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# The Luteinizing Hormone-releasing Hormone Inhibits the Anti-apoptotic Activity of Insulin-like Growth Factor-1 in Pituitary αT3 Cells by Protein Kinase Cα-mediated Negative Regulation of Akt*[S]

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**The luteinizing hormone-releasing hormone (LHRH) receptor is a G protein-coupled receptor involved in the synthesis and release of pituitary gonadotropins and in the proliferation and apoptosis of pituitary cells. Insulin-like growth factor-1 receptor (IGF-1R) is a tyrosine kinase receptor that has a mitogenic effect on pituitary cells. In this study, we used the αT3 gonadotrope cell line as a model to characterize the IGF-1R signaling pathways and to investigate whether this receptor interacts with the LHRH cascade. We found that IGF-1 activated the IGF-1R, insulin receptor substrate (IRS)-1, phosphatidylinositol 3-kinase, and Akt in a time-dependent manner in αT3 cells. The MAPK (ERK1/2, p38, and JNK) pathways were only weakly activated by IGF-1. In contrast, LHRH strongly stimulated the MAPK pathways but had no effect on Akt activation. Cotreatment with IGF-1 and LHRH had various effects on these signaling pathways. 1) It strongly increased IGF-1-induced tyrosine phosphorylation of IRS-1 and IRS-1-associated phosphatidylinositol 3-kinase through activation of the epidermal growth factor receptor. 2) It had an additive effect on ERK1/2 activation without modifying the phosphorylation of p38 and JNK1/2. 3) It strongly reduced IGF-1 activation of Akt. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and cell cycle analysis revealed that, in addition to having an additive effect on ERK1/2 activation, cotreatment with IGF-1 and LHRH also had an additive effect on cell proliferation. The LHRH-induced inhibition of Akt stimulated by IGF-1 was completely blocked by Safingol, a protein kinase C (PKC) α-specific inhibitor, and by a dominant negative form of PKCα. Finally, we showed that the inhibitory effect of LHRH on IGF-1-induced PKCα-mediated Akt activation was associated with a marked reduction in Bad phosphorylation and a substantial decrease in the ability of IGF-1 to rescue αT3 cells from apoptosis induced by serum starvation. Our results demonstrate for the first time that several interactions take place between IGF-1 and LHRH receptors in gonadotrope cells.**

Luteinizing hormone-releasing hormone (LHRH)[1] is a hypothalamic decapeptide, which plays a crucial role in normal reproductive function. In the pituitary, LHRH-I (mammalian LHRH) stimulates the synthesis and the release of the gonadotropins, LH and FSH, and promotes cell proliferation and apoptosis in pituitary cells (1, 2). The effects of LHRH-I are mediated by a cell surface receptor (LHRH-R) belonging to the G protein-coupled receptor superfamily (3, 4). LHRH-I binding to its cognate receptor leads to interaction of the receptor with heterotrimeric G proteins, including $G_{q/11}$. These G proteins in turn activate phospholipase C, leading to the production of diacylglycerol and the subsequent activation of protein kinase C (PKC) (5–9). Activation of PKC then leads to activation of downstream protein kinases belonging to the MAPK family (9–15). Activation of LHRH-R also triggers calcium influx, resulting in an increase in intracellular calcium (16) and cAMP levels (17, 18) and leading to the activation of other protein kinases, such as the c-Jun N-terminal kinase (19, 20). This LHRH-R-mediated pathway acts independently of the PKC and MAPK signaling pathways (21). Thus, multiple signal transduction pathways may mediate LHRH-I action in the pituitary gland.

Like LHRH-I, IGF-1 is a mitogen in pituitary cells (22–24) and an anti-apoptotic factor in several cell lines (25, 26). IGF-1 activates the intrinsic protein-tyrosine kinase activity of the IGF-1 receptor (IGF-1R) (27). This activation results in autophosphorylation of the receptor and phosphorylation of various intracellular substrates, including several insulin receptor substrate (IRS) proteins (IRS-1 to -4) and the Src homology collagen protein (28). Tyrosine-phosphorylated IRS-1 is a multisite docking protein for numerous Src homology 2 domain-containing proteins, including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and the adapter protein, Grb2 (29, 30). Downstream effectors of PI3K include the serine/threonine protein kinases, Akt/PKB and p70/S6 (31). The bind-

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[S] The on-line version of this article (available at http://www.jbc.org) contains Fig. 1.

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[1] The abbreviations used are: LHRH, luteinizing hormone-releasing hormone; LHRH-R, LHRH receptor; JNK, c-Jun N-terminal kinase; PI3K, phosphatidylinositol 3-kinase; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; LH, Luteinizing hormone; FSH, follicle-stimulating hormone; EGF, epidermal growth factor; EGFR, EGF receptor; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PH, pleckstrin homology; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; IRS, insulin receptor substrate; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; FAK, focal adhesion kinase.

ing of the IRS and Src homology collagen proteins to Grb2 and its associated guanine nucleotide exchange protein, mSos, leads to the activation of the Ras-Raf-MAPK pathway (27). The role of the different pathways activated by IGF-1 during proliferative and anti-apoptotic responses is cell type-specific. For example, in human intestinal smooth muscle cells (32, 33), IGF-1 induces growth by activating both the PI3K and MAPK-ERK1/2 pathways. In contrast, only the PI3K pathway is essential for IGF-1 signaling in proliferating MCF-7 cell lines (derived from human breast tumor) (34).

IGF-1 and IGF-1R are both expressed in pituitary cells (35). Like LHRH-I, IGF-1 treatment increases LH secretion by male rat pituitary cells (36). Moreover, IGF-1 enhances the LH response to LHRH-I in pituitary cells from male rats (37), pigs (38), fish (39), and sheep (40). IGF-1 also regulates growth in pituitary gonadotrope cells (41). LHRH-I agonists inhibit cell proliferation in prostate cancer cells by interfering with some of the cellular mechanisms required for the mitogenic activity of IGF-1 (42). The degree of interaction between LHRH-I and IGF-1 signaling pathways in the pituitary gland remains unclear. In this study we have investigated these interactions by using $\alpha$T3–1 gonadotrope cells derived from murine pituitary tumor cells. These cells produce the glycoprotein hormone $\alpha$-subunit, the LHRH-R and SF-1. However, these cells do not produce LH$\beta$, as they were derived from cells at an early stage of development during which LH$\beta$ was not produced. Although these cells do not express the $\alpha$, LH$\beta$, and FSH$\beta$ subunits (43, 44), they proliferate and express the LHRH receptor. Thus, $\alpha$T3 cells provide a good model system for determining whether interactions exist between the LHRH-I and IGF-1 signaling pathways.

In this study, we characterized interactions taking place between the IGF-1R and LHRH-R signaling pathways during cell proliferation and apoptosis in $\alpha$T3 cells. We demonstrate for the first time that LHRH-I treatment enhances IGF-1-induced, MAPK-ERK1/2 and PKC signaling pathway-mediated cell proliferation, whereas it strongly diminishes the anti-apoptotic effect of IGF-1 through inhibition of PKC$\alpha$-mediated Akt activation.

## EXPERIMENTAL PROCEDURES

*Materials*—[$\alpha$-$^{32}$P]dCTP (6000 Ci/mmol) and [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) were obtained from PerkinElmer Life Sciences. Recombinant human IGF-1 was purchased from R & D (Minneapolis, MN). Recombinant human LHRH-I, buserelin, and the LHRH antagonist, trifluoroacetate, were purchased from Sigma. Recombinant human EGF was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and trypsin were purchased from Invitrogen. PI3K-specific inhibitor (LY294002) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma. MEK1/2-specific inhibitor (U0126), p38 MAPK-specific inhibitor (SB202190), PKC-specific inhibitor (GF109203X), PKC$\delta$-specific inhibitor (Rottlerin), PKC$\alpha$-specific inhibitor (Safingol), EGF receptor tyrosine kinase-specific inhibitor (AG1470), and the calcium chelator, BAPTA, were obtained from Calbiochem. The JNK1/2 MAPK-specific inhibitor SP600125 was purchased from Biomol Research Laboratories (Plymouth Meeting, MA). All inhibitor stock solutions were prepared in MeSO. These stock solutions were prepared so that the concentration of MeSO added to the culture medium was below 0.1%.

Rabbit polyclonal antibodies against phospho-Bad (phospho-Ser-136), Bad, phospho-Akt (phospho-Ser-473), Akt, phospho-ERK1/2 (phospho-Thr-202/Tyr-204), phospho-p38 (phospho-Thr-180/Tyr-182), phospho-JNK1/2 (phospho-Thr-183/Tyr-185), phospho-GSK3$\alpha/\beta$ (phospho-Ser-21/9), phospho-FKHR (phospho-Ser-256), phospho-Elk-1 (phospho-Ser-383), and cleaved caspase-3 were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibodies to EGFR (1005), phospho-EGFR (Tyr-1173), ERK2 (C14), p38 (C20), JNK2 (N18), IGF-1R (C20), and FAK (C20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to rabbit IRS-1, IRS-2, and the p85 regulatory subunit of mouse PI3K ($\alpha$p85) were purchased from Upstate Biotechnology, Inc. Monoclonal anti-actin (clone AC) and monoclonal anti-phosphotyrosine (PY20) antibodies were obtained from Sigma and Transduction Laboratories (Lexington, KY), respectively. All antibodies were used at 1:1000 dilution.

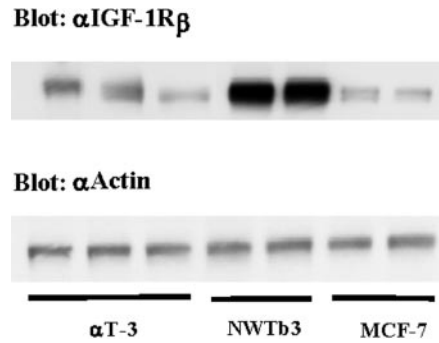


FIG. 1. **IGF-1R expression in $\alpha$T3 cells.** Cells ($\alpha$T3, MCF-7, and NWTb3) were lysed and protein extracts were subjected to SDS-PAGE. Proteins were probed using the IGF-1R$\beta$ antibody. Sample loading was evaluated by reprobing each membrane with an $\alpha$-actin antibody. All samples contained equal amounts of protein.

*Preparation of Rat Anterior Pituitary Cells and Cell Cultures*—Rat pituitary cells were prepared from 5-week-old female Wistar rats. Freshly removed anterior pituitary glands were washed twice in B1 buffer (0.25% bovine serum albumin, 0.01% deoxyribonuclease, 137 mM NaCl, 5 mM KCl, 25 mM HEPES, 0.6 mM $NaH_2PO_4$, 0.7 mM $Na_2HPO_4$ $12H_2O$, and 6 mM glucose) before being incubated with type I collagenase (4 mg/ml) and grade II dispase (2 mg/ml) in buffer B1 at 37 °C for 30 min. Cells were centrifuged for 4 min at 200 × *g* and then incubated with 0.8% type V neuraminidase in B1 buffer for 10 min at 37 °C. After centrifugation (200 × *g*, 4 min), dispersed cells were cultured in DMEM without red phenol and supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 $\mu$g/ml), fungizone (1.25 $\mu$g/ml), and L-glutamine (200 mM).

Mouse gonadotrope $\alpha$T3 cell lines and L$\beta$T2 cells were provided by Dr. P. Mellon (La Jolla, CA). MCF-7, MDA-MB-231, OVCAR, and SKOV cells were obtained from the ATCC (Manassas, VA). All cell lines were routinely cultured in a humidified atmosphere of 95% air and 5% $CO_2$ in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, and 300 $\mu$g/ml L-glutamine.

*Immunoprecipitation and Immunoblotting*—Cell lysates were homogenized on ice in lysis buffer A (10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40) supplemented with various protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 $\mu$g/ml leupeptin, and 10 $\mu$g/ml aprotinin) and phosphatase inhibitors (100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate). Lysates were incubated on ice for 30 min and then centrifuged at 12,000 × *g* for 20 min at 4 °C. The protein concentration in the resulting supernatants was then determined using the BCA protein assay. Extracts containing 200 $\mu$g of protein were incubated with $\alpha$IGF-1R, $\alpha$IRS-1, or $\alpha$IRS-2 (1:1000 dilution) for 16 h at 4 °C. Immune complexes were precipitated by incubation with protein G-agarose for 1 h at 4 °C as described previously (45). Immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked by incubation for 1 h at room temperature with 5% nonfat milk dissolved in Tris-buffered saline supplemented with 0.1% Tween 20 and then probed with the appropriate antibodies. After extensive washing, immune complexes were detected using horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) and an enhanced chemiluminescence (ECL) detection system. The radiographs were scanned, and the optical density of each band was measured using the MacBas version 2.52 software (Fuji PhotoFilm). Western blotting was performed without immunoprecipitation using cell extracts containing 50 $\mu$g of protein.

*PI3K Assay*—PI3K activity was determined as described previously (45). Cell lysates were prepared on ice in extraction buffer B (20 mM Tris (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM $Na_3VO_4$, 1% Nonidet P-40, 10% glycerol (v/v), 2 mM phenylmethylsulfonyl fluoride, and 10 $\mu$g/ml aprotinin in phosphate-buffered saline (PBS)). Cell lysates were cleared by centrifugation for 35 min at 40,000 × *g* at 4 °C. IRS-1, IRS-2, IGF-1R, or p85 were immunoprecipitated by incubating aliquots (containing 200 $\mu$g of total protein) of each of the supernatants with the appropriate antibodies (1:1000) overnight at 4 °C. Immunoprecipitates were collected using protein G-agarose beads and washed as follows: once in PBS supplemented with 1% Nonidet P-40 and 100 $\mu$M $Na_3VO_4$; twice in a buffer containing 100 mM Tris-HCl (pH 7.5), 500 mM

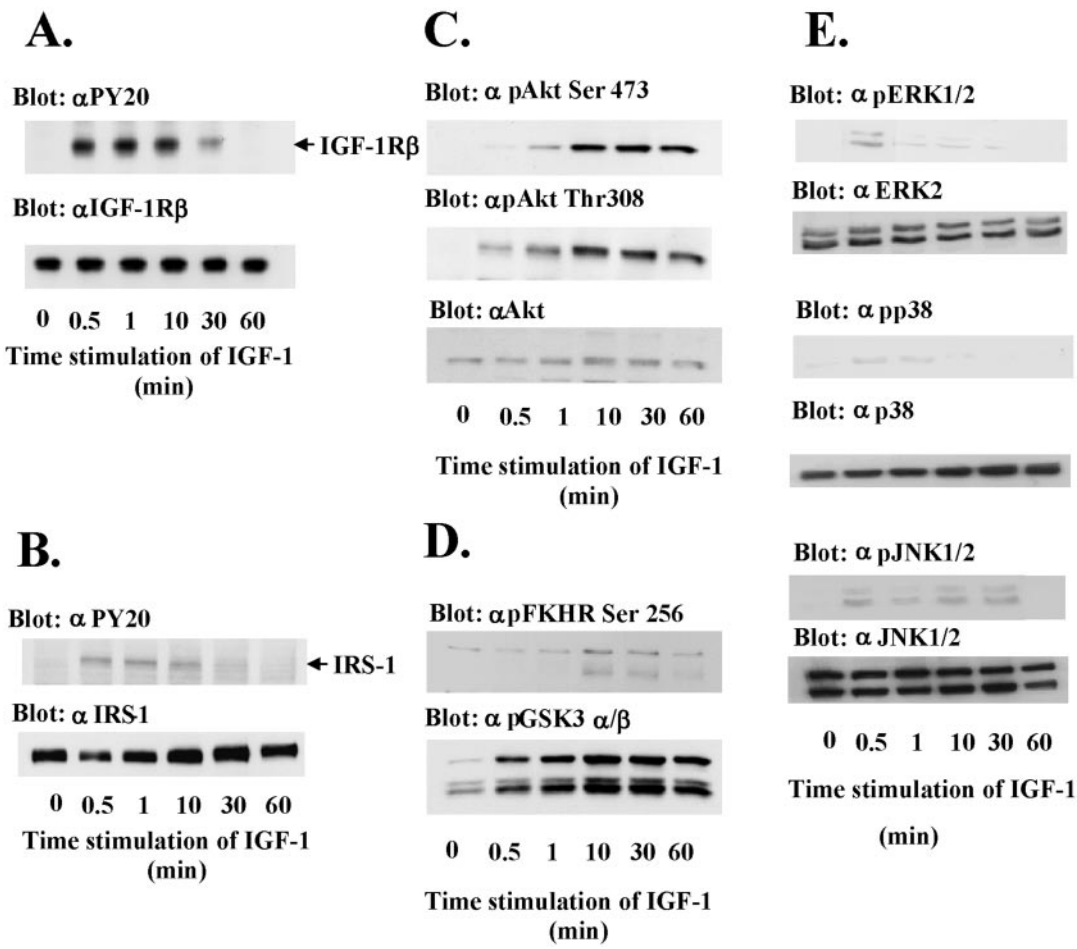


FIG. 2. **IGF-1 signaling pathways in αT3 cells.** Time course of IGF-1-stimulated IGF-1R (*A*), IRS-1 (*B*), Akt (*C*), GSK3α/β and FKHR (*D*), and MAPKs (ERK1/2, p38, and JNK1/2) (*E*) activation in αT3 cells. αT3 cells were incubated in serum-free DMEM overnight before treatment with 10 nM IGF-1 for 0, 0.5, 1, 10, 30, or 60 min. Whole cell lysates were separated by SDS-PAGE and immunoblotted with different antibodies (phosphotyrosine, phospho-MAPK (ERK1/2, p38, and JNK1/2), phospho-Akt Ser and Thr, phospho-GSK3α/β, and phospho-FKHR). The blots were then stripped and reblotted with appropriate antibodies to determine total proteins loading. The blots are typical examples of an experiment that was replicated three to four times.

$LiCl_2$, 100 μM $Na_3VO_4$; and finally once in a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, and 100 μM $Na_3VO_4$. Precipitates were then resuspended in 40 μl of a buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. $MnCl_2$ (10 μl of a 100 mM stock solution) and phosphatidylinositol (20 μg) were then added to each sample. PI3K assays were initiated by adding 10 μl of a 440 μM ATP solution containing 30 μCi of [γ-$^{32}$P]ATP. Reactions were performed at room temperature and allowed to proceed for 10 min. Reactions were stopped by adding 20 μl of HCl (8N) and 160 μl of $CHCl_3$/$CH_3OH$ (1:1). The reaction mixtures were then centrifuged at 3,000 × *g* for 4 min at 4 °C. The resulting organic phase was collected and subjected to silica gel TLC. TLC plates were developed by incubation in $CHCl_3$/$CH_3OH$/$H_2O$/$NH_4OH$ (120:94:22.6:4) and then left to dry. Radioactivity was quantified using a PhosphorImager (Storm, Amersham Biosciences).

*Akt and ERK1/2 MAPK Activity Assays*—Akt and ERK1/2 MAPK kinase activities were measured using the appropriate assay kits (Cell Signaling Technology, Beverly, MA). αT3 cells were stimulated as described in the figure legends and lysed using cell lysis buffer. Cell lysates containing 200 μg of total protein were immunoprecipitated by incubation with immobilized Akt or phospho-ERK1/2 monoclonal antibodies, with gentle rocking for 3 h at 4 °C. After two washes with cell lysis buffer followed by two washes with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM $Na_3VO_4$, 10 mM $MgCl_2$), the immunoprecipitates were resuspended in 40 μl of kinase buffer supplemented with 200 μM ATP and 1 μg of GSK-3 or Elk-1 fusion proteins. Samples were incubated for 30 min at 30 °C. The reaction was terminated by adding 20 μl of 3× SDS loading buffer. Samples were analyzed by probing with the phospho-GSK-3α/β (Ser-21/9) or phospho-Elk-1 (Ser-383) antibodies.

*Cell Proliferation Assay*—For growth studies, αT3 cells were seeded in 96-well plates (10,000 cells per well) in DMEM supplemented with 10% of FBS. After 1 day, cells were transferred to serum-free medium and allowed to grow for 24 h before being incubated with various stimulants (see figure legends for details). Growth was analyzed using the MTT assay as described previously (45).

*Flow Cytometry and Cell Cycle Analysis*—αT3 cultures, in which ~75% of the cells were confluent, were washed and transferred to serum-free DMEM and allowed to grow for 24 h. Cells were subjected to various treatments (see figure legends for details). After treatment with IGF-1 (10 nM) and/or LHRH (10 nM) for 18 h, cells were trypsinized, centrifuged, and washed twice with PBS. Cell pellets were resuspended in citrate buffer (250 mM sucrose, 40 mM trisodium citrate (pH 7.6), 5% $Me_2SO$) and stored at −70 °C. Nuclei were obtained by incubating the cells first in 300 μl of solution A (3.4 mM trisodium citrate (pH 7.6), 1 mM Tris, 3 mM spermine tetrahydrochloride, 0.2% Nonidet P-40, 100 μg/ml trypsin) for 5 min at room temperature and then in 300 μl of solution B (3.4 mM trisodium citrate (pH 7.6), 1 mM Tris, 3 mM spermine tetrahydrochloride, 0.2% Nonidet P-40, 100 μg/ml ribonuclease A, and 500 μg/ml trypsin inhibitor) for 5 min at room temperature. Nuclei were stained with 30 μl of propidium iodide solution (1 mg/ml), and DNA staining was analyzed by flow cytometry using a FACSCalibur system with the CellQuest software. Cell cycle analysis was performed using the ModfitLT™ software (version 2).

*Cell Death Assays*—Apoptotic cells were detected by using terminal dUTP nick-end labeling assay with ApopTag Plus Peroxidase, according to the manufacturer's instructions (Intergen Co., Purchase, NY). One thousand cells were counted, and the values obtained were used to calculate the percentage of labeled cells.

*Transient Transfection of MCF-7 and αT3 Cells*—MCF-7 and αT3 cells were plated out the day before transfection. Cells were transiently transfected using the LipofectAMINE transfection reagent (Invitrogen)

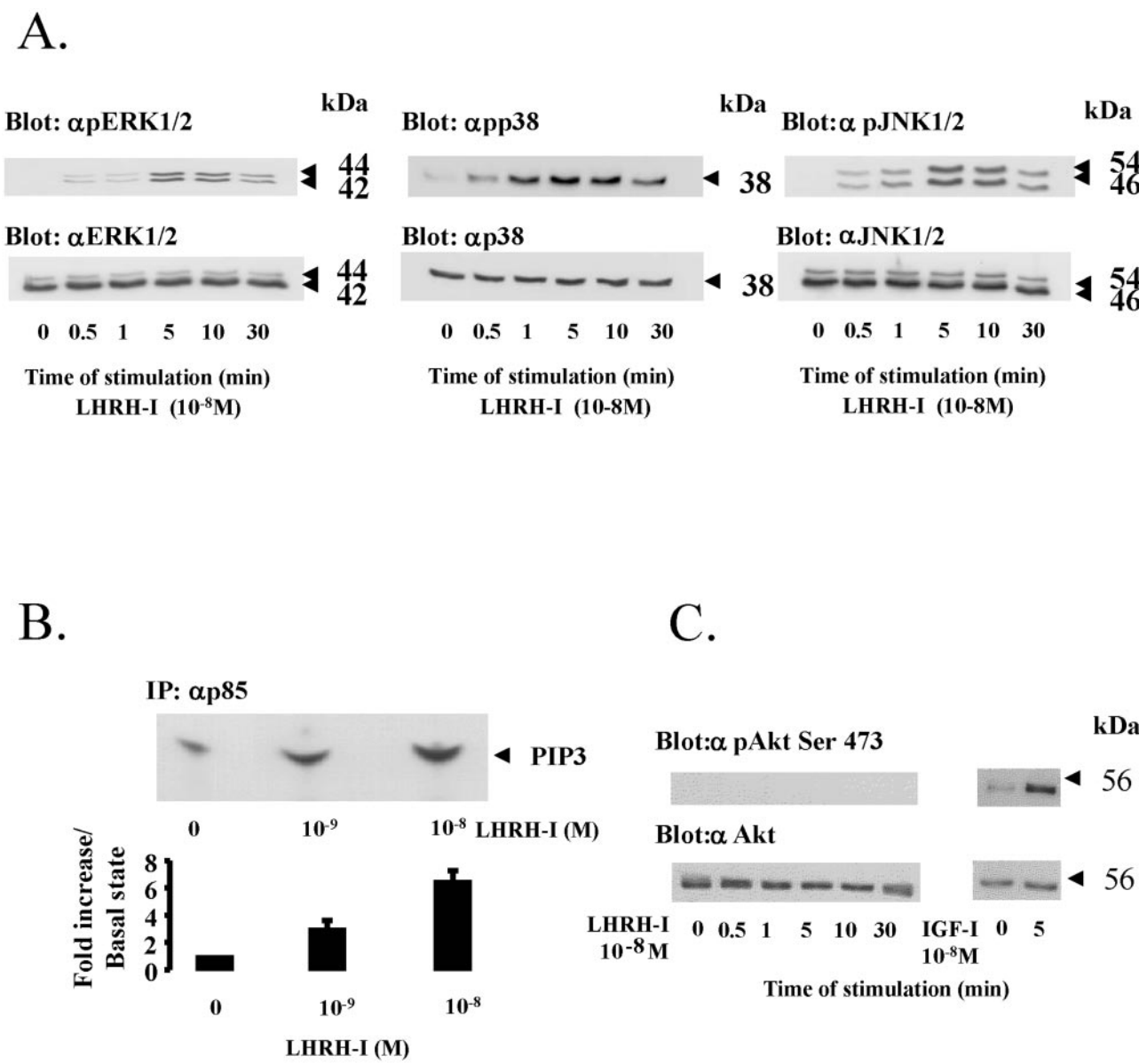


FIG. 3. **LH-RH type I activates MAPK family and PI3K in αT3 cells.** *A*, time course of LHRH type I-mediated activation of ERK1/2, p38, and JNK1/2. αT3 cells were incubated in serum-free DMEM overnight and then treated with LHRH-I (10 nM) for the different periods of time indicated. Whole cell lysates were subjected to SDS-PAGE, and proteins were detected using the appropriate antibodies (phospho-ERK1/2, phospho-p38, and phospho-JNK1/2). Sample loading was assessed by stripping and reprobing the membranes with antibodies to ERK1/2, p38, and JNK1/2. The blots shown are representative of those obtained from three or four independent experiments. *B*, PhosphorImager analysis of $^{32}$P-labeled inositol 1,3,4,5-triphosphate binding to p85, the regulatory subunit of the PI3K, in LHRH-I-treated αT3 cells. The results shown are representative of those obtained from three independent experiments. Values are the mean ± S.E. *C*, a comparison of LHRH-I-mediated and IGF-1-mediated activation of Akt phosphorylation in αT3 cells.

according to the manufacturer's instructions. Cells were transfected with 1 μg of each DNA construct (mouse LHRH-R construct for MCF-7 cells and PKCα DN or focal adhesion kinase dominant negative for αT3 cells) or 1 μg of empty pcDNA3.1 vector (negative control). Twenty four hours after transfection, cells were transferred to serum-free DMEM and allowed to grow for 16 h before being stimulated with various ligands, see figure legends for details. Cells were then washed with ice-cold PBS, and total protein was extracted.

*Statistics*—Data were analyzed by one-way analysis of variance using the Statview 5.0 software. Values are expressed as means ± S.E. Values of $p < 0.05$ were considered statistically significant.

## RESULTS

*IGF-1R Signaling Pathways in αT3 Gonadotrope Cells*—We first determined whether αT3 cells expressed the IGF-1 receptor (Fig. 1). Immunoblot analysis using an antibody recognizing the β-subunit of the IGF-1R revealed that αT3 cells did express the IGF-1R. NWTb3 (NIH-3T3 overexpressing the human IGF-1R) (46) and the human breast cancer cell line, MCF-7, were used as positive controls (Fig. 1). We then investigated the functionality of the IGF-1 receptor in αT3 cells. Serum-starved cells were exposed to 10 nM IGF-1 for different times (Fig. 2). We found that IGF-1 induced a rapid and substantial increase in tyrosine phosphorylation of the IGF-1R β-subunit (15-fold increase after 1 min of treatment) and of IRS-1 (5-fold increase after 1 min of treatment). Tyrosine phosphorylation levels then declined over the next 30 min. IGF-1R is involved in two main signaling pathways: the PI3K/Akt pathway and the MAPK (ERK1/2, p38, and JNK1/2) pathway (28). Thus, we tested whether these kinases were activated in response to treatment of αT3 cells with IGF-1. IGF-1 stimulated PI3K activity (data not shown) and resulted in a 5-fold increase in the levels of phosphorylated Ser-473 and Thr-308 Akt after 1 min of treatment. The level of phosphorylated Akt increased with the

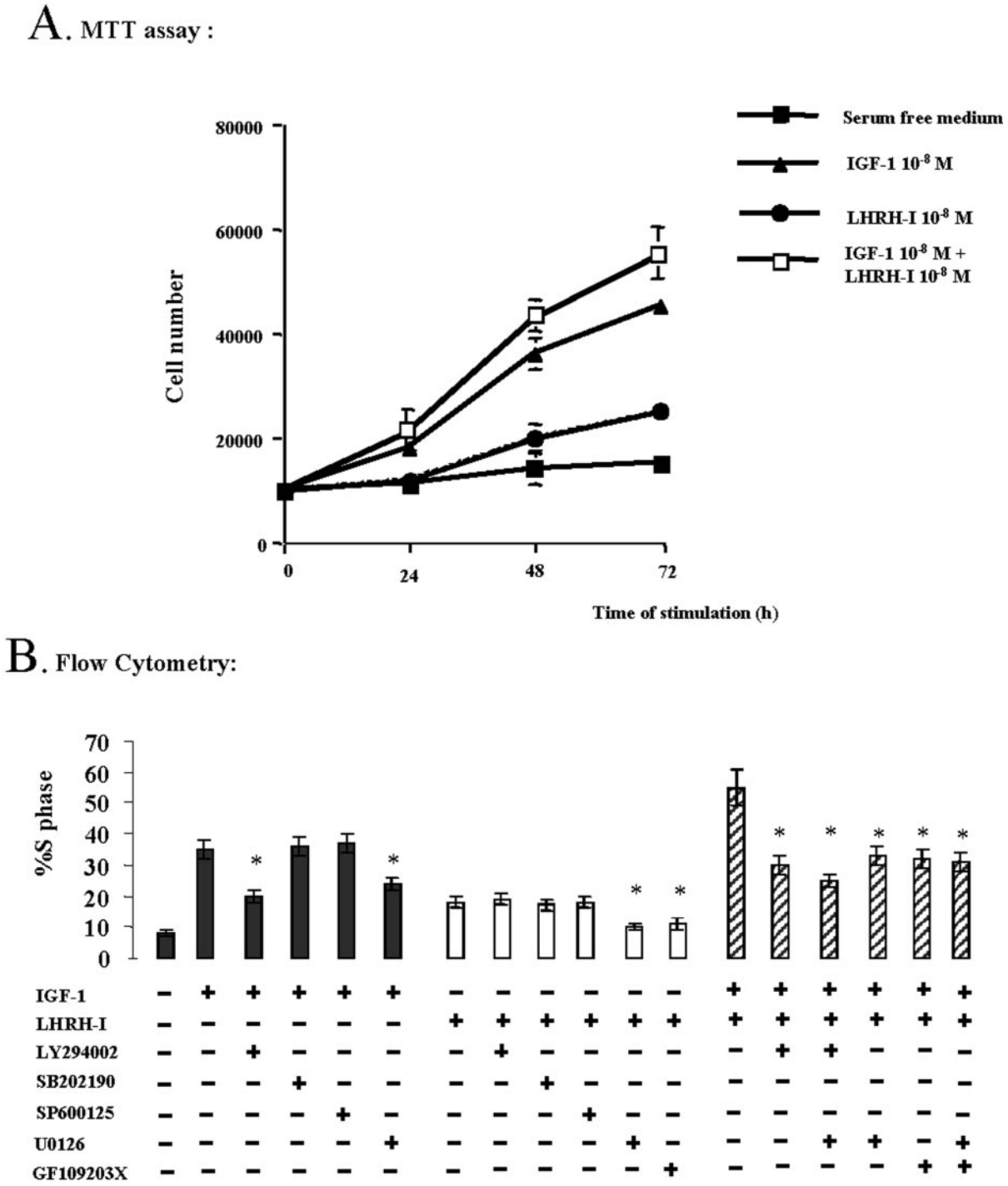


FIG. 4. *A*, additive effect of IGF-I and LHRH-1 on cell growth in αT3 cells. αT3 cells were maintained for 3 days in serum-free medium in the absence or presence of IGF-1 (10 nM), LHRH-I (10 nM), or a combination of these hormones. Cell number was determined indirectly by MTT assays. Values are the mean cell number (±S.E.) from three independent experiments. Five measurements were performed per experiment for each condition. *B*, determination of the intracellular signaling pathways involved in IGF-1- and LHRH-I-induced αT3 cell proliferation. Serum-starved αT3 cells were preincubated for 1 h with the following inhibitors: 50 μM LY294002, 1 μM U0126, 50 μM SB202190, 50 μM SP600125, or 10 nM GF109203X and then incubated in the absence or presence of IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both stimulants for 18 h. The percentage of cells in S phase was determined after each treatment. The *error bars* represent the mean ± S.E. from three independent experiments. * indicates $p < 0.05$ (significant difference between the effect of IGF-1 alone, LHRH-1 alone, or IGF-1 and LHRH-1, and the effect of these treatments plus the various inhibitors).

length of the treatment, reaching a maximum of 20-fold after 10 min (Fig. 2*C*). IGF-1 also stimulated phosphorylation of two downstream targets of Akt: the transcription factor FKHR and the kinase GSK3 α/β (Fig. 2*D*). Although the MAPKs, ERK1/2, p38, and JNK1/2, were present in the αT3 extracts, we detected only a small increase in the level of phosphorylation of these kinases in response to IGF-1 (Fig. 2*E*). Thus, our results indicate that αT3 cells are equipped with a functional IGF-1 receptor.

*LHRH-I Signaling Pathways in αT3 Gonadotrope Cells*—Previous studies (48) have shown that αT3 cells express a functional LHRH receptor. We found that, unlike IGF-1, treatment with LHRH-I (10 nM) strongly activated the ERK1/2, p38, and JNK1/2 MAP kinases in these cells (Fig. 3*A*). LHRH-1

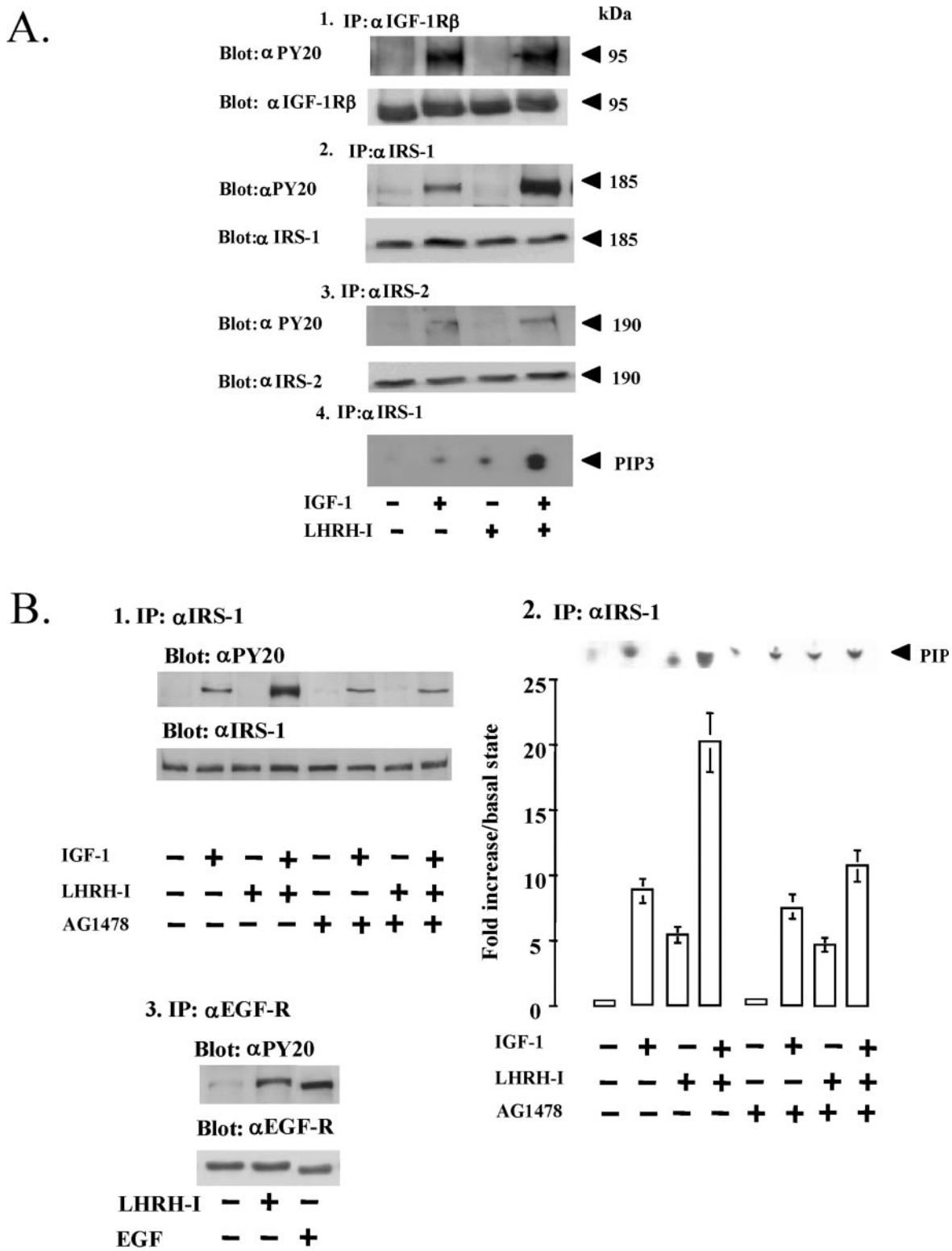


FIG. 5. **Effect of IGF-1 and LHRH-I on IRS-1-associated PI3K activity and the phosphorylation of IRS-1, IRS-2, and the IGF-1Rβ subunit.** *A,* serum-starved αT3 cells were stimulated with either IGF-1 (10 nM), LHRH-I (10 nM), or both agents for 5 min. Cells were lysed, and tyrosine phosphorylation of IRS-1, IRS-2, and the IGF-IRβ was determined. IGF-1Rβ (*panel 1*), IRS-1 (*panel 2*), and IRS-2 (*panel 3*) were immunoprecipitated (*IP*) from whole cell lysates. Samples were then subjected to Western blotting with antibodies recognizing phosphotyrosine (PY20, *panels 1–3*). IGF-1Rβ, IRS-1, and IRS-2 levels were evaluated by reprobing the membranes with αIGF-1Rβ, αIRS-1, and αIRS-2 antibodies. The results presented are representative of those obtained from three independent experiments. Typical results from the PhosphorImager analysis of $^{32}$P-labeled inositol 1,3,4,5-triphosphate binding to IRS-1 are shown in *panel 4*. *B,* role of the EGFR tyrosine kinase in the LHRH-I-mediated increase in IGF-1-induced IRS-1 phosphorylation and IRS-associated PI3K activity. *Panels 1* and *2,* serum-starved αT3 cells were either left untreated or preincubated with AG1478 (10 μM) for 1 h before stimulation with either IGF-1 (10 nM), LHRH-I (10 nM), or both agents for 5 min. IRS-1 phosphorylation and IRS-1-associated PI3K activity were determined as described above for *A. Panel 3,* LHRH-I-mediated activation of the EGFR tyrosine kinase. Serum-starved αT3 cells were stimulated with either EGF (10 nM) or LHRH-I (10 nM). Cells were lysed, and the levels of EGFR tyrosine phosphorylation were determined. EGFR was immunoprecipitated from whole cell lysates. Samples were then subjected to Western blotting with antibodies to phosphotyrosine. EGFR levels were determined by reprobing the membranes with αEGFR antibodies. The results presented are representative of three independent experiments.

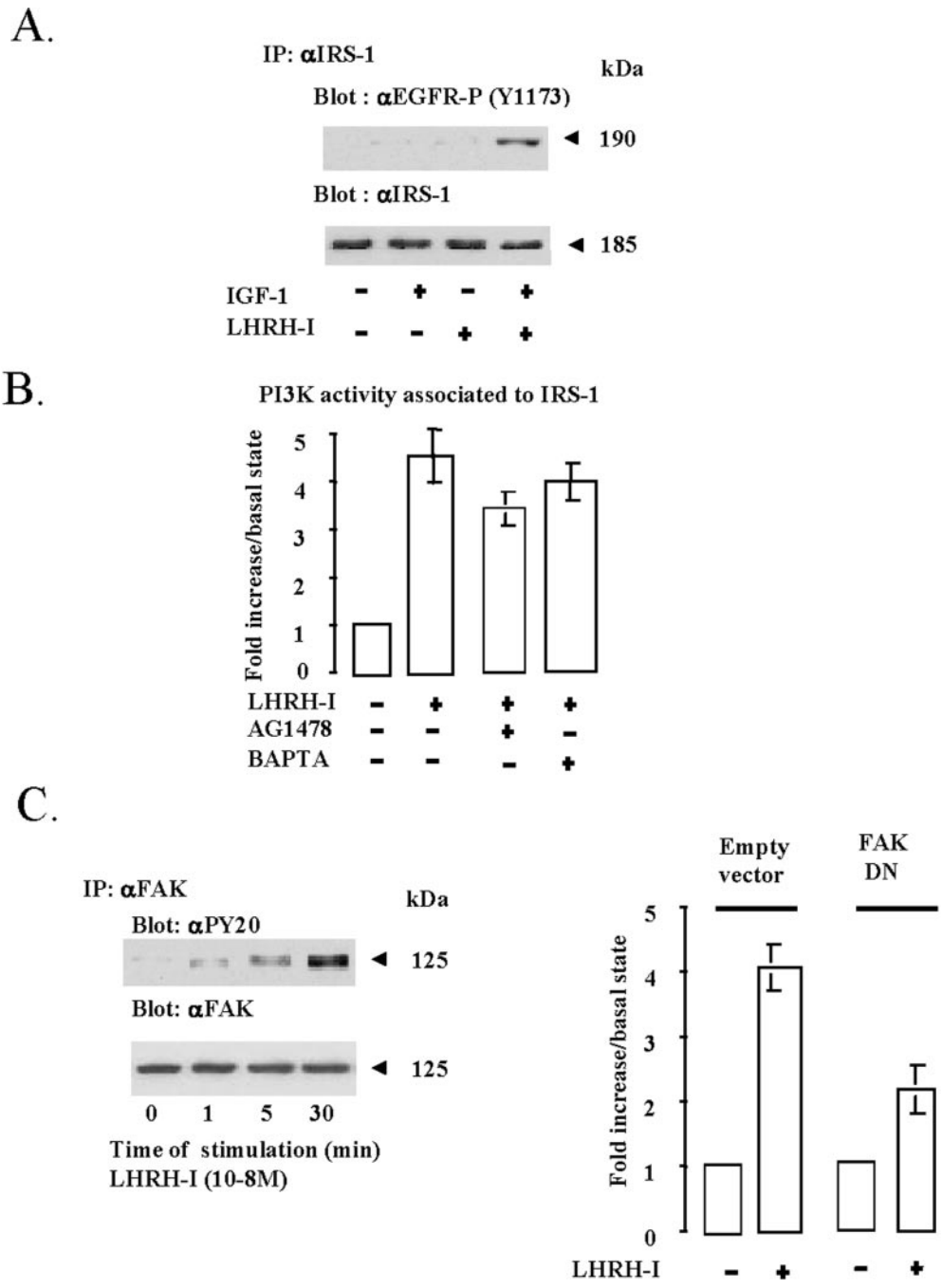


FIG. 6. **Role of the p125 FAK in the LHRH-I effect on the PI3K activity associated to IRS-1.** *A*, coimmunoprecipitation of IRS-1 with the tyrosine-phosphorylated EGF receptor. Serum-starved αT3 cells were stimulated with IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents for 5 min. Cells were lysed, and IRS-1 was immunoprecipitated from whole cell lysates. Samples were then subjected to Western blotting with antibodies to the tyrosine (residue 1173)-phosphorylated EGFR. IRS-1 levels were evaluated by reprobing with αIRS-1. The results shown are representative of those obtained with three independent experiments. *B*, role of calcium and the EGFR in the effect of LHRH-I on IRS-1-associated PI3K activity. Effect of AG1478 (10 μM) and the calcium chelator, BAPTA (10 μM), on LHRH-I-induced IRS-1-associated PI3K activity. Cells were incubated in the presence or absence of the inhibitors for 1 h and then stimulated with LHRH (10 nM) for 5 min. Cells were lysed, and IRS-1-associated PI3K activity was determined as described in Fig. 5*A*. *C*, LHRH-I activation of p125 FAK (*right panel*). Serum-starved αT3 cells were either left untreated or stimulated with LHRH-I (10 nM) for 1, 5, or 30 min. Cells were lysed, and the level of FAK tyrosine phosphorylation was determined. FAK was immunoprecipitated (*IP*) from whole cell lysates. Samples were then subjected to Western blotting with antibodies to phosphotyrosine. FAK levels were evaluated by reprobing the membrane with an αFAK antibody. The results shown are representative of those obtained in three independent experiments. *Left panel,* effect of a FAK dominant negative construct on LHRH-I-induced IRS-1-associated PI3K activity. αT3 cells were transiently transfected with either a dominant negative of FAK (*FAK DN*) construct or the empty vector (negative control). Serum-starved αT3 cell transformants were stimulated for 5 min with IGF-1 (10 nM), LHRH-I (10 nM), or with a combination of both agents. Cells were lysed, and the level of IRS-1-associated PI3K activity was determined as described in Fig. 5*A*. Values (mean ± S.E., $n = 3$) are presented fold differences between stimulated and unstimulated cells.

treatment resulted in a significant increase in PI3K activity (Fig. 3*B*), but it did not affect Akt phosphorylation (Fig. 3*C*). These results demonstrate that the LHRH and IGF-1 receptors share some signaling pathways in αT3 cells.

*Effect of a Combination of IGF-1 and LHRH-I on αT3 Cell Growth*—We examined the effect of 3-day treatments with IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents on αT3 cell growth. Cell proliferation was measured indirectly

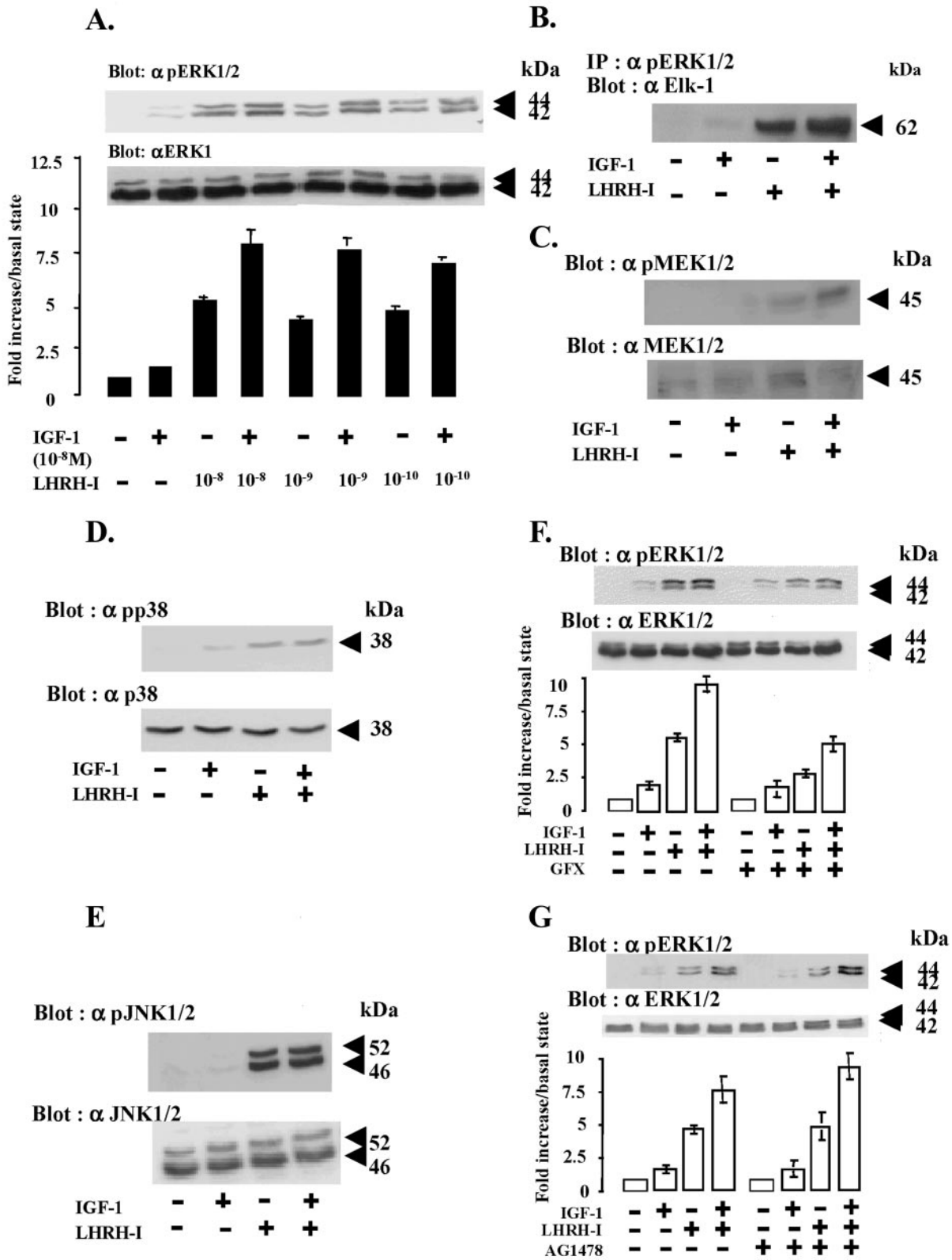


FIG. 7. **Additive effect of IGF-1 and LHRH-I on activation of the ERK1/2 MAPK in αT3 cells.** Serum-starved αT3 cells were stimulated for 5 min with different concentrations (1, 10. or 100 nM) of LHRH-I or IGF-1 (10 nM) or a combination of both agents. *A*, ERK1/2 phosphorylation. Cells were lysed and proteins separated by SDS-PAGE. Proteins were detected using antibodies to phospho-ERK1/2 and ERK1. The ERK1 cross-reacts to a small extent with ERK2. The values (mean ± S.E., $n = 3$) are expressed as fold differences between stimulated and unstimulated cells. *B*, ERK1/2 activity. The activity of the ERK1/2 MAPK was measured by using the Elk-1 fusion protein as a substrate. The levels of phosphorylated Elk-1 were evaluated by SDS-PAGE, transferred to a nitrocellulose membrane, and then probed with a phospho-Elk-1 antibody. *C*, MEK1/2 phosphorylation. Whole cell lysates were subjected to SDS-PAGE, and then proteins were detected using antibodies to phospho-MEK1/2 and MEK1/2. The results shown are representative of those obtained in three independent experiments. *D* and *E*, MAPK p38 and phosphorylation of the p38 JNK1/2 MAPKs. Whole cell lysates were subjected to SDS-PAGE, and proteins were probed with antibodies to phospho-p38 and p38 (*E*) or with antibodies to phospho-JNK1/2 and JNK1/2 (*F*). The results shown are representative of those obtained with three independent experiments. *F* and *G*, signaling pathways involved in LHRH-I induced the MAPK ERK1/2 phosphorylation. Serum-starved αT3 cells were

by performing MTT assays, and the percentage of cells in S phase was determined by flow cytometry. We found that cell proliferation increased by 1.5-, 3.2-, and 4.2-fold after 24, 48, and 72 h of treatment with IGF-1, respectively (Fig. 4*A*). LHRH-I also induced cell proliferation but to a lesser extent than IGF-1; the total number of cells increased by only 1.2-, 1.9-, and 2.2-fold after 24, 48, and 72 h of LHRH-I treatment, respectively. Treatment with both IGF-1 and LHRH-I for 48 or 72 h had an additive effect on cell proliferation (Fig. 4*A*).

To investigate the molecular mechanisms involved in the additive effect of LHRH-I and IGF-1 on cell proliferation, we analyzed αT3 cell growth after exposure to pharmacological inhibitors. Serum-starved cells were pretreated for 1 h with the PI3K inhibitor LY294002 (50 μM), the MEK inhibitor U0126 (1 μM), the p38 inhibitor SB202190 (50 μM), the JNK1/2 inhibitor SP600125 (50 μM), or the PKC inhibitor GF109203X (10 nM) and then treated with IGF-1 (10 nM), LHRH-I (10 nM), or both stimulants for 18 h. The optimal concentration of inhibitor necessary to block the targeted signaling pathway in αT3 cells was determined for each compound (data not shown). The percentage of cells in S phase was significantly lower for cells treated with LHRH-I and the MEK and PKC inhibitors than for cells treated with LHRH-I alone (Fig. 4*B*). The percentage of cells in S phase was lower for cells treated with IGF-1 and the PI3K and MEK inhibitors than for cells treated with IGF-1 alone. In agreement with these results, we found that cell proliferation was lower for cells treated with both IGF-1 and LHRH-I and the PI3K and MEK inhibitors than for cells treated with only IGF-1 and LHRH-I. No difference in the percentage of cells in S phase was observed between cells treated with either the PI3K inhibitor or the MEK inhibitor and cells treated with both inhibitors (Fig. 4*B*). Taken together, our results indicate that in the αT3 cell line, LHRH-I induces cell proliferation through the MAPK ERK1/2 and PKC signaling pathways, whereas IGF-1 stimulates cell proliferation mainly through the PI3K/Akt and MAPK ERK1/2 pathways.

*Effects of LHRH-I on IGF-1 Receptor Signaling in αT3 Gonadotrope Cells*—We then investigated whether any interactions took place between the various components of IGF-1 and LHRH-I receptor signaling cascades. αT3 cells were starved overnight and then exposed to IGF-1 (10 nM), LHRH-I (10 nM), or both stimulants for 5 min. PI3K activity and the phosphorylation state of IRS-1, IRS-2, and the IGF-1R were then determined (Fig. 5*A*). Treatment with IGF-1 (10 nM, 5 min) resulted in phosphorylation of the IGF-1R β subunit (Fig. 5*A, panel 1*), IRS-1 (Fig. 5*A, panel 2*), and IRS-2 (Fig. 5*A, panel 3*), whereas treatment with LHRH-I alone (10 nM, 5 min) did not promote phosphorylation of these proteins. However, the levels of IRS1 phosphorylation and PI3K activity were higher in cells cotreated with IGF-1 and LHRH-I than in cells treated with IGF-1 alone (Fig. 5*A, panel 4*). Most surprisingly, cotreatment with IGF-1 and LHRH-I did not have a synergistic or additive effect on IGF-1R and IRS-2 phosphorylation (Fig. 5*A, panels 1* and *2*).

It is well established that transactivation of receptor tyrosine kinases, such as the EGF receptor, contributes to G protein-coupled receptor-mediated ERK1/2 activation in some cell types (49). As αT3 cells express the EGF receptor, we used this cell type to evaluate whether the EGF receptor was involved in the synergistic effect of LHRH-I on IGF-1-induced IRS-1 tyrosine phosphorylation and IRS-1-associated PI3K activity. We pretreated cells with AG1478 (10 μM), an EGF receptor tyrosine kinase-specific inhibitor, for 1 h and then stimulated them for 5 min by adding LHRH-I (10 nM), IGF-1 (10 nM), or a combination of the two agents. The increase in IGF-1-induced IRS-1 tyrosine phosphorylation and IRS-1-associated PI3K activity was less dramatic for cells treated with LHRH-I and AG1478 than for cells treated with LHRH-I alone (Fig. 5*B, panels 1* and *2*). These results indicate that these two phenomena were dependent on transactivation of the EGF receptor. Moreover, LHRH-I also stimulated EGF receptor tyrosine phosphorylation in αT3 cells (Fig. 5*B, panel 3*). Immunoprecipitation studies and immunoblot analysis revealed that IRS-1 and the activated (tyrosine-phosphorylated) EGFR were more tightly associated in cells cotreated with LHRH-1 and IGF-1 than in cells treated with only one of the stimulants (Fig. 6*A*). These data demonstrate that transactivation and phosphorylation of the EGF receptor are critical for the synergistic effect of LHRH-I on IGF-1-induced IRS-1-associated PI3K activation in αT3 cells.

We then determined whether a calcium chelator (BAPTA) and a dominant negative focal adhesion kinase (p125 FAK) modified the effect of LHRH-I on IRS-1-associated PI3K activity. BAPTA did not alter the response to LHRH-I (Fig. 6*B*). In contrast, LHRH-I induced up-regulation of IRS-1-associated PI3K activity to a lesser extent in cells transformed with the FAK mutant than in control cells (Fig. 6*C, right panel*). Moreover, we found that the level of FAK tyrosine phosphorylation was higher in LHRH-I-treated αT3 cells than in unstimulated cells (Fig. 6*C, left panel*). These data indicate the following. 1) LHRH-I stimulates IRS-1-associated PI3K activity without increasing IRS-1 phosphorylation. 2) This effect is inhibited by the presence of a dominant negative FAK but not by BAPTA or AG1478. 3) LHRH-I enhances FAK phosphorylation. 4) In the presence of IGF-1, LHRH-I enhances both IRS-1 phosphorylation and IRS-1-associated PI3K activity through transactivation of the EGF receptor. 5) LHRH-I stimulation transactivates the EGF receptor.

We also examined the effects of IGF-1 and LHRH-I on the activation of several MAP kinases (ERK1/2, p38, and JNK1/2, Fig. 7) and Akt (Fig. 8). As shown in Fig. 7*A*, The levels of ERK1/2 phosphorylation were higher in αT3 cells treated with LHRH-I for 5 min than in unstimulated cells. In contrast, IGF-1 had only a small effect on ERK1/2 phosphorylation. Treatment with both IGF-1 and LHRH-I had an additive effect on ERK1/2 phosphorylation (Fig. 7*A*). This additive effect was confirmed by measuring MAPK activity using Elk-1 as a substrate (Fig. 7*B*). Cotreatment had a similar effect on MEK1/2 phosphorylation (Fig. 7*C*). However, treatment with both LHRH-I and IGF-1 did not have an additive effect on p38 and JNK1/2 phosphorylation (Fig. 7, *D* and *E*).

We then treated the cells with AG1478 (10 μM) and the PKC inhibitor GF109203X (10 nM) to investigate whether the EGF receptor and the PKC signaling pathway were involved in LHRH-I-mediated activation of the ERK1/2 MAPKs and in the additive effect of LHRH on IGF-1 stimulation of ERK1/2 phosphorylation. AG1478 had no effect on LHRH-I stimulation (Fig. 7*F*). However, LHRH-I stimulation of ERK1/2 phosphorylation and LHRH-I up-regulation of IGF-1-induced stimulation of MAPK ERK1/2 phosphorylation occurred to a lesser extent in cells treated with the PKC inhibitor than in control cells (Fig. 7*G*, $p < 0.05$). Thus, our results suggest that the PKC signaling

preincubated in the presence or absence of AG1478 (10 μM, *F*) or GF109203X (10 nM, *G*) for 1 h prior to stimulation with IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents for 5 min. Cells were lysed and proteins separated by SDS-PAGE. Proteins were detected by probing with antibodies to phospho-ERK1/2 and ERK1. Values (mean ± S.E., $n = 3$) are expressed as fold differences between stimulated and unstimulated cells. *IP*, immunoprecipitation.

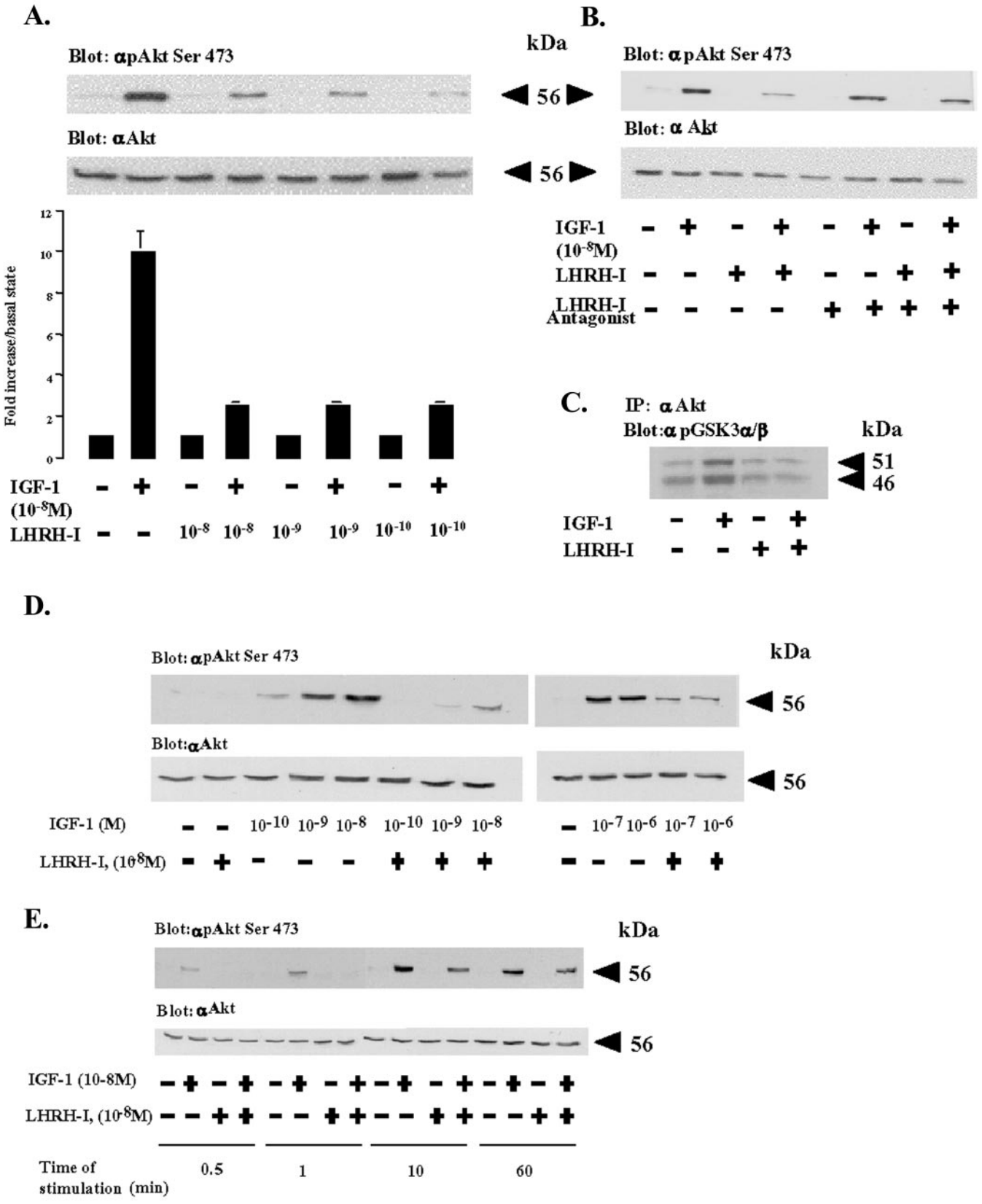


FIG. 8. **LHRH-I-mediated inhibition of IGF-1-induced Akt activation in αT3 cells.** Serum-starved αT3 cells were stimulated with different concentrations (0.1, 1, or 10 nM) of LHRH, IGF-1 (10 nM), or a combination of both for 5 min. *A*, Akt Ser-473 phosphorylation. Cells were lysed and proteins separated by SDS-PAGE. Proteins were detected by probing with antibodies to phospho-Akt (Ser-473) and Akt. Values (mean ± S.E., $n = 3$) are expressed as fold differences between stimulated and unstimulated cells. *B*, effect of an LHRH-I antagonist. Serum-starved αT3 cells were stimulated for 5 min with IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents in the absence or presence of a LHRH-I antagonist. Whole cell lysates were subjected to SDS-PAGE and then probed with antibodies to phospho-Akt (Ser-473) and Akt. The results shown are representative of three independent experiments. *C*, Akt activity. Akt activity was measured using the GSK-3 fusion protein as an artificial substrate. The substrate was by SDS-PAGE and transferred to nitrocellulose membrane. The protein was detected by probing with a phospho-GSK3α/β (Ser-21/9) antibody. *D*, effect of IGF-1 concentration on LHRH-I-mediated inhibition. Serum-starved αT3 cells were stimulated for 5 min

pathway, and not the EGF receptor, is involved in LHRH-I signaling in αT3 gonadotrope cells.

Phosphorylation of Akt, one of the downstream targets of PI3K, was also investigated. We found that IGF-1 induced phosphorylation of the Ser-473 residue of Akt. Unlike PI3K activity, Akt phosphorylation was unaffected by treatment with LHRH-I (Fig. 8*A*). However, LHRH-I treatment inhibited IGF-1-induced stimulation of Akt phosphorylation. LHRH-I also had an inhibitory effect on IGF-1-induced phosphorylation of the Thr-308 residue of Akt (data not shown). The inhibitory effects of LHRH-I were abolished by treatment of the cells with an LHRH-I antagonist (Fig. 8*B*). We also measured Akt kinase activity using a GSK-3 fusion protein as a substrate. We found that LHRH-I strongly inhibited IGF-1-induced phosphorylation of the Ser-21/9 residue of GSK3 (Fig. 8*C*). The inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation was observed at all concentrations of IGF-1 tested. In addition, the inhibitory effect of LHRH-I was very rapid, and 30 s of incubation with LHRH-I was sufficient to reduce IGF-1-induced Akt phosphorylation by 2-fold (data not shown). The inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation was still observed 1 h after treatment (Fig. 8*E*). Buserelin, an agonist of LHRH-I, also inhibited IGF-1-induced Akt phosphorylation (data not shown). We also investigated the effect of different types of LHRH on the IGF-1 response. We found that LHRH type II (chicken LHRH), but not LHRH type III (Lamprey LHRH), mimicked LHRH type I inhibition of IGF-1-induced Akt phosphorylation (data not shown).

To determine whether the inhibitory effect of LHRH on Akt phosphorylation was cell-specific, we treated different cell lines with IGF-1, LHRH-I, or a combination of both agents for either 5 min or 1 h. The following cell lines were tested: two ovarian (OVCAR and SKOV) and two mammary cancer cell lines (MCF-7 and MDA-MB-231) expressing both the LHRH-I and IGF-1 receptors (50, 51), the gonadotrope αT3 and Lβ T2 cell lines, and a rat primary pituitary cell line. LHRH-I only led to down-regulation of IGF-I-induced Akt phosphorylation in the gonadotrope cell lines (supplemental Fig. 1). This effect was more potent in αT3 cells than in Lβ T2 or rat primary pituitary cells. Our finding that LHRH-I did not have an inhibitory effect in ovarian and mammary cancer cell lines may be explained by the fact that these cells line express relatively low levels of the LHRH-I receptor. To check this hypothesis, we transiently transfected MCF-7 cells with either an empty vector (control) or a mouse LHRH-I receptor construct (R-LHRH+/+). Transfectants were then treated with LHRH-I, IGF-1, or a combination of the two agents for 5 min. LHRH-I significantly inhibited IGF-1-induced Akt phosphorylation in R-LHRH+/+ cells but not in MCF-7 control cells (data not shown). Thus, the inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation is dependent on the amount of LHRH-I receptor expressed by the cell.

*Involvement of PKCα in the LHRH-I Inhibition of IGF-1-induced Akt Phosphorylation*—A panel of pharmacological inhibitors and activators was used to identify the signaling pathways involved in LHRH-I inhibition of IGF-1-induced Akt phosphorylation. Serum-starved cells were pretreated for 1 h with the following: 1 μM okadaic acid (a potent type I and 2A protein phosphatase-specific inhibitor), MAPK ERK1/2, p38, and JNK1/2 inhibitors (U0126 (1 μM), SB202190 (50 μM), and SP600125 (50 μM)), 10 μM ionomycin (an inducer of maximal calcium influx), and the PKC inhibitor GF109203X (10 nM). Cells were then incubated with IGF-1, LHRH-I, or a combination of the two agents for 5 min. LHRH-I inhibition of IGF-1-induced Akt phosphorylation was not affected by okadaic acid, the MAPK inhibitors, or ionomycin (data not shown). However, the inhibitory effect of LHRH-I was abolished by treatment with the PKC inhibitor, suggesting that the PKC signaling pathway is involved in LHRH-I-mediated inhibition (Fig. 9*A*). In agreement with this result, we demonstrated that PMA (1 μM), a PKC activator, mimicked the inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation (Fig. 9*B*), an effect that was reversed by adding the PKC inhibitor (Fig. 9*B*). To investigate which PKC was involved, we investigated the effect of adding PKCα (Safingol, 40 μM) and PKCδ-specific (Rottlerin, 5 μM) inhibitors to the cell cultures. We found that the PKCα inhibitor completely abolished LHRH-I inhibition of IGF-1-stimulated Akt phosphorylation, whereas the PKCδ inhibitor had no effect (Fig. 9, *C* and *D*). Together these results suggest that PKCα is involved in the inhibitory effect of LHRH-I on IGF-1-mediated Akt activation.

To confirm these results, we transiently transfected αT3 cells with a dominant negative PKCα construct (K368A (52), Fig. 10). The level of phosphorylation of the Ser-473 residue of Akt was substantially higher (about 16-fold) in IGF-1-treated control (empty vector) transformants than in unstimulated cells. As expected, cotreatment with LHRH-I led to inhibition (by at least 3-fold) of IGF-1-induced Akt phosphorylation. The inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation was substantially weaker in αT3 cells transfected with a dominant negative PKCα construct (Fig. 10).

*LHRH-I-induced PKCα Activation Inhibits the Anti-apoptotic Effect of IGF-1 by Inhibiting Akt Activation*—LHRH agonists have been shown to induce apoptosis in tumor cells (53). To begin to explore the biological significance of the inhibitory effect of LHRH-I on IGF-1 signaling, we tested the effect of IGF-1, LHRH-I, or a combination of both agents on apoptosis induced by serum starvation of αT3 cells. Cells were subjected to serum starvation for 48 h and then incubated in the presence or absence of IGF-1 (10 nM) and/or LHRH-I for an additional 24 h. The degree of apoptosis was then determined by terminal dUTP nick-end labeling staining. Apoptosis levels were substantially higher (about 3.5-fold; $p < 0.05$) in serum-starved cells than in control cells (Fig. 11*A*). Incubation with IGF-1 for 24 h led to a 2-fold inhibition of the apoptosis induced by serum starvation, whereas incubation with LHRH-I led to a 1.4-fold stimulation of apoptosis in serum-starved cells (Fig. 11*A*). Cotreatment with both IGF-1 and LHRH-I abolished the anti-apoptotic effect of IGF-1. LHRH-I inhibition of the anti-apoptotic effect of IGF-1 was prevented by treatment with GF109203X or Safingol, suggesting that PKCα is involved the LHRH-I-mediated inhibition.

Caspase-3 cleavage is a critical step in the control of the apoptotic DNA fragmentation and is involved in the activation of some caspases (54). We investigated whether this process was involved in the LHRH-I and IGF-1 responses. Serum starvation for 48 h resulted in an increase in the level of caspase-3 cleavage (Fig. 11*B*). Treatment of these serum-starved cells with 10 nM IGF-1 for 24 h prevented cleavage of caspase-3, whereas treatment with LHRH-I promoted cleavage. The level of cleaved caspase in cells treated with both LHRH-I and IGF-1 was similar to that in cells treated with LHRH alone. Our

with different concentrations (0.1–1000 nM) of IGF-1, LHRH-I alone (10 nM), or a combination of both agents. Western blotting was performed as described for *A* and *B*. *E*, effect of the time of stimulation on the inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation. Serum-starved αT3 cells were stimulated with IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents for different lengths of time (0.5, 1, 10, and 60 min). Western blotting was performed as described for *A* and *B*. The results shown are representative of those obtained for three independent experiments.

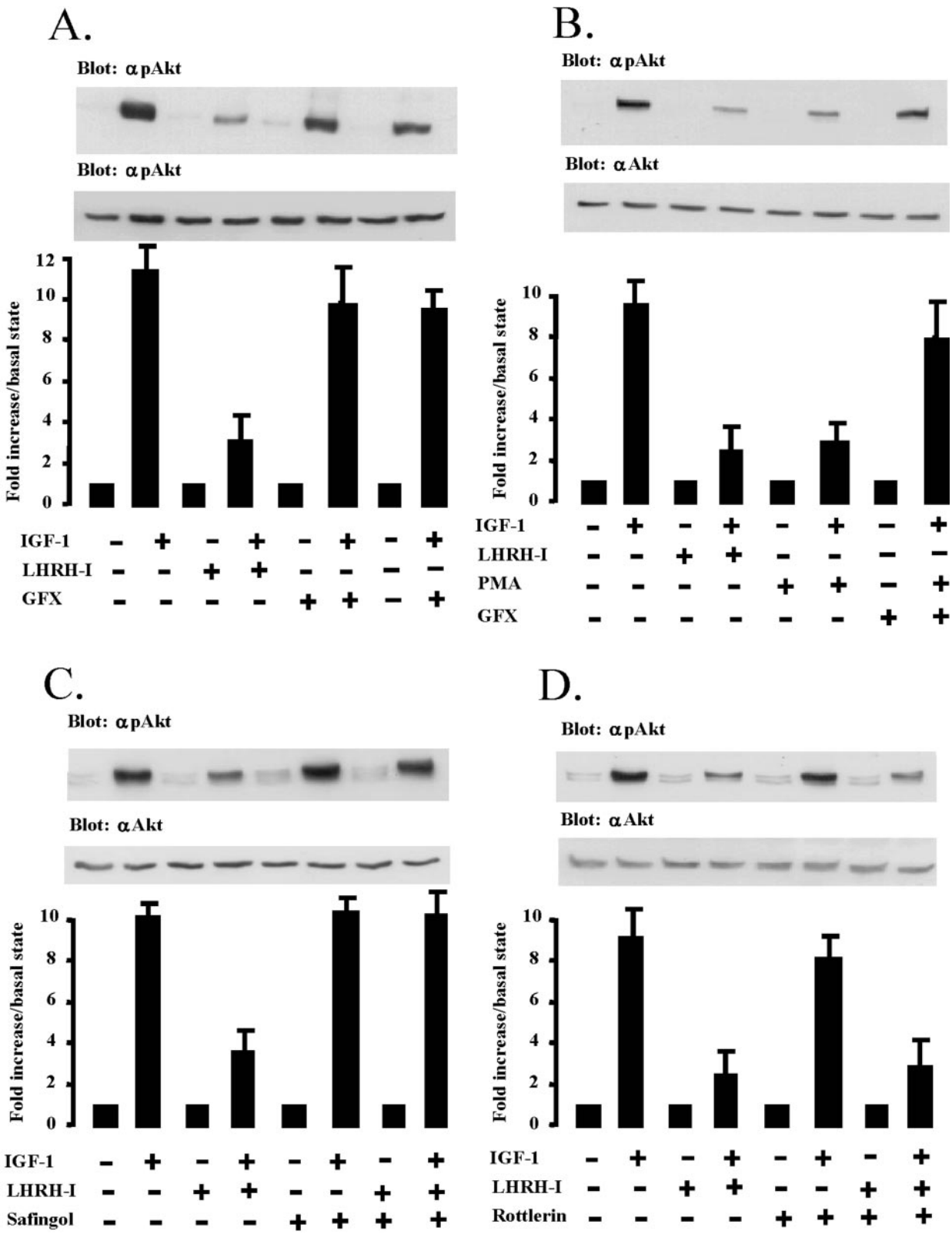


FIG. 9. **Role of the PKC signaling pathway in the LHRH-I-mediated inhibition of IGF-1-induced Akt activation in αT3 cells.** Serum-starved αT3 cells were preincubated for 1 h with the following inhibitors or activators: GF109203X (10 nM, *A* and *B*), PMA (1 μM, *B*), Safingol (40 μM, *C*), and Rottlerin (5 μM, *D*). Cells were then incubated in the absence or presence of IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents for 5 min. Whole cell lysates were subjected to SDS-PAGE, and proteins were detected with antibodies to phospho-Akt (Ser-473) and Akt. Values (mean ± S.E., $n = 3$) are expressed as fold differences between stimulated and unstimulated cells.

results indicate that LHRH-I treatment inhibits the anti-apoptotic activity of IGF-1 in serum-starved αT3 cells.

We then investigated the mechanisms involved LHRH-I inhibition of the anti-apoptotic effect of IGF-1 in αT3 cells. Growth factors may play a role in protecting cells from apoptosis. Phosphorylation and inactivation of the pro-apoptotic Bcl2 family member, Bad, may be involved in this process (55). Akt has been shown to promote cell survival by a mechanism in-

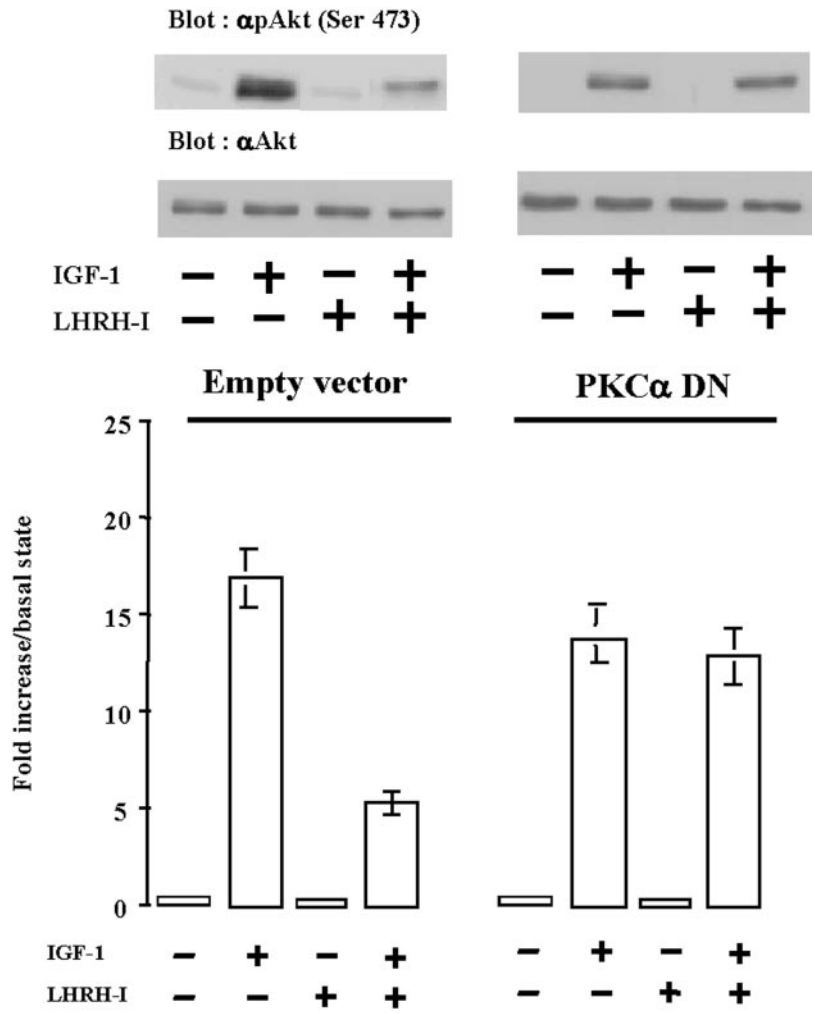


FIG. 10. **A dominant negative PKC$\alpha$ construct abolished the inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation in $\alpha$T3 cells.** $\alpha$T3 cells were transiently transfected with either the dominant negative PKC$\alpha$ (*PKC$\alpha$ DN*) construct or the empty vector (negative control). Serum-starved $\alpha$T3 cells were stimulated for 5 min by treatment with IGF-1 (10 nM), LHRH-I (10 nM), or a combination of both agents. Cells were lysed, and proteins were separated by SDS-PAGE. Proteins were then detected using antibodies to phospho-Akt (Ser-473) and Akt. Values (mean ± S.E., $n = 3$) are expressed as fold differences between stimulated and unstimulated cells.

volving the phosphorylation of the Ser-136 residue of Bad (56). We examined the phosphorylation status of Bad by using an antibody recognizing phosphorylated Ser-136. We found that phosphorylation of Bad was IGF-1-dependent and that the level of Bad phosphorylation correlated with that of Akt phosphorylation (Fig. 11*B*). Pretreatment with LY294002 inhibited IGF-1-induced phosphorylation of the Ser-136 residue of Bad, supporting the idea that Akt is necessary for Bad activation (data not shown). To confirm whether Bad phosphorylation had an anti-apoptotic effect, we determined the level of cleaved caspase-3. We found that IGF-1 treatment prevented caspase-3 cleavage. This inhibition of caspase-3 cleavage correlated with an up-regulation of Bad phosphorylation. We also found that serum starvation for 48 h promoted caspase-3 cleavage and down-regulated Bad phosphorylation. Serum starvation for 48 h followed by IGF-1 treatment for 24 h led to up-regulation of phosphorylation of the Ser-136 residue Bad, whereas caspase-3 cleavage was down-regulated (Fig. 11*B*). Cotreatment with LHRH-I and IGF-1 led to down-regulation of Bad and Akt phosphorylation and up-regulation of caspase-3 cleavage. These effects were abolished by adding GF109203X and Safingol (Fig. 11*B*). These data indicate that LHRH-I inhibits the anti-apoptotic effect of IGF-1 by inhibiting PKC$\alpha$-mediated Akt and Bad phosphorylation.

## DISCUSSION

We showed that in the pituitary gonadotrope $\alpha$T3 cells, IGF-1 stimulates cell proliferation mainly via the PI3K and MAPK ERK1/2 pathways. The effect of IGF-1 on cell proliferation in $\alpha$T3 cells is consistent with results described in several previous reports. In humans (57), mice (24), and rats, IGF-1 is synthesized in the pituitary *in vivo* (35). The IGF-1R is expressed in rat and mouse pituitary glands, and IGF-1 has been shown to stimulate the proliferation of pituitary cells (24). In addition, IGF-1 leads to an increase in basal and LHRH-stimulated release of LH from rat anterior pituitary explants and dispersed cells *in vitro* (36, 37, 58, 59). IGF-1 increases FSH content and LHRH-stimulated FSH release in Coho salmon pituitary cells *in vitro* (60). IGF-1 also stimulates LHRH release *in vivo* (61) and LHRH gene expression in immortalized cell lines (62, 63) and in peripubertal mice (64). Although previous studies have indicated that interactions between the IGF-1 and LHRH signaling pathways do take place in pituitary gonadotrope cells, the underlying mechanisms remain to be established. We used the $\alpha$T3 gonadotrope-derived cell line to investigate these interactions. $\alpha$T3 cells express high levels of LHRH receptors exhibiting binding characteristics similar to those found in normal mouse and rat pituitary glands (7, 65).

We demonstrated that LHRH, like IGF-1 stimulates proliferation of $\alpha$T3 cells. However, this stimulation is mediated by different signaling pathways. We found that LHRH uses both the MAPK ERK1/2 and the PKC signaling pathways. The effects of LHRH on cell proliferation are cell-specific. LHRH agonists (66, 67) and an LHRH antagonist (68) have been shown to have an antiproliferative effect on the human androgen-independent prostate cell lines, DU-145 and PC-3. In DU-145 prostate cancer cells, the antiproliferative effects of LHRH agonists are mediated through negative regulation of the EGFR, which is inactivated through phosphorylation by PKC

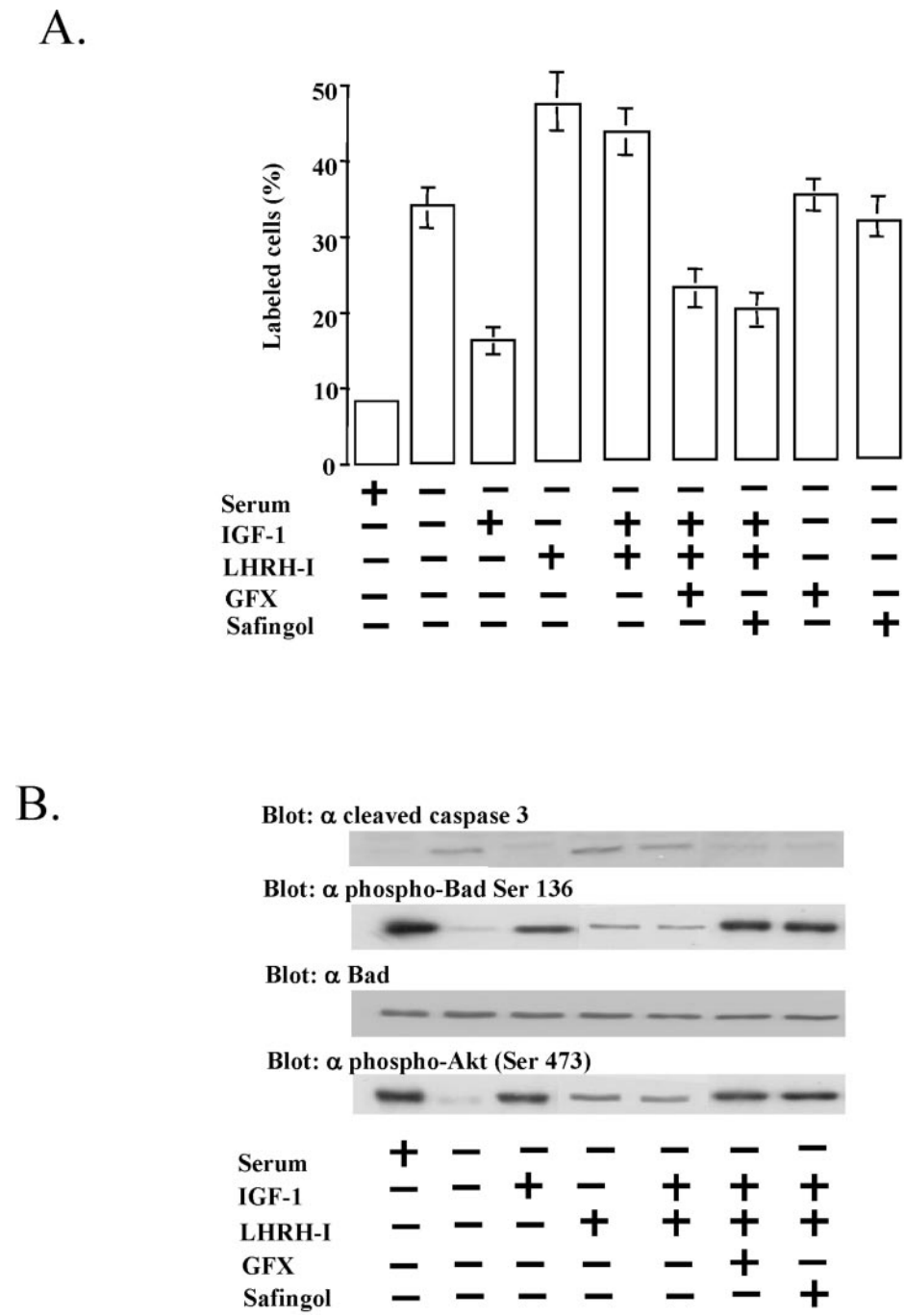


FIG. 11. **LHRH inhibits the anti-apoptotic effect of IGF-1 through the inhibition of Akt and the activation of PKCa.** Cells were plated on Lab-Tek (*A*) or 100-mm dishes (*B*) containing normal culture medium. After 18 h, the medium was changed to serum-free DMEM for 48 h. Cells were then incubated with IGF-1, LHRH-I, or a combination of both agents for 24 h. For treatment with the inhibitors, GF109203X (10 nM) and Safingol (40 $\mu$M), cells were preincubated for 1 h prior to the addition of IGF-1 and/or LHRH-I. *A,* cells were fixed and apoptotic nuclei were identified by terminal dUTP nick-end labeling staining. The percentage of labeled cells ± S.E. was determined by counting ~1,000 nuclei. Means were compared by analysis of variance. *B,* cells were lysed and proteins were separated by SDS-PAGE. The levels of Ser-136-phosphorylated Bad, Bad, phospho-Akt, and Akt were analyzed by probing with the appropriate antibodies.

(69). By ng the same cell line, Marelli *et al.* (42) also showed hat an LHRH agonist inhibits IGF-1-induced cell proliferation by interfering with some of the cellular mechanisms mediating the IGF-1-induced stimulation. The LHRH agonist Zoladex strongly reduces the level of IGF-1R expression and tyrosine phosphorylation (42). In our study, we found that LHRH promoted cell growth. This result is consistent with those found in previous reports showing that LHRH induces cell proliferation in pituitary gonadotrope cells (1, 22). Moreover, recent studies using primary mouse pituitary cells and the gonadotrope-derived L$\beta$T2 cell line have shown that addition of a LHRH agonist increased the number of cells at S phase and decreased the number of cells at $G_1$ phase after 2 and 4 days of culture (71). In addition, in primary rat pituitary cell cultures (a gonadotrope-enriched population), LHRH is a mitogenic factor for both LH and FSH gonadotrope cells (1, 22). Thus, LHRH appears to be a positive regulator of pituitary gonadotrope cell proliferation. However, this effect is dependent on cell type.

Our results suggest that activation of the ERK1/2 MAPK is dependent on PKC. This hypothesis was confirmed by pretreating $\alpha$T3 cells with GF109203X. Indeed, we found that ERK1/2 phosphorylation was 50% lower in pretreated cells than in controls (data not shown). Previous studies have reported that the ERK (11, 12), JNK (71), and p38 MAPK (21) cascade is activated in a PKC-dependent manner by LHRH in $\alpha$T3 cells. Benard *et al.* (72) showed that LHRH-mediated activation of ERK involves two distinct signaling pathways, which converge at the step involving Raf-1. The main pathway involves direct activation of Raf-1 by PKC. This step is partially dependent on a second pathway involving dynamin-dependent Ras activation downstream of Src (72). Our results were consistent with these observations. Moreover, we showed that the EGF receptor is not involved in the activation of ERK1/2 MAPK in response to LHRH-I. This result is consistent with those obtained by

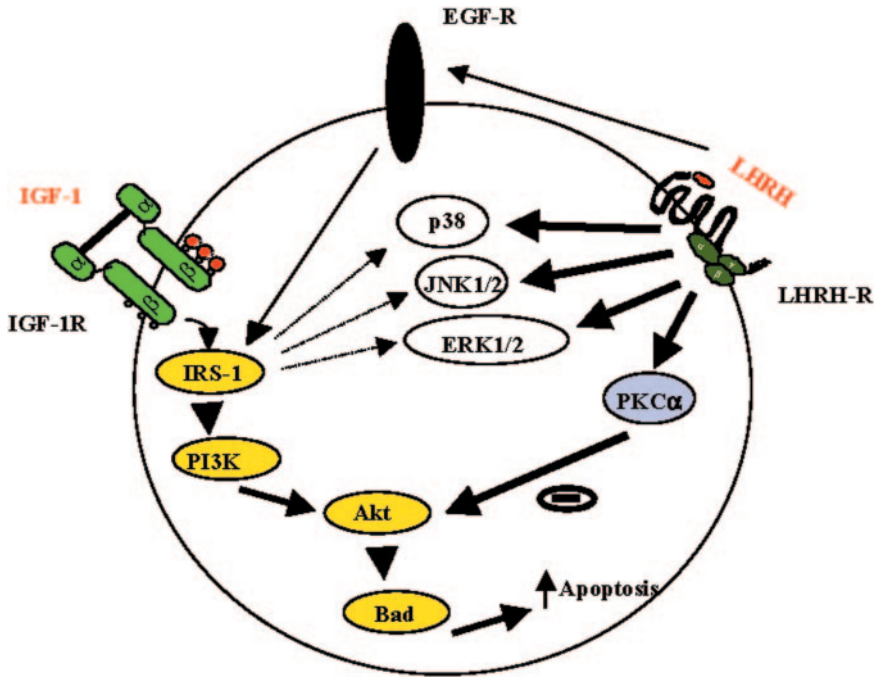


FIG. 12. **Schematic representation of a cross-talk between IGF-1R, LHRH-R, and EGFR in $\alpha$T3 cells.** IGF-1 activates the PI3K/Akt pathway but only weakly the MAPK pathway (ERK1/2, p38, and JNK1/2), whereas LHRH-I activates mainly the MAPK pathway. LHRH-I interacts with several components of the IGF-1R signaling cascade different. First, it stimulates IGF-1-induced tyrosine phosphorylation of IRS-1 and IRS-1-associated PI3K activity by facilitating transactivation of the EGFR. LHRH-I also has an inhibitory effect on IGF-1-induced PKC$\alpha$-mediated Akt phosphorylation, preventing Bad phosphorylation in the $\alpha$T3 gonadotrope cell line and thus leading to inhibition of the anti-apoptotic effects of IGF-1.

Benard *et al.* (72). In addition, we demonstrated that LHRH-I and IGF-1 have an additive effect on cell proliferation. The ERK1/2 MAPK signaling pathway is partially responsible for IGF-1-induced cell proliferation in $\alpha$T3 cells. Thus, the additive effect of LHRH and IGF-1 on cell proliferation is probably the result of the additive effect of these two agents on ERK1/2 phosphorylation and activity. We found that LHRH-I enhances IGF-1-induced IRS-1 phosphorylation and IRS-1-associated PI3K activity without affecting IGF-1-induced IGF-1R $\beta$ tyrosine phosphorylation. Furthermore, we showed that LHRH-I transactivates the EGF receptor, which phosphorylates IRS-1. Other tyrosine kinase-like platelet-derived growth factor receptors have also been reported to be involved in the transmission of signals from G protein-coupled receptors (73). For example, Grosse and co-workers. (74, 75) showed that LHRH-I treatment resulted in a rapid increase in MAPK ERK activity in $\alpha$T3 gonadotrope cells. This activity was enhanced by the EGFR-specific tyrosine kinase inhibitor AG1478 (74, 75). We showed that the EGFR only played a minor role, if any, in LHRH-I activation of IRS-1-associated PI3K activity. However, we found that LHRH-I promoted phosphorylation of the focal adhesion kinase (p125 FAK or FAK), which is known to bind the p85, the regulatory subunit of PI3K.

We showed, in $\alpha$T3 cells and to a lesser extent in L$\beta$T2 cells and primary rat pituitary cells, that LHRH-I strongly inhibited the IGF-1-induced phosphorylation of the Ser-473 and Thr-308 residues and thus the activity of Akt. A wide variety experimental approaches have shown that Akt is activated via at least two pathways, one of which is PI3K-dependent and the other is PI3K-independent (76–80). Stress, okadaic acid, and cAMP regulate the activation of Akt independently of PI3K. Our results support the idea that LHRH-I inhibits IGF-1-induced Akt activation via a PI3K-independent pathway. Indeed, LHRH-1 has a synergistic effect on IGF-1-induced IRS-1-associated PI3K activity. Moreover, both PDK1/2 phosphorylation and IGF-1R- and IRS-2-associated PI3K activity were up-regulated in response to IGF-1. Both of these responses were also slightly up-regulated following LHRH-1 treatment (data not shown). We hypothesize that the inhibitory effect of LHRH-I on IGF-1-induced Akt phosphorylation is a result of LHRH-mediated activation of a phosphatase that dephosphorylates both the Ser and Thr residues of Akt. Protein phosphatases are known to play a critical role in the regulation of the PI3K/Akt cascade. For example, granulocyte-macrophage colony-stimulating factor-induced inhibition of Akt/PKB by ceramide in human erythroleukemic TF-1 cells (81) and insulin-induced inhibition by palmitate in C2C12 skeletal muscle cells (82) both lead to PI3K-independent Ser-473 dephosphorylation by phosphatases. Inhibitors of protein phosphatases, such as okadaic acid for PP1 and PP2A, FK506 for PP2B, and sodium orthovanadate for protein-tyrosine phosphatase, are useful tools for studying the significance of protein phosphorylation. We found that none of these inhibitors blocked LHRH inhibition of IGF-1-induced Akt phosphorylation, suggesting that phosphatases, such as PP2A/B and PP1, were not involved in this process (data not shown for FK506 and sodium orthovanadate).

However, we showed that the conventional isoform-specific PKC inhibitor (GF109203X) did block the inhibitory effect of LHRH-1 on IGF-1-induced Akt activation. Several recent reports have shown that some PKCs inhibit Akt activity. For example, Wen *et al.* (82) used a nonselective PKC inhibitor to demonstrate that PKC negatively regulates Akt in a human airway epithelial cell culture (A549). The PKC family consists of at least 12 members divided into three groups as follows: 1) the conventional PKCs ($\alpha$, $\beta$I, $\beta$II, and $\gamma$); 2) the novel PKCs ($\delta$, $\epsilon$, $\eta$, and $\theta$); and 3) the atypical PKCs ($\zeta$ and $\iota/\lambda$). As all of these PKC isoforms are expressed in $\alpha$T3 cells (84, 85), it was necessary to establish whether all or just one of these isoforms was involved in the LHRH-1 inhibition of IGF-1-induced Akt activation. We investigated the roles of the different isoforms using a variety of isoform-specific PKC inhibitors. Our data revealed that PKC$\alpha$ plays a major role in LHRH-1 inhibition of IGF-1-induced Akt activation.

The inhibition of Ser/Thr phosphorylation of Akt through a PKC pathway can be mediated by either a PI3K-dependent or PI3K-independent pathway depending on the ligand and cell lineage analyzed. For example, the involvement of PKC in insulin-stimulated responses has been demonstrated by studies of various steps in the signaling cascade, including those analyzing the effect of the binding of substrates like IRS-1 and PI3K to the receptor (23, 86, 87). In contrast, our results indicated that the inhibition of Akt activity probably occurs up-

stream of PKC, possibly involving PI3K activity. Doornbos *et al.* (23) reported that PKC$\zeta$ is a negative regulator of Akt/PKB activity and that this regulation is PI3K-independent. The PH domain of Akt has been proposed to be involved in protein-protein interactions, such as binding of PKC and the $\beta/\gamma$ subunits of heterotrimeric G proteins (47). Konishi *et al.* (70) used glutathione *S*-transferase fusion proteins containing the three different Akt PH domains to demonstrate that PKC$\alpha$ and -$\delta$ bind to the PH domain of Akt1, -2, and -3 and that PKC$\zeta$ binds to the PH domain of Akt1 and -2 *in vitro*. We found that GF10923X did not prevent LHRH from enhancing IGF-1-induced IRS-1 phosphorylation or the association of this phosphorylated form with PI3K (data not shown). We hypothesize that PMA and LHRH-mediated inhibition of IGF-1-induced Akt stimulation is mediated, at least in part, by a direct interaction between PKC$\alpha$ and Akt. It is possible that PKC-mediated phosphorylation of Akt leads to a change in the conformation the protein, which subsequently inactivates the kinase and blocks further phosphorylation of Akt by PDK1/2.

In conclusion, our results provide strong evidence that LHRH-I regulates several steps of the IGF-1R signaling pathway in the gonadotrope cell line, $\alpha$T3. LHRH-I enhances IGF-1-induced IRS-1 tyrosine phosphorylation and promotes the association of this protein with PI3K. In contrast, LHRH-I strongly inhibits Akt activation through PKC$\alpha$ (Fig. 12). This LHRH-I-mediated inhibition of IGF-1-induced Akt phosphorylation strongly inhibits the anti-apoptotic effect IGF-1. Our study provides a novel insight into the mechanisms involved in LHRH and IGF-1 signaling during growth and proliferation of gonadotrope cells.

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# The Luteinizing Hormone-releasing Hormone Inhibits the Anti-apoptotic Activity of Insulin-like Growth Factor-1 in Pituitary αT3 Cells by Protein Kinase Cα-mediated Negative Regulation of Akt

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