in the context of cancer, the potential to activate PI3K/ AKT through mutated GPCRs may account for the altered metabolism, inhibited apoptosis, and enhanced proliferation associated with tumorigenesis. Thus, the molecular link between GPCRs and the PI3K/ AKT pathway is a valuable contribution to the ongoing development of targeted therapeutic approaches to treating a broad range of cancers and other pathologies (9, 75). Clearly, further studies are warranted to evaluate both the extent to which our proposed model is conserved among PKA-dependent systems and its therapeutic potential.

**Experimental Procedures**

**Materials**—Materials were described previously (31) with the following additions: antibody recognizing total MYPT1 (for immunoblot and immunoprecipitation) was purchased from BD Biosciences. Pregnant mare’s serum gonadotropin and forskolin were purchased from Sigma-Aldrich. Human CG was purchased from Abraxis Pharmaceutical Products. The Enzchek® phosphatase assay kit containing DiFMUP, OptiMEM, and Lipofectamine® 2000 were purchased from Thermo Fisher Scientific, Inc. The following antibodies were purchased from Cell Signaling Technology (primary dilution is indicated): GAPDH (1:1000), phospho-IRS1(Ser318) (1:1000 in 5% BSA + 0.1% Tween), phospho-IRS1(Ser635) (1:750), phospho-IRS1(Ser668) (1:333), and phospho-MYPT1(Ser669) (1:333).

**Granulosa, FRTL, and MCF7 Cell Culture—**Preantral GCs were isolated from 24-day-old immature Sprague-Dawley rats primed with estrogen and cultured under serum-free conditions as described previously (31). PO GCs were isolated from 24-day-old immature rats injected with pregnant mare’s serum gonadotropin 48 h prior to isolation and cultured under serum-free conditions (76). Rats were from a breeder colony (originally from Charles River Laboratories) maintained in accordance with the Animal Welfare Act by protocols approved by the Washington State University Animal Care and Use Committee. Transduction of preantral and PO GCs with adenoviruses followed procedures used previously (67).

FRTL cells were obtained from the ATCC, and MCF7 luminal A subtype, estrogen receptor-positive cells were provided by Dr. Ruth Keri (Case Western Reserve University). FRTL and MCF7 cells were subcultured no more than three times to avoid changes because of passing and were cultured according to protocols established by the ATCC, but insulin was omitted from MCF7 cell complete medium. FRTL cells were plated at ~2 × 10⁶ cells/well in 3 ml of medium and MCF7 cells at ~1 × 10⁶ in 3 ml followed by overnight incubation. Serum-containing medium over FRTL and MCF7 cells was then removed and...
replaced with serum- and insulin-free medium. Cells were then incubated overnight prior to treatments. Notably, FRTL and MCF7 cells were treated with Myr-PKI as opposed to adenoviral PKI because overnight transduction was lethal (data not shown).

**Immunoblot, Immunoprecipitation, and Cell-free PKA Phosphorylation Assay**—Following treatments, cells were collected and processed according to procedures described previously (31, 77).

**Phosphatase Assay**—GCs were treated as indicated and collected in a previously described (31) immunoprecipitation lysis buffer without the Ser/Thr phosphatase inhibitors NaF and Na$_3$P$_2$O$_7$. Pulldown (“bound”) samples were washed three times as described previously (31) and resuspended in 100 µl of 1× phosphatase assay buffer containing 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl. Resuspended immunoprecipitates were loaded into a black 96-well clear-bottom plate (Corning, Inc.) and incubated on a rotator at room temperature for 3 h. Pellets were resuspended in 1 ml of phosphatase assay buffer according to the protocol of the manufacturer and combined with immunoprecipitation samples in the 96-well plate on ice. Phosphatase activity was then assayed at room temperature from 455-nm fluorescence emission (360-nm excitation) measured over time on a Victor X5 multilabel plate reader (PerkinElmer Life Sciences). Accumulation of fluorescence within the linear range was used to calculate phosphatase activity.

**Cell-free Dephosphorylation of IRS1—MYPT1 and IRS1 were isolated by immunoprecipitation using antibodies against MYPT1 and IGF1R, respectively. Immunoprecipitations were separately collected in the absence of NaF and Na$_3$P$_2$O$_7$ as in the phosphatase assay procedure above. Washed MYPT1 pulldown samples were treated with H$_2$O or recombinant PKA catalytic subunit according to previous protocols (31). MYPT1 and IGF1R immunoprecipitations were then resuspended in 1× phosphatase assay buffer, combined, and incubated on a rotator at room temperature for 3 h. Pellets were analyzed by immunoblot.

**Transfection of Preantral GCs**—Prior to plating, 2.5 × 10$^6$ isolated preantral GCs were resuspended in 2 ml of Opti-MEM. Lipofectamine® 2000 and cDNA plasmids were combined according to the protocol of the manufacturer and added to the resuspended GCs, and the combined solution was immediately plated on fibronectin-coated plates. GCs were incubated for 3 days in Opti-MEM/Lipofectamine®/cDNA solution before the medium was removed and replaced with DMEM/F12 medium containing estrogen, penicillin, and streptomycin (31). GCs were incubated for >6 h, treated as indicated, and collected for immunoblot analysis.

**Statistical Analysis**—Densitometry from immunoblots was determined using Quantity One 1-D analysis software (Bio-Rad) and taken from images within the approximate linear range of detection. The densitometry signal was normalized to loading controls. Experiments were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test ($p < 0.05$) or Student’s t test ($p < 0.05$) using Prism software (GraphPad Software, Inc.). Each experimental replicate ($n$) represents an independent experiment performed on GCs collected from ovaries of approximately eight rats. Therefore, each $n$ represents a biological replicate.

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