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Acute Regulation of Translation Initiation by Gonadotropin-Releasing Hormone in the Gonadotrope Cell Line LβT2

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The hypothalamic neuropeptide hormone GnRH is the central regulator of reproductive function. GnRH stimulates the synthesis and release of the gonadotropins LH and FSH by the gonadotropes of the anterior pituitary through activation of the G-protein-coupled GnRH receptor. In this study, we investigated the role of translational control of hormone synthesis by the GnRH receptor in the novel gonadotrope cell line LβT2. Using immunohistochemical and RIA studies with this model, we show that acute GnRH-induced synthesis and secretion of LH are dependent upon new protein synthesis but not new mRNA synthesis. We examined the response to GnRH and found that activation of cap-dependent translation occurs within 4 h. LHβ promoter activity was also examined, and we found no increases in LHβ promoter activity after 6 h of GnRH stimulation. Additionally, we show that increased phosphorylation of translation initiation proteins, 4E-binding protein 1, eukaryotic initiation factor 4E, and eukaryotic initiation factor 4G, occur in a dose- and time-dependent manner in response to GnRH stimulation. Quantitative luminescent image analysis of Western blots shows that 10 nM GnRH is sufficient to cause a maximal increase in factor phosphorylation, and maximal responses occur within 30 min of stimulation. Further, we demonstrate that the MAPK kinase inhibitor, PD 98059, abolishes the GnRH-mediated stimulation of a cap-dependent translation reporter. More specifically, we demonstrate that PD 98059 abolishes the GnRH-mediated stimulation of a downstream target of the ERK pathway, MAPK-interacting kinase. Based on these findings, we conclude that acute GnRH stimulation of LβT2 cells increases translation initiation through ERK signaling. This may contribute to the acute increases in LHβ subunit production. (*Molecular Endocrinology* 18: 1301–1312, 2004)

THE REGULATION OF reproductive function requires coordination of signals from several cell types in tissues widely dispersed within the organism. In mammals, ovulation is highly regulated and depends upon precise interaction of positive regulatory signals converging at the level of the pituitary and regulating the release of LH and FSH. The production of these hormones is, in turn, centrally regulated by the hypothalamic neurosecretory cells that produce the releasing factor GnRH. Changes in the pulsatile release of GnRH from the hypothalamus into the hypophysial circulation are correlated with changes in LH and FSH production by the pituitary (1–4). Both GnRH pulse amplitude and frequency play a role in the synthesis and release of LH (1, 5).

Cell models of fully committed and differentiated gonadotropes αT3–1, LβT2, and LβT4 cells (6, 7), derived by targeted tumorigenesis in mouse pituitary, have been developed. These cell lines allow the characterization of signaling pathways activated in response to ligand binding and GnRH receptor activation. Studies using these gonadotrope cell models and primary rat pituitary cultures to investigate the transcriptional response of gonadotropin genes to GnRH have shown that transcriptional changes in gene expression require 6–24 h to reach maximal response levels (3, 8, 9). In addition, studies in pituitary fragments showed no transcriptional responses within a 24-h period of tonic GnRH treatment (10). Similarly, microarray analysis of LβT2 cells detected no significant changes (<1.3 fold change) in gonadotropin gene expression in response to either 1 or 6 h of tonic GnRH treatment (11–13). These observations corroborate *in vivo* analysis of steady-state LHβ mRNA levels in which a less than 50% increase (1.4-fold change) occurs in response to GnRH stimulation within 6 h (14). In

Abbreviations: CMV, Cytomegalovirus; 4E-BP, 4E-binding protein; eIF4E, eukaryotic initiation factor 4E; eIF4G, eukaryotic initiation factor 4G; EMCV, encephalomyocarditis virus; GPCR, G protein-coupled receptor; MEK, MAPK kinase; Mnk1, MAPK-interacting kinase; mTOR, mammalian target of rapamycin; PI3 kinase, phosphatidylinositol 3-kinase.

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contrast, this same study found maximal (100-fold) increases in serum gonadotropin levels within 6 h of GnRH treatment. Furthermore, it has been shown that increases in LHβ protein synthesis in response to GnRH occur within 4 h in LβT2 cells (15). The discrepancy between measurements of transcriptional activity and protein production may be attributed, in part, to translational regulation of protein synthesis.

Translational regulation through extracellular signaling mechanisms commonly occurs through activation of receptor tyrosine kinases such as the insulin and epidermal growth factor receptors (16, 17). Regulation of translation by these receptors proceeds through phosphatidylinositol 3-kinase (PI3 kinase)/AKT and/or ERK signaling pathways. These pathways target the function of the N^7-methylguanosine mRNA cap-binding protein eIF4E (eukaryotic initiation factor 4E) as well as eIF4G (eukaryotic initiation factor 4G), a scaffold protein required for the assembly of the translation initiation complex eIF4F. The association of these initiation factors with the mRNA cap is the rate-limiting step in translation initiation and is essential for initiation of capped mRNA translation (18). Phosphorylation of initiation factors controls the rate of mRNA binding to ribosomes. eIF4E is negatively regulated by a family of binding proteins known as the 4E-binding proteins (4E-BP) or protein, heat, and acid stable (PHAS) (19, 20). Phosphorylation of 4E-BP by activated receptor tyrosine kinase signaling cascades disables eIF4E binding activity, allowing eIF4E to associate with the N^7-methylguanosine cap and initiate translation (21).

Regulation of translation via G protein-coupled receptors (GPCRs) is not commonly observed. To date, the μ-opioid receptor and the receptors for endothelin, phenylephrine, angiotensin II, and lysophosphatidic acid have been shown to regulate translation in other cell systems (16, 22–24). We recently demonstrated that the GnRH receptor regulates translation in the gonadotrope-derived cell line, αT3–1, which expresses the GnRH receptor but not gonadotropin subunit genes (25). Therefore, to determine whether translational control is relevant to the production of gonadotropins, we examined the impact of GnRH-induced translational activation on LHβ synthesis in LβT2 cells, a cell line that endogenously expresses both the GnRH receptor and gonadotropin genes. Our studies address the reported discrepancy between acute increased gonadotropin production and mRNA synthesis.

Using the LβT2 cell model, we show that acute synthesis of LHβ and LH secretion in response to GnRH are not exclusively dependent on transcription. We further show that activation of the translational initiation proteins 4E-BP, eIF4E, and eIF4G occurs in a dose- and time-dependent manner in LβT2 cells in response to acute GnRH administration. Moreover, we demonstrate GnRH activation of translation is inhibited by the MAPK kinase (MEK) inhibitor, PD 98059. Based on these findings, we conclude that translational regulation is an important component of the acute response to GnRH receptor activation.

RESULTS

GnRH-Induced Synthesis of LHβ Protein and LH Secretion Are Independent of Transcription

Results reported by others suggest that the acute response of gonadotropes to GnRH is not exclusively dependent on increased gonadotropin mRNA synthesis but, instead, may involve a posttranscriptional component (9, 11, 12, 15). The increase in gonadotropin subunit synthesis as detected by immunocytochemical methods may be explained by an increase in synthesis of LHβ through increased utilization of the mRNA already present in the cell. To test this directly, we examined the response of LHβ synthesis and LH secretion to GnRH stimulation in the presence of the mRNA synthesis inhibitor actinomycin D or in the presence of the translational inhibitor cycloheximide (Fig. 1). LβT2 cells were incubated in the presence of vehicle, actinomycin D, or cycloheximide for 1 h. Media were then supplemented with vehicle or 10 nM GnRH. After 4 h, cells were processed for immunocytochemistry using antibody directed against the LHβ subunit (Fig. 1A). Alternatively, media were removed for analysis by LH immunoradiometric assay (Fig. 1C). Intensity of staining was compared between untreated and GnRH-treated cells. Quantification of digital images showed significantly increased staining intensity for LHβ subunit, after stimulation by GnRH in both vehicle and actinomycin D-treated cells (Fig. 1B). In contrast, cycloheximide significantly decreased GnRH induction of LHβ staining. Similarly, GnRH-stimulated LH secretion was not significantly decreased in the presence of actinomycin D but was significantly decreased in the presence of cycloheximide (Fig. 1C). Based on these observations, we conclude that increases in LHβ subunit protein and LH secretion after 4 h of GnRH treatment are more dependent on new protein synthesis than new mRNA synthesis.

GnRH Stimulates Cap-Dependent Translation, But Not LHβ Promoter Activity, in LβT2 Cells

We sought to confirm the data presented in Fig. 1 by evaluating the effects of GnRH stimulation on reporter genes designed to evaluate the differential role of transcriptional *vs.* translational stimulation. To this end, we examined the activation of a reporter gene under the transcriptional control of the rat 1.8-kb LHβ promoter in comparison with a bicistronic reporter gene responsive to increased cap-dependent translational activity. The bicistronic reporter gene directs the synthesis of a single mRNA encoding two independently translated reading frames. The first reading frame, encoding the firefly luciferase reporter enzyme, is translated by a

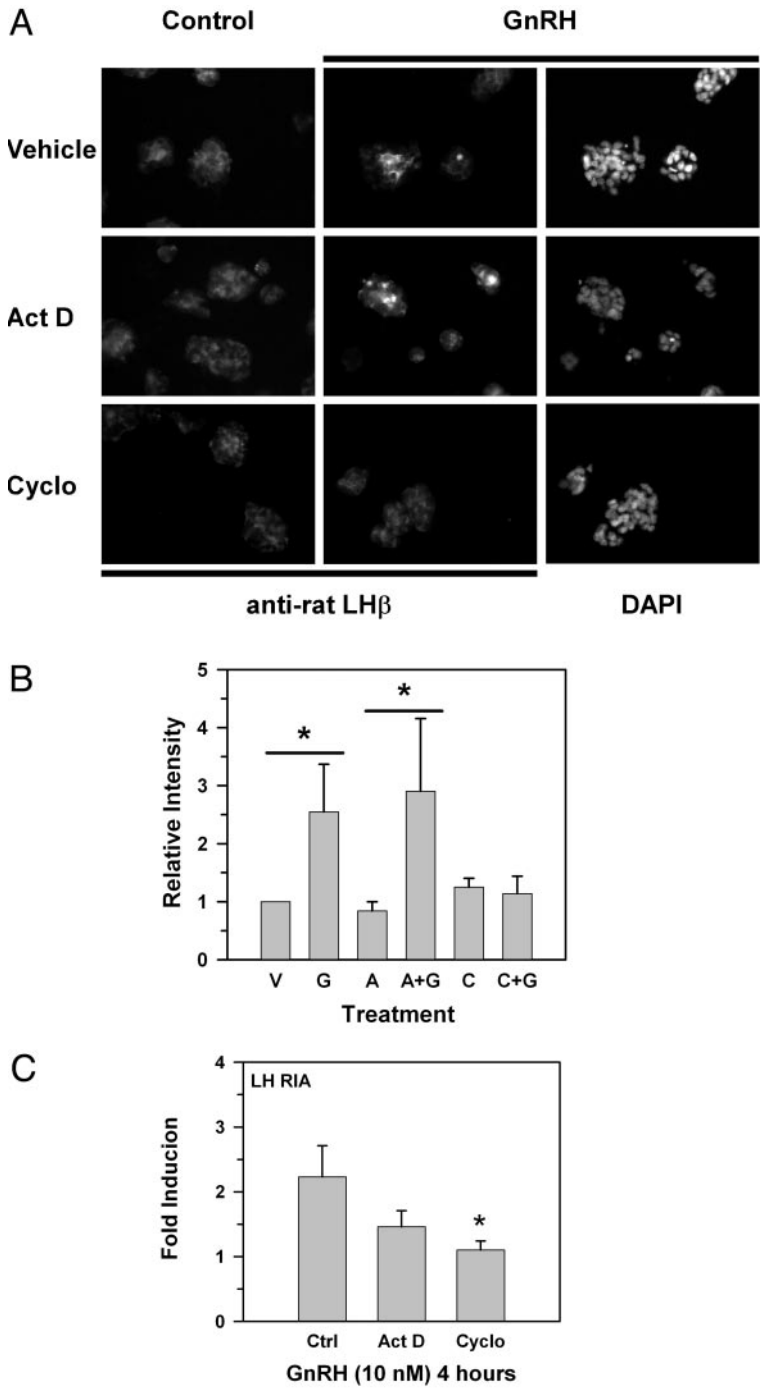

Fig. 1. GnRH-Induced Synthesis of LHβ Protein in the Presence of Actinomycin D

LβT2 cells were plated on chamber slides and serum starved for 12–26 h before actinomycin D, cycloheximide, or vehicle treatment. After 1 h, cells were further treated with GnRH (10 nM) or vehicle for 4 h. A, Representative images of fixed cells incubated with rabbit LHβ primary antibody and biotinylated antirabbit IgG secondary antibody with avidin-biotin fluorescein isothiocyanate conjugate and subsequently costained with 4′,6-diamidino-2-phenylindole. Cells were photographed under fluorescence (*center* and *right columns* are identical fields photographed under appropriate filter set). B, Quantification of total intensity of LHβ staining. Total intensity values were normalized to vehicle to yield relative intensity. Comparisons were made between multiple images of GnRH-treated and respective non-GnRH-treated controls from at least three independent experiments. The *asterisk* indicates significant difference in LHβ staining as compared with their respective non-GnRH-treated control ($P \leq 0.05$) as determined by ANOVA and *post hoc* Dunnett's comparison to control test. C, Fold induction of GnRH-stimulated LH secretion as determined by immunoradiometric assay. The *asterisk* indicates significant difference of fold induction compared with control ($P \leq 0.05$) as determined by ANOVA and *post hoc* Dunnett's comparison to control test. V, Vehicle; G, GnRH; A, actinomycin D; C, cycloheximide.

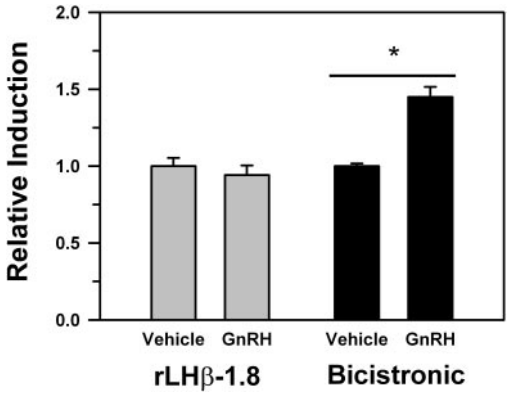

Fig. 2. GnRH Activation of Cap-Dependent Translation in the Absence of LHβ Promoter Activation

Cultured LβT2 cells were transiently cotransfected with pGL3-rLHβ 1.8 reporter plasmid and pGL3-CMV internal control plasmid (*gray*) or the pGL3-CMV-Luc-EMCV-Gal bicistronic reporter gene (*black*). Cells were treated with GnRH for 6 h before harvesting. The *histogram* represents one of three independent assays of each reporter showing the ratio of luciferase to β-galactosidase activity. The *asterisk* indicates significant difference of relative induction compared with vehicle-treated control ($P \leq 0.05$) as determined by ANOVA and *post hoc* Student's *t* tests. The interassay CV is 3.01% for pGL3-rLHβ 1.8 reporter plasmid and 28.5% for the bicistronic vector.

cap-dependent translation initiation mechanism. The second reading frame, encoding the β-galactosidase reporter enzyme, is translated independently of the first reading frame through internal ribosomal entry directed by the encephalomyocarditis virus (EMCV) 5′-noncoding region. Activity of the LHβ promoter in a vector containing the firefly luciferase gene was measured directly relative to the control cytomegalovirus (CMV) promoter in an identical vector background encoding the *Escherichia coli* β-galactosidase. After 6 h stimulation with 10 nM GnRH, no significant increase in rat 1.8-kb LHβ promoter activity was detected (Fig. 2); this is in agreement with observations by others examining stimulation of endogenous mRNA for similar time periods (11–14). However, 4 h activation of cap-dependent translation measured with the bicistronic reporter gene under the transcriptional control of the CMV promoter resulted in significant activation of cap-dependent translation as measured by the ratio of luciferase to β-galactosidase activity produced by the bicistronic mRNA (Fig. 2). These results confirm that increased synthesis of LHβ after GnRH stimulation may be attributed to activation of translation, rather than transcription directed by the LHβ promoter.

GnRH Activates Translational Initiation Factors 4E-BP1, eIF4E, and eIF4G

Upon observing the GnRH-induced increases in LHβ synthesis and translation, we investigated the mechanism of this activation. We previously showed that GnRH stimulation of the less differentiated gonadotrope cell line, αT3–1, causes phosphorylation of the translational regulatory factor 4E-BP1. The 4E-BP family of proteins are phosphorylated at five distinct serine/threonine residues causing a marked alteration in their electrophoretic mobility, with the γ-hyperphosphorylated inactive isoform showing the lowest mobility (26). It is known that phosphorylation of 4E-BP prevents interaction with eIF4E, thereby allowing eIF4E to associate with the scaffolding protein eIF4G (27–29). Phosphorylation of 4E-BP, eIF4E, and eIF4G has been shown to result in an increase of cap-dependent protein synthesis (19, 20, 30, 31). To determine whether GnRH activates these translational initiation factors, the phosphorylation status of each factor after 30 min of GnRH treatment (0.3–100 nM) in LβT2 cells was examined by Western blotting (Fig. 3). Quantitative luminescent image analysis shows that 3 nM GnRH was sufficient to maximally phosphorylate 4E-BP1; although 10 nM GnRH treatment resulted in the highest fold induction of 4E-BP1 phosphorylation, there were no significant differences found between 3 nM and 10 nM GnRH treatments (Fig. 3A). As for eIF4E, 10 nM GnRH caused maximal activation, and this was sustained even in the presence of 100 nM GnRH (Fig. 3B). Similar to 4E-BP1, eIF4G was maximally stimulated in the presence of 3 nM GnRH but at 100 nM, significant stimulation was not observed (Fig. 3C).

GnRH Differentially Activates Translational Initiation Factors 4E-BP1, eIF4E, and eIF4G

To determine the kinetics of the observed GnRH activation, the phosphorylation status of initiation factors was examined at indicated time points in response to 10 nM GnRH. The Western blots show that there is a time dependence and difference in each factor's response to GnRH. 4E-BP1 phosphorylation is increased within 5 min of stimulation, as demonstrated by the increased proportion of 4E-BP1 found in the γ-isoform (Fig. 4A). Analysis of phosphorylation of 4E-BP1 in comparison to control by quantitative luminescent image analysis shows that maximal stimulation occurs within 15 min of GnRH treatment and is maintained up to 60 min, indicating that 10 nM GnRH treatment results in sustained phosphorylation of 4E-BP1 in LβT2 cells. After 4E-BP1 phosphorylation, maximal activation of eIF4E occurs at 15 min and is also maintained for up to 60 min (Fig. 4B). Unlike 4E-BP1 and eIF4E, eIF4G is not maximally activated until 30 min of GnRH treatment. Additionally, eIF4G activation is not sustained by prolonged GnRH stimulation; rather, phospho-eIF4G is significantly decreased after 60 min of treatment (Fig. 4C). This implicates a time-sensitive response to GnRH stimulation that is specific to each initiation factor.

GnRH Mediates Translational Activation through the ERK Cascade

The MAPK and PI3 kinase signaling cascades have been implicated in translational initiation control,

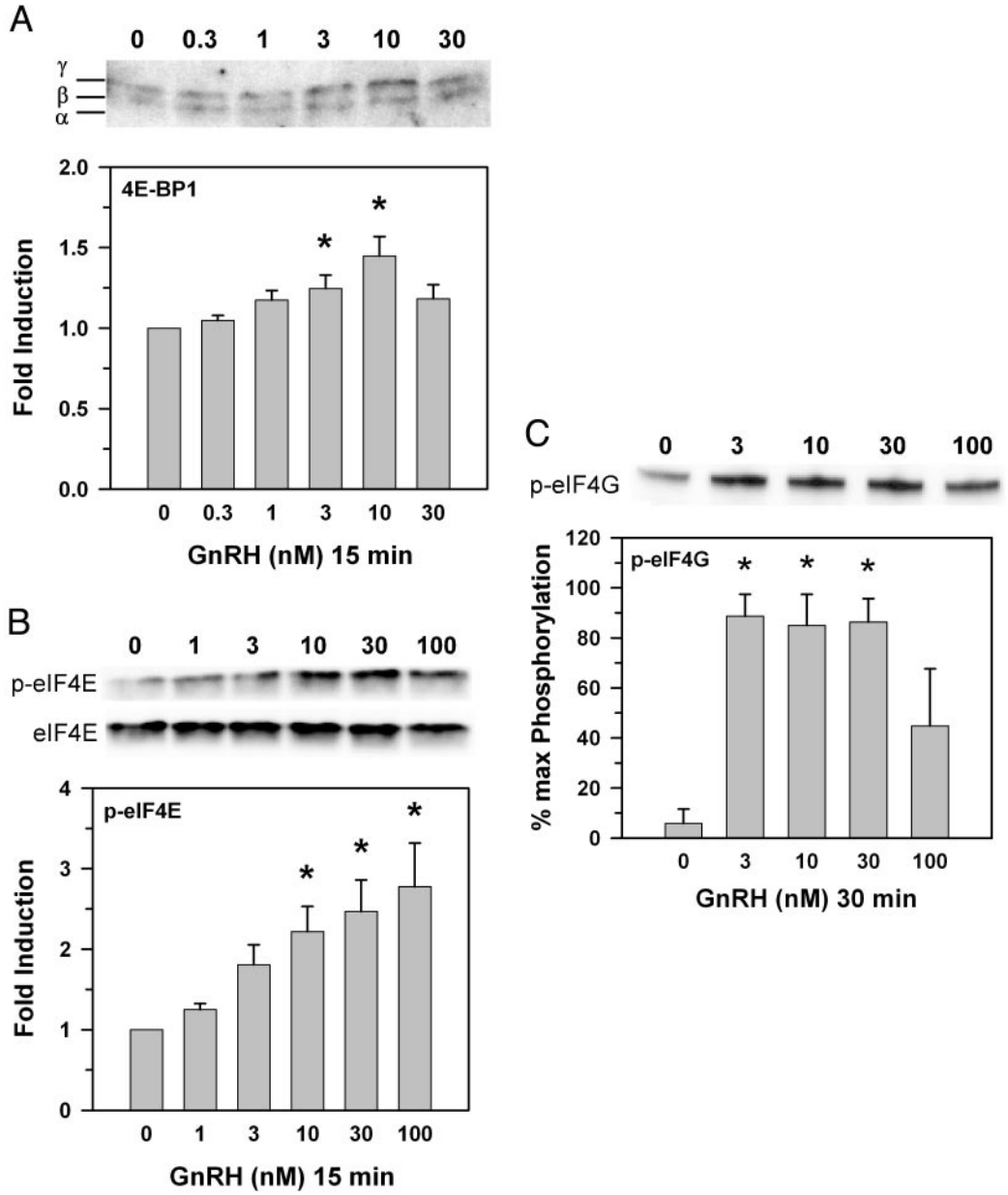

Fig. 3. Dose-Dependent Response of 4E-BP1, eIF4E, and eIF4G to GnRH

LβT2 cells were serum starved overnight (A–C) and amino acid starved 1 h (B and C) before GnRH treatment at indicated doses and time points. Extracts were subjected to SDS-PAGE followed by Western blotting with the following antibodies. A, Anti-4E-BP1 reveals three electrophoretic forms (γ, β, α) with the *histogram* representing the proportion of inactive γ-isoform relative to total 4E-BP1. B, Antiphospho-eIF4E (Ser 209) and eIF4E; *histogram* represents ratio of phospho-eIF4E relative to total eIF4E. C, Anti-phospho-eIF4G (Ser 1108); *histogram* represents values expressed as a percentage of maximal induction. Blots are representative images of each experiment. *Histograms* represent quantitative chemiluminescent image analysis of at least three independent experiments. The *asterisks* show significant difference from the control mean ($P \leq 0.05$) as determined by ANOVA and *post hoc* Dunnett's comparison to control test.

through activity of ERK and mammalian target of rapamycin (mTOR), respectively. We have previously shown that inhibiting the mTOR pathway in the αT3–1 cell line attenuates GnRH-stimulated translation, suggesting the involvement of mTOR in translational control (25). However, GnRH has also been shown to activate the ERK pathway in LβT2 cells, and inhibition of ERK activation by the MEK inhibitor PD 98059 prevents acute GnRH-induced increase in LHβ synthesis (15, 32). To determine whether the ERK pathway is involved in the GnRH regulation of translation, we measured GnRH activation of the cap-dependent translational reporter activity in the presence of the MEK inhibitor PD 98059 (Fig. 5). The

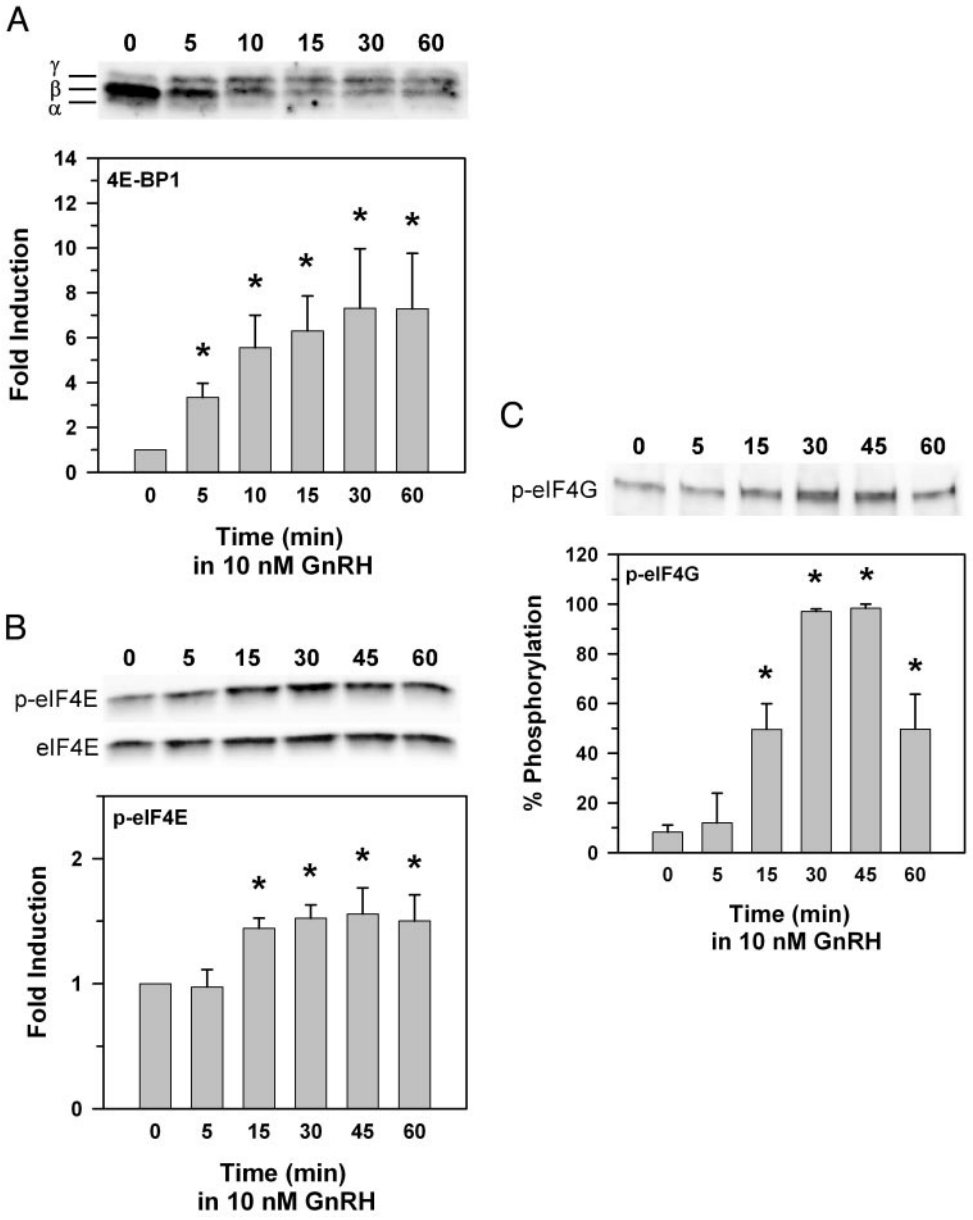

Fig. 4. Time-Dependent Response of 4E-BP1, eIF4E, and eIF4G to GnRH

LβT2 cells were serum starved overnight and amino acid starved for 1 h followed with 10 nM GnRH for the times shown. Extracts underwent SDS-PAGE and immunoblotted with the indicated antiserum. A, Three electrophoretic forms (γ, β, α) of 4E-BP1; the *histogram* depicts the proportion of inactive γ-isoform relative to total 4E-BP1. B, *Histogram* represents proportion of phospho-eIF4E relative to total eIF4E. C, Percent of maximal eIF4G phosphorylation. Blots are representative images. *Histograms* are the result of quantitative chemiluminescent imaging analysis of at least three separate experiments. The *asterisks* show significant difference from the control mean ($P \leq 0.05$) as determined by ANOVA and *post hoc* Dunnett's comparison to control test.

presence of 30 μM PD 98059 significantly represses the GnRH-induced activation of the translation reporter gene, as measured by the ratio of luciferase to β-galactosidase activity. Interestingly, translational reporter activation was not affected by rapamycin inhibition of mTOR, the upstream regulator of 4E-BP1 phosphorylation (data not shown). These findings implicate the ERK rather than mTOR as the key pathway in the GnRH activation of translation in LβT2 cells.

MAPK-Interacting Kinase 1 (Mnk1) and eIF4E Are Directly Involved in the GnRH Activation of the ERK Pathway

We further investigated the translation factors that might be affected by the ERK cascade. It is known that Mnk1 is a downstream target of ERK and that Mnk1-mediated phosphorylation of eIF4E is necessary for the recruitment of ribosomes to mRNA and the progression of translation initiation (28, 33, 34). Dominant-

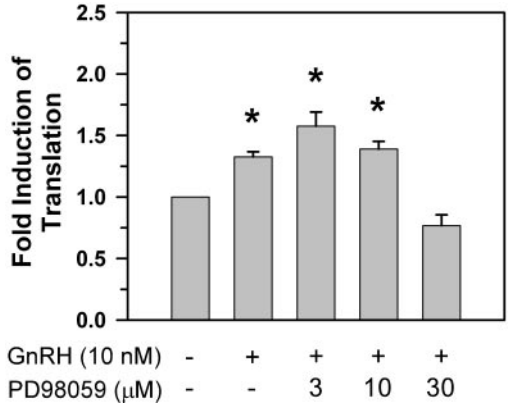

Fig. 5. MAPK Pathway Inhibitor PD98059 Prevents GnRH Activation of Translation

Cultured LβT2 cells were transiently transfected with the cap-dependent translation reporter gene, pGL3-CMV-Luc-EMCV-Gal. Cells were serum starved overnight, treated with PD 98059 at the indicated concentrations for 30 min, and subsequently stimulated with 10 nM GnRH for 4 h. Extracts were assayed for luciferase and β-galactosidase reporter activity. The *histograms* represents normalized ratio of luciferase to β-galactosidase activity of three independent experiments. The *asterisks* show significant difference from the control mean ($P \leq 0.05$), as determined by ANOVA and *post hoc* Dunnett's comparison to control test.

negative Mnk1 has been shown to inhibit PKC/ERK-activated phosphorylation of eIF4E (33). Therefore, we examined the phosphorylation status of Mnk1, eIF4E, and eIF4G in the presence of GnRH and the MEK inhibitor PD 98059; the PI3 kinase inhibitor LY 294002; or the mTOR inhibitor rapamycin (Fig. 6). Western blot analysis shows that GnRH activation of Mnk1 was significantly inhibited by the ERK inhibitor alone (Fig. 6A), whereas activation of eIF4E was significantly inhibited by the ERK, PI3 kinase, and mTOR inhibitors (Fig. 6B). Nevertheless, none of the inhibitors had an effect on GnRH activation of eIF4G (data not shown), suggesting that Mnk and eIF4E are the direct targets of the ERK pathway. This observation, along with the observation that the ERK and not mTOR inhibition leads to translational repression, suggests that Mnk may be the key factor in GnRH regulation of translation in LβT2 cells. This is consistent with the finding that a kinase-deficient Mnk1 results in the impairment of cap-dependent translation in the human embryonic kidney 293 cell line (31).

PI3 Kinase Activity Is Endogenously Active in LβT2 Cells

ERK involvement in translation regulation in LβT2 cells contrasts our previous observations in αT3–1 cells and other studies showing GPCR modulation of translation through 4E-BP1 and PI3 kinase (16, 22, 25). It is noted that significant levels of the γ-isoform of 4E-BP1 exist in unstimulated LβT2 cells (Fig. 3A). This may be a consequence of a high level of endogenous PI3 kinase

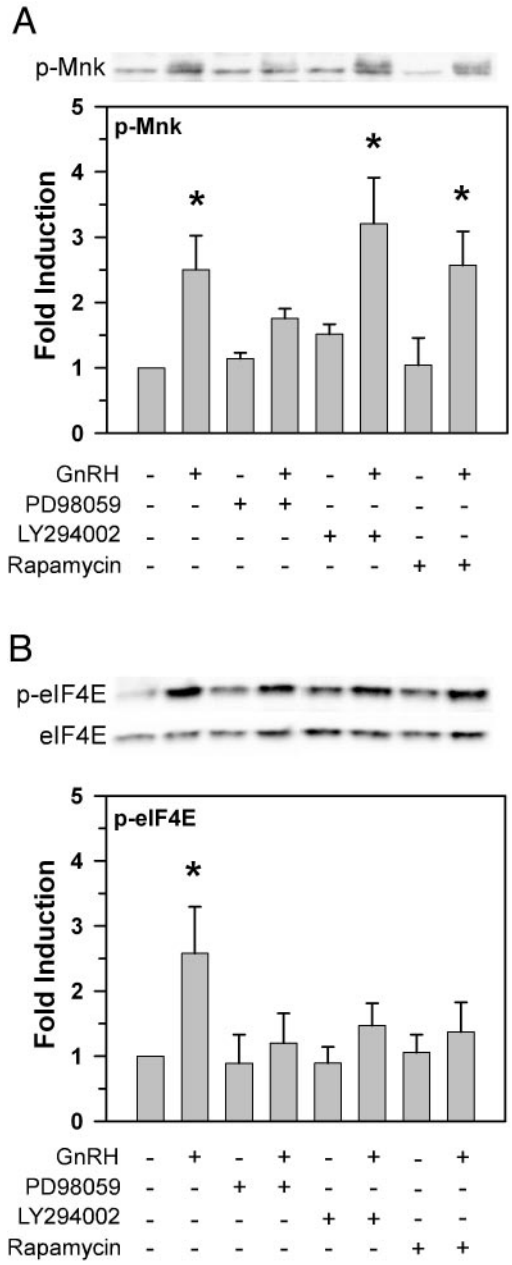

Fig. 6. eIF4E and Mnk Are Directly Activated in Response to GnRH Stimulation of MAPK

LβT2 cells were serum starved overnight, followed by amino acid starvation for 1 h and subsequently pretreated with 10 μM PD98059, 1.0 μM LY294002, or 10 nM rapamycin for 30 min, after which cells were treated with 10 nM GnRH for 15 min. Extracts were separated by SDS-PAGE and immunoblotted with the indicated antiserum. A, *Histogram* represents activation of phospho-Mnk. B, *Histogram* represents ratio of phospho-eIF4E to total eIF4E normalized to control. Blots are representative images and *histograms* are the result of quantitative chemiluminescent imaging analysis of at least three separate experiments. The *asterisks* show significant difference from the control mean ($P \leq 0.05$), as determined by ANOVA and *post hoc* Dunnett's comparison to control test.

activity providing sufficient basal levels of mTOR activity, leading to the maintenance of inactive 4E-BP1 in unstimulated cells. To test this hypothesis, we examined the endogenous levels of AKT, a target of PI3 kinase activity that lies upstream of mTOR. We examined AKT phosphorylation in serum-starved unstimulated LβT2 cells and found that, indeed, AKT is highly activated as determined by phosphorylation at Ser 473 (Fig. 7). Levels of AKT activity in untreated control extracts are as high as in GnRH-stimulated extracts and are inhibited in the presence of the PI3 kinase inhibitor, LY 294002. This finding suggests that PI3 kinase activity is endogenously active in our cell model and provides a possible explanation for the involvement of ERK rather than 4E-BP1/PI3 kinase in translational activation of LβT2 cells.

DISCUSSION

Recent analysis of GnRH action in gonadotropes, particularly the LβT2 cell model, has suggested that transcriptional responses of cell-specific genes such as LHβ subunit are secondary or later events subsequent to GnRH stimulation (11, 13). Various studies have shown that stimulation of gonadotropin hormone subunit mRNA or activation of gonadotropin subunit promoters does not reach maximal levels for several hours to 1 d after stimulation with GnRH (1, 9, 11, 35, 36). However, immunofluorescence examination of GnRH-stimulated LβT2 cells and *in vivo* studies of rat serum LHβ levels after GnRH stimulation show marked increases in hormone level in less than 6 h (14, 15). These observations offer the possibility that protein synthesis mechanisms contribute to this increase in hormone content. These nontranscriptional mechanisms may play a role in the rapid increase in gonadotropin hormone subunit production after acute GnRH stimulation. We have tested this hypothesis directly by examining the ability of GnRH to increase production of LHβ-subunit protein or LH secretion after treatment of the gonadotrope-derived cell line, LβT2, with the RNA polymerase II inhibitor actinomycin D or the translation inhibitor cycloheximide. Under these conditions, measurable increases in LHβ subunit protein and LH secretion are detected within 4 h of GnRH stimulation of cells pretreated with actinomycin D. However, GnRH is unable to elicit any changes in LHβ protein and LH secretion in cells pretreated with cycloheximide (Fig. 1). It is of interest that there are no significant differences between groups treated with GnRH in the presence of actinomycin D or cycloheximide in Fig. 1, B and C. If transcription were not involved in the response of GnRH activation, we would expect to see significant differences between these two groups. The lack of difference suggests a transcriptional component in the GnRH-stimulated increase of hormone production. This finding is consistent with other studies showing a small (≤1.4 fold) transcriptional activation at 6 h of GnRH stimulation (11–14).

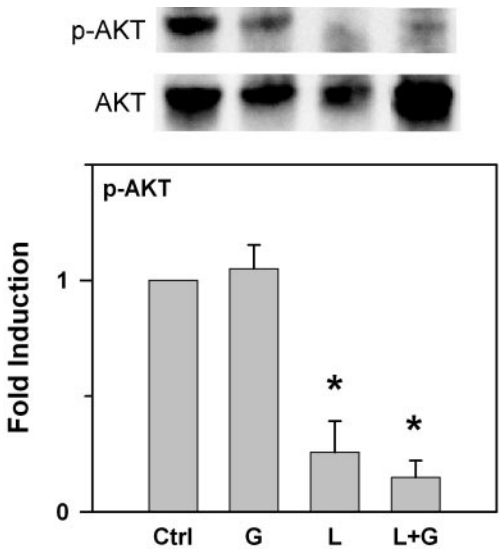

Fig. 7. AKT Endogenously Active in LβT2 Cells

LβT2 cells with overnight serum starvation were incubated in the presence of vehicle or 10 μM LY294002 (L) for 1 h. Cells were subsequently treated with 10 nM GnRH (G) for 15 min. Extracts were analyzed by Western blot using antiphospho-AKT (Ser 473) antibody, stripped, and reblotted with anti-AKT antibody. The *histogram* shows ratio of phospho-AKT to total AKT, normalized to the control of three independent experiments. The *asterisk* represents significant difference ($P \leq 0.05$) according to ANOVA and *post hoc* Dunnett's comparison to control test.

To further examine the significance of the translational component observed in Fig. 1, B and C, we performed transient tranfections using reporter genes that distinguish between specific activation of the LHβ promoter and activation of translation. We observed no significant increase in the rLHβ promoter activity driving the reporter gene in LβT2 cells at 4 h of GnRH stimulation (Fig. 2). However, increases in promoter activity are observed in 8 h of GnRH treatment (Lawson, M. A., unpublished observations). In spite of this, increases in translational activity are observed within 4 h of GnRH treatment when changes in cap-dependent translational activity of a bicistronic reporter gene under transcriptional control of the nonspecific CMV immediate-early gene promoter are measured (Fig. 2). These observations suggest that the transcriptional activation may not be as significant as translational activation within the 4-h time period. The differences between our data and previous observations of increased promoter activity reported (37) may be attributed to differences in GnRH treatment. The use of a GnRH analog rather than GnRH peptide and the use of truncated promoters to examine GnRH regulation of promoter activity in other studies may contribute to the observed differences.

To elucidate the mechanism of GnRH regulation of translation, we examined the phosphorylation status

of translation initiation factors, 4E-BP1, eIF4E, and eIF4G, in response to GnRH stimulation. Hyperphosphorylation of 4E-BP1 prevents binding to eIF4E, thereby activating translation through increased availability of eIF4E binding to eIF4G to form the cap-binding complex eIF4F. The formation of this cap-binding complex is necessary for ribosome assembly, recruitment, and initiation. Although it is not clearly understood how phosphorylation of eIF4E and eIF4G leads to increased translation, it is known that phosphorylation of eIF4E and eIF4G does not increase their association (28). Rather, it has been postulated that phosphorylation of eIF4E leads to the promotion of ribosome loading (28, 29). Analyses of these initiation factors show that GnRH stimulation results in increased phosphorylation of all three factors. We found this action to be dose dependent with 3–10 nM GnRH as optimal concentrations for maximal initiation factor activation. In addition, we also observed an order of maximal initiation factor activation in response to GnRH treatment: 4E-BP1 and eIF4E are the first to be maximally phosphorylated at 15 min, followed by eIF4G at 30 min (Fig. 4). These findings establish a dose- and time-sensitive factor response that will allow us to establish a pattern of differential regulation by GnRH.

Specificity was also established for the signal cascades that affect each factor's phosphorylation status. Functional tests of the pathways involved in translational regulation found that GnRH-stimulated cap-dependent translation was inhibited by blockade of the ERK pathway with PD 98059 (Fig. 5) but was unaffected when mTOR activity was blocked with rapamycin (data not shown). Only recently has evidence showing regulation of translation by GPCRs been presented (16, 22, 24, 25). However, these studies show GPCR translational activation through PI3 kinase, mTOR, and 4E-BP1. In contrast, our present study shows that, unlike other GPCRs, GnRH activation of translation in LβT2 cells is more dependent upon activation of eIF4E, rather than inactivation of 4E-BP1. The importance of eIF4E in translational activation has also been demonstrated in human embryonic kidney 293 cells, *i.e.* eIF4E mutants that cannot be phosphorylated have been found to drastically inhibit cap-dependent translation (31). The dependence of eIF4E rather than 4E-BP1 regulation in LβT2 cells may be attributed to the high level of endogenous PI3 kinase activity in these cells, as evidenced by the high level of AKT phosphorylation found in the unstimulated state (Fig. 7). Endogenously activated AKT may in turn provide sufficient mTOR activation and maintain a level of inactive 4E-BP1 that allows sufficient, unbound eIF4E to be available for interaction with eIF4G in unstimulated cells. Although not sequestered by 4E-BP1, eIF4E still requires activation by Mnk1 and thus renders eIF4E sensitive to inhibition of ERK activity.

GnRH-mediated control of translation initiation has implications for the control of gonadotropes and, ultimately, reproductive function. An important difference in measuring overall translational responses as opposed to measuring transcriptional responses of gonadotropin genes to GnRH is that the first mechanism is a very general response, whereas the second is a very specific response. The question remains whether a generalized stimulation of the translational control apparatus can elicit the specificity in the observed GnRH response. Although many eukaryotic genes are translationally regulated, it is not yet clearly understood how specific genes are controlled through this mechanism. However, regulation of specific genes through alteration of translation rather than transcription has been demonstrated in other model systems (38, 39). It is possible that the structure of the mRNA is a principal contributor to this specificity. Examination of mRNA structure or sequence similarities between genes sensitive to cap-dependent translational control may reveal a common mechanism of regulation.

Our observations suggest that translational control plays an important role in the acute GnRH response of the LβT2 gonadotrope cell line. The rapidity of the translational response explains the ability to detect increased LHβ subunit protein in cells within 4 h of GnRH treatment (15). We have confirmed that this activation may indeed be a result of translational regulation that occurs concomitantly with, but independently of, transcriptional activation. Our interpretation is that translational stimulation is a component of the acute GnRH response. In the absence of significant acute changes in LHβ gene transcription, cap-dependent translational activation provides a potent, direct, and nongenomic regulatory response to GnRH that results in a rapid increase in hormone synthesis. This regulatory mechanism is one that functions on a time scale that more closely matches the hourly changes that occur during the preovulatory phase of the estrous cycle and may therefore provide a regulatory pathway that is responsive to short-term changes in GnRH pulsatility.

In summary, we have demonstrated that the acute response to GnRH includes a component of translation regulation. We show that this involves GnRH regulation of translation initiation factors. We further show the ERK cascade as the key pathway in the GnRH activation of translation in the LβT2 cell line. Acute activation of translation by GnRH provides a mechanism for rapid response to hypothalamic signals controlling gonadotrope function, thereby providing a mechanism for fine temporal control of gonadotrope function. This may contribute to an increase in LHβ protein synthesis that is not solely dependent on transcriptional regulation.

MATERIALS AND METHODS

Cell Culture

The pituitary gonadotrope cell line, LβT2 (6), was maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supple-

mented with 4.5 mg/ml glucose, 10% fetal bovine serum, and 5% penicillin/streptomycin and incubated in a humidified atmosphere of 5% carbon dioxide.

Immunocytochemistry

For immunocytochemistry, LβT2 cells were plated at a density of 7×10^4 cells/cm^2 on Lab-Tek chamber slides (Nalge Nunc, Naperville, IL) in DMEM containing 10% fetal bovine serum. After 48 h of incubation, medium was changed to serum-free DMEM. After overnight incubation, the media were replaced with fresh serum-free DMEM containing vehicle, 400 μM actinomycin D, or 100 μg/ml cycloheximide. Effectiveness of actinomycin D inhibition of transcription was verified by blockade of increased early-growth response factor-1 mRNA expression in response to GnRH stimulation using Northern blot analysis (40, 41). Cycloheximde inhibition of translation was verified by blockade of protein synthesis as measured by metabolic labeling with ^{35}S-methionine or ^{35}S-cystine. After 1 h of inhibitor treatments, media were adjusted to 10 nm GnRH or an equivalent volume of vehicle. After 4 h, cell viability was verified using Trypan Blue, and no effects by inhibitor treatments were observed. Alternatively, after 4 h of treatment, cells were washed 5 min in PBS once and fixed with 4% formaldehyde in PBS for 20 min. The cells were subsequently washed twice and blocked with 2.0% normal goat serum and 0.3% Triton X-100 in PBS blocking buffer for 20 min. Fixed cells were then washed and incubated for 1 h with 1:2000 dilution of rabbit antirat LHβ primary antibody (supplied by the National Hormone and Pituitary Program), washed three times, and incubated with biotinylated antirabbit IgG 1.5 μg/ml (Vector Laboratories, Burlingame, CA) for 30 min. After antibody treatments, fluorescein isothiocyanate Avidin D was then applied at a final concentration of 1 μg/ml (Vector Laboratories). Finally, cells were cover slipped with Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories) for visualization of nuclei and examined by fluorescence microscopy on a Nikon E800 microscope (Nikon, Melville, NY). Images of cells were acquired using a Microfire digital camera (Olympus Corp., Lake Success, NY) using the manufacturer's software. Image analysis, correction for background fluorescence, and measurement of cell staining intensity was carried out using Sigma Scan Pro v5.0 Software (SPSS, Inc., Chicago, IL). Approximately 10 images per treatment were analyzed for each experiment. Total intensity of staining was normalized to control to yield relative intensity.

LH Immunoradiometric Assay

For the analysis of LH secretion, 1.2×10^6 cells were plated in six-well plates and treated as described for the immunohistochemistry experiments. After the 4-h GnRH treatment, 150 μl of media were collected and desalted using MicroSpin G-25 columns (Amersham Biosciences Corp, Piscataway, NJ). To prevent nonspecific protein binding to the beads, spin columns were rinsed twice with 0.1% BSA/PBS before sample application. Samples were collected according to the manufacture's instructions. Samples were lyophilized and resuspended in 75 μl of sterile H_2O. LH secretion levels were determined by immunoradiometric assay, performed at the Ligand Assay & Analysis Core Laboratory (University of Virginia, Charlottesville, VA).

Plasmids and Transfections

The bicistronic reporter plasmid was previously described (25). Briefly, the 664-bp *Spe*I fragment from pcDNAI (Invitrogen, Carlsbad, CA) containing the immediate-early CMV promoter was inserted into the *Nhe*I site of pGL3-basic (Promega Corp., Madison, WI) creating a new vector, pGL3-CMV. Downstream of the luciferase reporter gene coding sequence, the 5′-noncoding region of the EMCV was inserted, followed by the *E. coli* β-galactosidase coding sequence derived from pSDK LacZ pA. The resultant plasmid directs the synthesis of a single transcript encoding the luciferase reporter followed by the EMCV 5′-untranslated region containing the internal ribosome entry site, and the β-galactosidase coding sequence. Each reading frame is translated independently by either cap-dependent (luciferase) or cap-independent (β-galactosidase) mechanisms (42).

The 1.8-kb rat LHβ promoter-driven reporter plasmid previously described (43) was removed by *Hin*DIII-*Xba*I restriction digest and inserted into the multiple cloning site of pBluescriptII KS+ (Stratagene, La Jolla, CA). This new plasmid, pBKS+-rLHβ1.8, was then digested with *Kpn*I and *Xba*I to liberate the 1.8-kb promoter fragment. This fragment was inserted into *Kpn*I- and *Nhe*I-digested pGL3-Basic. The resultant plasmid, pGL3-rLHβ-1.8, was then sequenced to confirm identity. The proximal sequence of this promoter matches that of the 797 bases reported in GenBank accession no. AF020505. To construct the internal control plasmid pGL3-CMV-β-Gal, the luciferase coding sequences from *Nco*I to *Bam*HI of pGL3-CMV were then substituted by replacement with the *Nco*I to *Bam*HI region of the plasmid pSDK-Lac Z pA encoding *E. coli* β-galactosidase.

For transfection, cells were plated at a density of 50,000 cells/cm^2 and incubated 24 h before transfection. Cells were then changed into serum-free medium and transfected using Fugene 6 (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions using 0.1 μg total DNA per cm^2 plate area and incubated 16–18 h before treatment with GnRH. For inhibitor experiments, cells were pretreated with PD 98059, LY 294002, or rapamycin (Calbiochem, San Diego, CA) at indicated concentrations for 30 min before 10 nM GnRH treatment (4 h). Cells were harvested by lysis in 100 mM PBS containing 0.1% Triton X-100, vortexed, and clarified by centrifugation. Cell lysates were assayed directly for luciferase and β-galactosidase activity using the glow-type luciferase assay kit (Promega Corp.) and the Galacto-Light Plus kit (Tropix, Bedford, MA), respectively. Luminescence was measured in a 96-well plate using 20 μl of lysate in a LB96V luminometer (EG&G Berthold, Gaithersburg, MD). Plate transfection assays were performed in quadruplicate.

Western Blotting

For analysis of protein derived from static cultures, 1×10^7 cells were plated in 10-cm dishes and incubated for 24 h before treatment. LβT2 cells were placed in serum-free medium 24 h before harvest to eliminate hormone and growth factor influences on translation (44). To remove amino acid influences on initiation factor phosphorylation, cells were incubated in Earle's balanced salt solution (Sigma Chemical Co., St. Louis, MO) to amino acid starve (with the exception of 4E-BP1 dose response and AKT experiments) for 1 h (44) before stimulation with GnRH at the indicated doses and times. For inhibitor experiments, cells were treated with 10 μM PD 98059, 10 nM rapamycin, or 1.0 μM LY 294002 (Calbiochem, San Diego, CA) for 30 min and treated with 10 nM GnRH for 15 min.

For 4E-BP1, after cell treatment, the medium was removed and cells were washed once with ice-cold buffer A (50 mM Tris, pH 7.5; 150 mM KCl; 1 mM dithiothreitol; and 1 mM EDTA with phosphatase inhibitors, 50 mM β-glycerophosphate, 1 mM EGTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, and 50 nM okadaic acid). Cells were then harvested into 1 ml of the same buffer and pelleted briefly. The supernatant was removed, and 150 μl fresh buffer A were added to the cell pellet. For eIF4E, Mnk, eIF4G, and AKT, cells were washed with ice-cold PBS after cell treatment, harvested with Laemmli sample buffer, and sonicated for 15–30 sec. Cell lysates were assayed for protein content using the BIO-RAD DC Protein Assay (Bio-Rad

Laboratories, Hercules, CA). All samples were boiled for 5 min before 50–100 μg of protein were separated by SDS-PAGE on a 15% (4E-BP1), 10% (eIF4E, AKT, Mnk), or 7.5% (eIF4G) gel and transferred onto polyvinylidene difluoride membrane by semidry transfer. The membranes were blocked in 2× casein (Vector Laboratories) and incubated with primary antibodies. 4E-BP1, phospho-eIF4E, and AKT antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) whereas phospho-eIF4G, -AKT, -Mnk, and -eIF4E antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit anti-4EBP1-R113 was used in a 1:400 dilution; rabbit phospho-eIF4G Ser1108 and phospho-AKT Ser 473 antibody were used at a 1:1000 dilution; and AKT antibody was used at 1:2000 dilution at room temperature for 60 min. Rabbit eIF4E, rabbit phospho-Mnk 1 Thr197/202, and phospho-eIF4E Ser209 antibodies were used in a 1:1000 dilution overnight at 4 C. Blots were developed by enhanced chemiluminescence using a 1:5000 dilution of biotinylated antirabbit secondary antibody and horseradish peroxidase-conjugated avidin-biotin complex (Vector Laboratories), and visualized using GeneSnap Bio Imaging System (Syngene, Frederick, MD). Chemiluminescent analysis was performed using GeneTools software (Syngene).

Statistical Analysis

All statistical analysis was performed using JMP v. 4.0 or v. 5.0 software (SAS Institute, Cary, NC). Data are expressed as means ± SEM of at least three samples per group. Results were analyzed for significant differences using ANOVA. *Post hoc* group comparison was made using Student's *t* test, Dunnett's comparison to control test, or Tukey's honestly significant difference where appropriate. Analysis was conducted using untransformed data or data optimally transformed by the method of Box and Cox as indicated (45). A $P \leq 0.05$ was the requirement for declaring significance.

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