Gonadotropin-Inhibitory Hormone Inhibits GnRH-Induced Gonadotropin Subunit Gene Transcriptions by Inhibiting AC/cAMP/PKA-Dependent ERK Pathway in LβT2 Cells

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A neuropeptide that directly inhibits gonadotropin secretion from the pituitary was discovered in quail and named gonadotropin-inhibitory hormone (GnIH). The presence and functional roles of GnIH orthologs, RF-amide-related peptides (RFRP), that possess a common C-terminal LPXRF-amide (X = L or Q) motif have also been demonstrated in mammals. GnIH orthologs inhibit gonadotropin synthesis and release by acting on pituitary gonadotropes and GnRH neurons in the hypothalamus via its receptor (GnIH receptor). It is becoming increasingly clear that GnIH is an important hypothalamic neuropeptide controlling reproduction, but the detailed signaling pathway mediating the inhibitory effect of GnIH on target cells is still unknown. In the present study, we investigated the pathway of GnIH cell signaling and its possible interaction with GnRH signaling using a mouse gonadotrope cell line, LβT2. First, we demonstrated the expression of GnIH receptor mRNA in LβT2 cells by RT-PCR. We then examined the inhibitory effects of mouse GnIH orthologs [mouse RFRP (mRFRP)] on GnRH-induced cell signaling events. We showed that mRFRP effectively inhibited GnRH-induced cAMP signaling by using a cAMP-sensitive reporter system and measuring cAMP levels, indicating that mRFRP function as an inhibitor of adenylate cyclase. We further showed that mRFRP inhibited GnRH-stimulated ERK phosphorylation, and this effect was mediated by the inhibition of the protein kinase A pathway. Finally, we demonstrated that mRFRP inhibited GnRH-stimulated gonadotropin subunit gene transcriptions and also LH release. Taken together, the results indicate that mRFRP function as GnIH to inhibit GnRH-induced gonadotropin subunit gene transcriptions by inhibiting adenylate cyclase/cAMP/protein kinase A-dependent ERK activation in LβT2 cells. **(*Endocrinology* 153: 2332–2343, 2012)**

The hypothalamic neuropeptide, GnRH, is the primary factor regulating gonadotropin secretion (1, 2; for a review, see Ref. 3). An inhibitory hypothalamic neuropeptide for gonadotropin secretion was, until recently, unknown, although gonadal sex steroids and inhibin can modulate gonadotropin secretion. In 2000, we identified a novel hypothalamic dodecapeptide (SIKPSAYLPLRF-NH_2), which directly acts on the anterior pituitary to in-

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Abbreviations: AC, Adenylate cyclase; 8-CPT, 8-(4-chlorophenylthio); CRE, cAMP-response element; F, forward; FSK, forskolin; GF, GF109203X; GnIH, gonadotropin-inhibitory hormone; GnIH-R, GnIH receptor; GnRH-R, GnRH receptor; GPR, G protein-coupled receptor; H89, H-89 dihydrochloride; IBMX, 3-isobutyl-1-methylxanthine; MDL, MDL-12330A; mRFRP, mouse RFRP; PKA, protein kinase A; PKC, protein kinase C; qPCR, real-time quantitative PCR; R, reverse; Raf, Raf serine/threonin-protein kinase; RFRP, RF-amide-related peptide.

hibit gonadotropin release in quail, and termed it gonadotropin-inhibitory hormone (GnIH) (4). Subsequently, GnIH orthologs were found in various mammals (for reviews, see Refs. 5–7). GnIH and its mammalian orthologs, RF-amide-related peptides (RFRP), belong to the peptide group that possesses an LPXRF-amide carboxyl peptide consensus sequence (X = L or Q) (for reviews, see Refs. 5–7). Mammalian GnIH orthologs have also been identified in the brains of various mammalian species (8–13), and the inhibitory effect of GnIH orthologs on gonadotropin release has been demonstrated (13–19). GnIH and its orthologs also inhibit gonadotropin common α-subunit and specific β-subunit transcription in the pituitary (20–22). Accordingly, GnIH inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release (20). GnIH and its mammalian GnIH orthologs appear to act similarly across vertebrate species to regulate reproduction (for reviews, see Refs. 6, 7).

Although the role of GnIH in gonadotropin synthesis and release and the functional significance of GnIH in gonadal development and maintenance have been established, inhibitory mechanisms of GnIH actions are still unknown. GnIH can act directly on gonadotropes in the pituitary (4) via its specific G protein-coupled receptor (GPR), which is also expressed in multiple brain regions, including GnRH neurons (23) as well as the pituitary gland (10). Two receptors, GPR147 and GPR74, for GnIH have been identified (12, 24–29). However, to date, limited information regarding the GnIH signaling pathway through GnIH receptor (GnIH-R) in gonadotropes as well as in GnRH neuron is available (7, 14, 21, 30). In the present study, therefore, we investigated the pathway of GnIH cell signaling and its possible interaction with GnRH signaling pathway using the LβT2 cell line, a widely used mouse gonadotrope model. To investigate the interactions between GnIH and GnRH signalings, we examined the effects of mouse GnIH orthologs [mouse RFRP (mRFRP)-1, mRFRP-3L, and mRFRP-3S] on GnRH signaling pathways in the LβT2 cell line. LβT2 cells exhibit all the characteristics of a fully differentiated gonadotrope, including expressions of LH, FSH, and GnRH receptor (GnRH-R), as well as appropriate responses to GnRH (31). We first investigated the expression of GnIH-R mRNA in LβT2 cells. We then conducted detailed mechanistic analyses of the inhibitory effects of mRFRP on GnRH-induced cell signaling pathways. At the first step of our analyses of cell signaling, we demonstrated the inhibitory effect of mRFRP on GnRH-induced adenylate cyclase (AC)/cAMP/cAMP-dependent protein kinase A (PKA) activation. We further demonstrated the inhibitory effects of mRFRP on ERK phosphorylation and gonadotropin subunit gene transcriptions stimulated by GnRH in LβT2 cells. We also found that mRFRP inhibited GnRH-induced LH release in LβT2 cells. The present results indicate that the inhibitory effects of mRFRP on GnRH-induced gonadotropin subunit gene transcriptions are mediated by an inhibition of AC/cAMP/PKA-dependent ERK pathway. This is the first study showing the detailed signaling pathway mediating GnIH action on gonadotropin subunit gene transcriptions in the gonadotrope cell line, LβT2.

Materials and Methods

Reagents

GnRH (LH-releasing hormone human acetate salt; Sigma-Aldrich Co., St Louis, MO), forskolin (FSK) (Santa Cruz Biotechnology, Santa Cruz, CA), 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), phorbol 12-myristate 13-acetate (also known as 12-*O*-tetradecanoylphorbol-13-acetate; LC Laboratories, Woburn, MA), H-89 dihydrochloride (H89) (Cayman Chemical Co., Ann Arbor, MI), GF109203X (GF) (Enzo Life Sciences, Farmingdale, NY), MDL-12330A (MDL) (Sigma-Aldrich), and 8-(4-chlorophenylthio) (8-CPT)-cAMP (Wako, Osaka, Japan) were purchased from the manufacturer. mRFRP (mRFRP-1, mRFRP-3L, and mRFRP-3S) were synthesized with an automated solid-phase peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan) and purified by reversed phase HPLC.

Cell culture

LβT2 cells were kindly given by Pamela Mellon (University of California, San Diego, CA). αT3-1 cells were given by Salk Institute for Biological Studies (La Jolla, CA). The cells were grown in DMEM (GIBCO/Invitrogen, Carlsbad, CA) supplemented with high glucose (4.5 g/liter) containing 10% fetal bovine serum (GIBCO/Invitrogen) and 1% penicillin/streptomycin antibiotics (GIBCO/Invitrogen) in a humidified 5% CO_2 atmosphere at 37 C. Cells were starved overnight in serum-free DMEM before the experiment. For experimental protocols, dispersed cells were used between passages 4 and 15.

RNA extraction and RT-PCR analysis

Total RNA was extracted by using Sepazol-RNA I Super (Nacalai Tesque, Kyoto, Japan) and subjected to RT using Prime Script RTase (Takara, Shiga, Japan). The expression of mouse GnIH-R was confirmed by RT-PCR analysis using *Taq* polymerase (Ex *Taq* polymerase; Takara). The sequences of the primers were as follows: 5′-CCGAGTCTGAACGAGAGTGA-3′ forward (F) and 5′-CGGTTCTTAAGCACGATGAA-3′ reverse (R) to amplify the partial mouse GPR147 cDNA (173 bp); 5′-GCCCTCCTTTTCATCCTTTC-3′ (F) and 5′-TGGAAAGCATCTTGGAAACC-3′ (R) to amplify the partial mouse GPR74 cDNA (209 bp); and 5′-ACAACTTTGGCATTGTGGAA-3′ (F) and 5′-GATGCAGGGATGATGTTCTG-3′ (R) to amplify the partial mouse glyceraldehyde-3-phosphate dehydrogenase cDNA (133 bp). The amplification of specific DNA regions was monitored by agarose gel electrophoresis and ethidium bromide staining. The PCR products were sequenced by using a DNA sequencer (3130 Genetic Anaylzer; Applied

Biosystems, Foster City, CA), and the correct amplifications of the target genes were confirmed.

Transient transfection and dual-luciferase reporter assay system

Duplicate samples of LβT2 cells were plated in 24-well plates and grown to 70–80% confluence for 24 h before transfection. Cells were then cotransfected with 500 ng of the pGL4.29[luc2P/CRE/Hygro] (firefly luciferase reporter construct; Promega, Madison, WI) and 15 ng of pRL-CMV (renilla luciferase reporter construct; Promega) using TransIT-LT1 (Mirus Bio Corp., Madison, WI) according to the manufacturer's instructions. Cells were starved overnight in serum-free DMEM before the experiment and then stimulated by GnRH with or without mRFRP for 6 h to allow accumulation of luciferase mRNA/protein. Cell extracts were prepared, and luciferase activity was measured using the dual-luciferase reporter assay system (Promega). The ratio of firefly luciferase activity to renilla luciferase activity was used as the results to coordinate the differences in transfection efficiency among samples.

Measurement of cAMP accumulation

LβT2 cells were plated at a density of approximately 1.5×10^5 in 24-well plates and grown to 80% confluence. To determine the intracellular content of cAMP, cells were starved overnight in serum-free DMEM and then preincubated for 30 min in serum-free DMEM containing 1 mM IBMX to inhibit cAMP phosphodiesterase activity. After preincubation, cells were treated with indicated concentrations of mRFRP for 30 min and then stimulated with GnRH for 45 min. Intracellular cAMP concentrations were measured using a Direct cAMP ELISA kit (Enzo Life Sciences) (32) following the manufacturer's protocol.

Protein extraction and Western blot analysis

LβT2 cells were grown to 80% confluence in six-well plates. After starvation for overnight in serum-free DMEM, cells were treated with reagents in various combinations. Cells were then washed with cold PBS and lysed in radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate] supplemented with protease inhibitor cocktail (Nacalai USA, San Diego, CA). Equal amounts of protein samples were precleared by centrifugation (15,000 rpm for 10 min at 4 C) and separated by 12.5% SDS-PAGE. After electrotransfer onto polyvinylidene difluoride membrane, detections of phosphorylated ERK-1/2 and total ERK were achieved by using a rabbit polyclonal antiphosphorylated ERK antibody (Cell Signaling, Danvers, MA) at 1:1000 and a rabbit polyclonal antitotal ERK-1/2 (Cell Signaling) at 1:1000, respectively, followed by a horseradish peroxidase-conjugated antirabbit IgG secondary antibody (Santa Cruz Biotechnology) at 1:1000. Detection was accomplished by using the Amersham ECL Plus TM Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ).

Real-time quantitative PCR (qPCR) analysis

To quantify the mRNA expression of mouse gonadotropin subunit genes (common α-subunit, LHβ-subunit, and FSHβ-subunit) in LβT2 cells, qPCR was conducted with slight modification of our previous method (20). In this study, we used the StepOnePlusTM Real-Time PCR System (Applied Biosystems) according to the recommendations of the manufacturer. β-Actin, a housekeeping gene, was used for the internal standard. The sequences of the primers for qPCR were as follows: 5′-TCTGGTCATGCTGTCCATGT-3′ (F) and 5′-GGAGAAGCAACAGCCCATAC-3′ (R) for mouse common α-subunit (amplifying 154-bp product); 5′-TGTCCTAGCATGGTCCGAGTACT-3′ (F) and 5′-AGGGCTACAGGAAAGGAGACTATG-3′ (R) for mouse LHβ-subunit (145 bp); 5′-CTGGTGCTGGAGAGCAATCT-3′ (F) and 5′-CCGAGCTGGGTCCTTATACA-3′ (R) for mouse FSHβ-subunit (157 bp); and 5′-GGAATGGGTCAGAAGGACTC-3′ (F) and 5′-CATGTCGTCCCAGTTGGTAA-3′ (R) for mouse β-actin (111 bp). The final reaction mixture contained 1 μM each forward and reverse primer, 50 ng of cDNA, and SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan). The PCR condition was 95 C for 2 min, followed by 45 cycles of 95 C, 15 sec; 60 C, 15 sec; and 72 C, 15 sec. An external standard curve was generated by various concentrations of the target PCR product, which had been purified and its concentration measured previously. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis and gel electrophoresis. The mouse gonadotropin subunit gene expressions in each reaction were normalized by the expression of β-actin, and the results were represented as fold activation with respect to the experimental control.

Measurement of LH release

LβT2 cells were grown to 80% confluence in 24-well plates and starved overnight in serum-free DMEM. For determination of the inhibitory effect of mRFRP on GnRH-induced LH release, cells were treated with two concentrations (10^{-7} and 10^{-6} M) of mRFRP for 30 min, and further incubated with 10^{-8} M GnRH for 1.5 h. After treatment, the supernatant of the culture media was collected, and the concentration of LH in LβT2 culture media, was determined using a Rat LH ELISA kit (Shibayagi Co., Ltd., Gunma, Japan) following the manufacturer's protocol.

Statistical analysis

Statistical significance was assessed by one-way ANOVA followed by Tukey's multiple comparisons test using Prism statistical software (GraphPad Software, Inc., La Jolla, CA).

Results

Expression of GnIH-R mRNA in LβT2 cells

We first examined the expression of GnIH-R mRNA in LβT2 cells by RT-PCR using intron-spanning primers designed to specifically amplify the mouse GPR147, also known as neuropeptide FF1 receptor 1, cDNA. We used mouse diencephalon and liver cDNA as positive and negative controls, respectively (24). As shown in Fig. 1, single PCR products of the expected size were detected for GnIH-R cDNA in LβT2 cells. The PCR product was sequenced and confirmed to be the partial mouse GPR147 cDNA (from 182 to 347 bp; GenBank, NM_001177511). We also examined the expression of GPR74, also known as neuropeptide FF1 receptor 2, in LβT2 cells. DNA se-

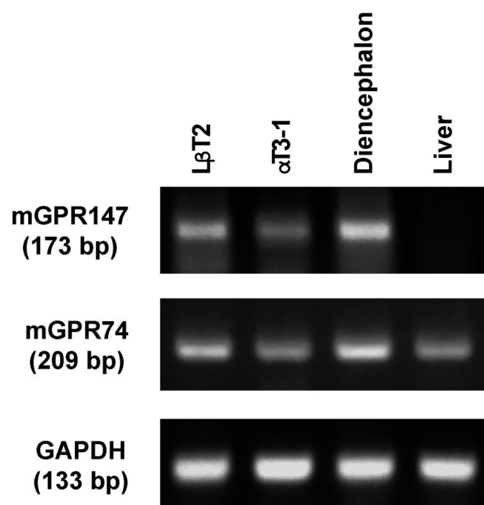

FIG. 1. RT-PCR analysis of GnIH-R in gonadotrope cells. Total RNA were extracted from LβT2, αT3-1, mouse diencephalon (positive control), and mouse liver (negative control) and reverse transcribed into cDNA. After PCR amplification with primers for mouse GPR147, GPR74, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), amplicons were electrophoresed on a 1.5% agarose gel.

quencing of the PCR product confirmed it to be the partial mouse GPR74 cDNA (from 895 to 1103 bp; GenBank, NM_133192). Low level of expression was also found in liver (26). The results indicate that the LβT2 cell line is an appropriate cell model system to investigate the interaction between GnIH and GnRH signalings. GnIH-R was also expressed in another mouse gonadotrope cell line, αT3-1, confirming general expression of GnIH-R in gonadotropes.

Synthesis of mRFRP

In mammals, the RFRP precursor gene is translated and cleaved into at least two peptides, RFRP-1 and RFRP-3 (for reviews, see Refs. 6, 7). The N terminus of each RFRP may be generated by proteolytic cleavage at the first or second basic residue from the C terminus. There are two basic residues in the N-terminal portions of mRFRP-1 and mRFRP-3 (Fig. 2A). mRFRP-1 was predicted as the short form because of its similarity to the identified human RFRP-1, MPHSFANLPLRF-NH_2 (Fig. 2B) (10). On the contrary, the N-terminal sequence of mRFRP-3 exhibited low similarity to the human RFRP-3, VPNLPQRF-NH_2 (10). Accordingly, we synthesized both the long form of mRFRP-3 (mRFRP-3L) and short form of mRFRP-3 (mRFRP-3S) and used both peptides for the experiment (Fig. 2B).

Alignment of avian GnIH precursor polypeptide and mammalian RFRP precursor polypeptide shows that avian GnIH peptide corresponds to RFRP-2 in mammals. However, the RFRP precursor polypeptide of rodents does not encode RFRP-2 (for reviews, see Ref. 6, 7). Accordingly, both RFRP-1 and RFRP-3 do not correspond to avian GnIH in terms of their position in the precursor polypeptide.

A

MEIISLKRFILLTVATSSFLTSNTFCTDEFMMPHFHSKEGDGKYSQLRGI

PKGEKERSVSFQELKDWGAKNVI***K***MSPAPAN***K***VPHSAANLPLRFGRTIDE
mRFRP-1

KRSPAA***R***VNMEAGT***R***SHFPSLPQRFGRTTARSPKTPADLPQKPLHSLGSS
mRFRP-3

ELLYVMICQHQEIQSPGGKRTRRGAFVETDDAERKPEK

B

Sequence	Name
VPHSAANLPLRF-NH_2	mRFRP-1
VNMEAGTRSHFPSLPQRF-NH_2	mRFRP-3 long (mRFRP-3L)
SHFPSLPQRF-NH_2	mRFRP-3 short (mRFRP-3S)

FIG. 2. The amino acid sequence of mRFRP. A, Amino acid sequence of mRFRP preproprotein. The sequence of mRFRP-1 and mRFRP-3 are underlined. The first and the second basic amino acids at the N termini of mRFRP-1 and mRFRP-3 are shown in *bold italic letters*. B, Three kinds of mRFRP, mRFRP-1, mRFRP-3L (long form), and mRFRP-3S (short form), were synthesized and used in this study.

Inhibitory effect of mRFRP on GnRH-induced AC/cAMP/PKA activation

Because GnIH-R, GPR147, and GPR74 couple with $G\alpha_i$, it was hypothesized that activation of GnIH-R inhibits the AC, thus reduces intracellular cAMP levels and PKA activity (AC/cAMP/PKA pathway) (for a review, see Refs. 7, 33). On the contrary, it is established in LβT2 cells that GnRH can stimulate $G\alpha_s$-mediated AC/cAMP/PKA pathway as well as the classical $G\alpha_{q/11}$-mediated pathway (34–38). Accordingly, we examined the effect of mRFRP on AC/cAMP/PKA pathway stimulated by GnRH in LβT2 cells, using a cAMP-response element (CRE) reporter system. To determine the specificity of this reporter system, specific chemical activator or inhibitors for the AC/cAMP/PKA pathway were used in control experiments. The CRE-luciferase reporter system was significantly activated by FSK, an AC activator, in a dose-dependent manner (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). This FSK-induced CRE-luciferase activity was specifically inhibited by the inhibitors for AC (MDL) and PKA (H89) but not by the PKC inhibitor (GF) (Supplemental Fig. 1B). Using this AC/cAMP/PKA-specific reporter system, we found that GnRH can induce a dose-dependent stimulation of CRE-luciferase activity in LβT2 cells (Fig. 3A). Although GnRH failed to stimulate CRE-luciferase activity at the concentrations of 10^{-10} to 10^{-8} M, increases in CRE-luciferase activity were detected in cells stimulated by 10^{-7} and 10^{-6} M GnRH (2.3- and 5.7-fold over basal). Notably, we could not observe any

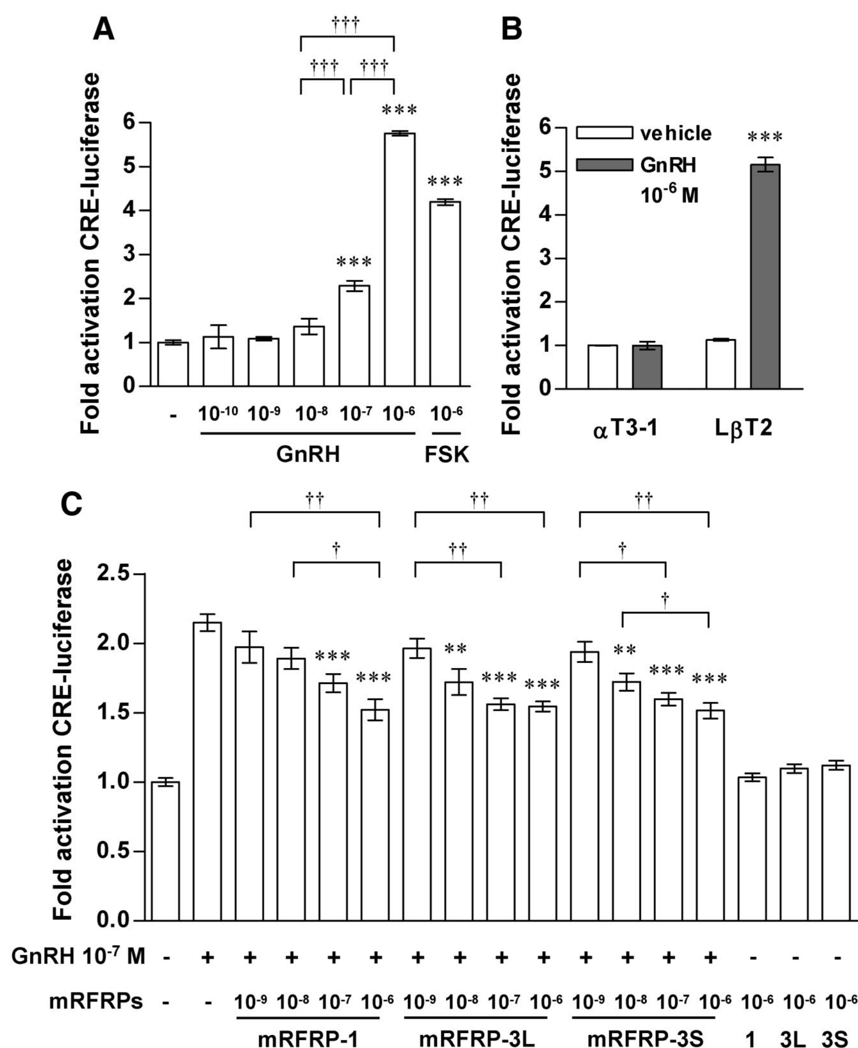

FIG. 3. Inhibitory effect of mRFRP on GnRH-induced cAMP/PKA activation in LβT2 cells. A, GnRH-induced transcriptional activity of the cAMP-dependent CRE reporter gene in LβT2 cells. LβT2 cells were transiently transfected with the CRE-luciferase reporter. Cells were then stimulated with GnRH (at the concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M) for 6 h; 10^{-6} M FSK, an AC activator, was used as a positive control. The luciferase activity was measured from cell lysates and expressed as fold activation over its respective basal. Data shown are mean ± SEM of repeated experiments. ***, $P < 0.001$ *vs.* basal (−); †††, $P < 0.001$ between 10^{-8}, 10^{-7}, and 10^{-6} M GnRH; n = 4. B, Activation of cAMP/PKA pathway by GnRH is dependent on cell context. Both αT3-1 and LβT2 cells were transiently transfected with the CRE-luciferase reporter and then stimulated with 10^{-6} M GnRH for 6 h. ***, $P < 0.001$ *vs.* basal (vehicle); n = 4. C, All mRFRP (1, 3L, and 3S) inhibited GnRH-induced CRE-luciferase activity in dose-dependent manners. LβT2 cells were transiently transfected with the CRE-luciferase reporter and then stimulated by 10^{-7} M GnRH in the presence of mRFRP-1, mRFRP-3L, and mRFRP-3S at the concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M for 6 h; 10^{-6} M mRFRP were also treated alone. **, $P < 0.01$; ***, $P < 0.001$ *vs.* cells only stimulated by GnRH; †, $P < 0.05$; ††, $P < 0.01$ between different concentrations of mRFRP; n = 5–9.

significant increase in CRE-luciferase activity in αT3-1 cells even at the concentration of 10^{-6} M GnRH (Fig. 3B), consistent with the previous reports showing that αT3-1 cells were unable to substantiate any GnRH-induced cAMP production (39–41) and GnRH-R exclusively couples to $G\alpha_{q/11}$ in αT3-1 cells (42).

Next, we coadministered the synthetic mRFRP with GnRH to examine their effects on GnRH-induced CRE-luciferase activity. As shown in Fig. 3C, 10^{-7} M GnRH induced approximately 2-fold increase in luciferase activity over basal. This increase was inhibited by cotreatment with various concentrations of mRFRP-1, mRFRP-3L, and mRFRP-3S. Increasing the concentrations of mRFRP from 10^{-9} to 10^{-6} M resulted in a dose-dependent reduction of CRE-luciferase activity (Fig. 3C), suggesting that all mRFRP effectively inhibit GnRH-induced cAMP/PKA activation by inhibition of AC. These inhibitory effects of mRFRP were similar to the effect of an AC inhibitor, MDL (data not shown). Notably, mRFRP alone did not alter basal CRE-luciferase activity (Fig. 3C), indicating that mRFRP alone do not have significant inhibitory effect on AC/cAMP/PKA activation in LβT2 cells.

To confirm that mRFRP inhibit cAMP-mediated signaling pathway in LβT2 cells, we directly measured intracellular cAMP levels in response to mRFRP. First, we showed that 10^{-7} M GnRH significantly increased cAMP levels in LβT2 cells as previously reported (Fig. 4) (36, 43). Next, we examined the inhibitory effect of mRFRP on GnRH-induced cAMP accumulation. We treated mRFRP at the concentrations from 10^{-7} to 10^{-6} M (these concentrations were determined based on CRE-luciferase reporter assay, see Fig. 3C). As shown in Fig. 4, mRFRP also inhibited GnRH-induced cAMP production, consistent with the results of CRE-reporter assay, demonstrating that mRFRP directly inhibit cAMP production induced by GnRH.

Inhibitory effect of mRFRP on GnRH-induced ERK activation

In gonadotropes, GnRH stimulation leads to the activation of MAPK pathways, such as ERK, c-Jun N-terminal kinase, and p38. Among these pathways, ERK pathway appears to play a central role in the regulation of gonadotropin gene expression as defined by gonadotrope-derived cellular models (for a review, see Ref. 44). Thus, we investigated ERK activation within the GnIH signaling pathway. Before assessing the effect of

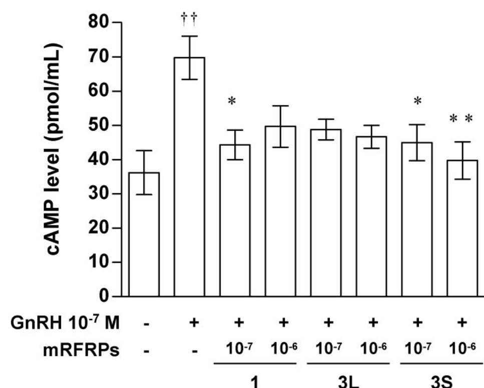

FIG. 4. Inhibitory effect of mRFRP on GnRH-induced cAMP production. LβT2 cells were treated with the indicated combinations and concentrations of GnRH and mRFRP (pretreated with mRFRP for 30 min and then stimulated with GnRH for 45 min) after a 30-min preincubation in the presence of the phosphodiesterase inhibitor IBMX (1 mM), the intracellular cAMP produced was determined by ELISA. Data shown are mean ± SEM of repeated experiments. ††, $P < 0.01$ *vs.* basal (−); *, $P < 0.05$; **, $P < 0.01$ *vs.* cells only stimulated by GnRH; n = 3.

mRFRP on GnRH-induced ERK activation in LβT2 cells, we first characterized the pathway activating ERK in LβT2 cells using various inhibitors of intracellular signaling cascades (Fig. 5A). Although Gα_q-mediated protein kinase C (PKC) pathway was demonstrated for GnRH-induced ERK activation (for a review, see Ref. 45), the PKC inhibitor (GF) did not significantly decrease GnRH-stimulated ERK phosphorylation in LβT2 cells (Fig. 5B). On the contrary, the inhibitor for AC (MDL) and PKA (H89) eliminated GnRH-stimulated ERK phosphorylation to the basal level (Fig. 5B). The concentrations of these inhibitors (10^{-4} M MDL, 10^{-6} M H89, and 10^{-6} M GF) were determined according to previous reports. These inhibitors alone had no inhibitory effect on basal ERK activity (Supplemental Fig. 2A). These results indicate that, in LβT2 cells, AC/cAMP/PKA pathway is involved in GnRH-induced ERK activation, and GnRH signaling toward ERK is transmitted mainly through PKA rather than PKC in LβT2 cells.

We then examined whether mRFRP inhibit ERK activation by GnRH in LβT2 cells. As shown in Fig. 5B, all mRFRP effectively inhibited GnRH-stimulated ERK activation with no change in the total amount of ERK as judged by the equal probing with antitotal ERK antibody. Consistent with the result of CRE-luciferase assay, mRFRP had no inhibitory effect on the basal ERK activity without GnRH stimulation (Supplemental Fig. 2A).

The inhibitory effect of mRFRP on ERK activation is mediated by the AC/cAMP/PKA-dependent pathway but not by the PKC pathway

To confirm that mRFRP inhibit ERK activation by interfering AC/cAMP/PKA pathway in LβT2 cells, we used an AC activator, FSK. We found that an artificial activation of Gα_s-AC by FSK can induce ERK activation (Fig. 6A), confirming the involvement of Gα_s-AC/cAMP/PKA pathway in ERK activation in LβT2 cells. We then demonstrated that mRFRP can also effectively inhibit this FSK-induced ERK activation in a similar manner to MDL and H89, inhibitors of the AC/cAMP/PKA pathway (Fig. 6A, also refer to Fig. 5, A and B). On the contrary, we could not observe any inhibitory effect of mRFRP on PKC-dependent ERK activation (Supplemental Fig. 2B). The PKC activator, phorbol 12-myristate 13-acetate, effectively activated ERK phosphorylation, and this activation was significantly inhibited by a PKC-specific inhibitor, GF, in a dose-dependent manner but not by mRFRP as well as MDL (inhibitor of AC) and H89 (inhibitor of PKA) (Supplemental Fig. 2B). These results indicate that mRFRP specifically inhibit

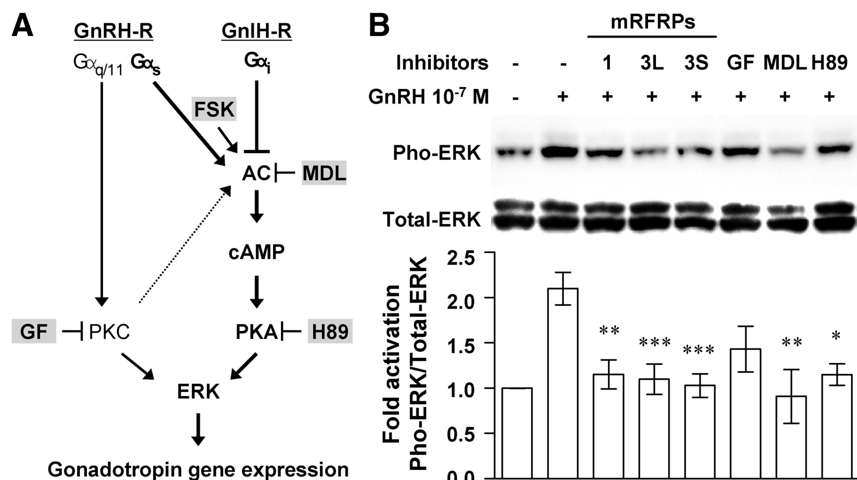

FIG. 5. Inhibitory effect of mRFRP on the phosphorylation of ERK in LβT2 cells. A, Model for the inhibitory effect of GnIH on GnRH-induced ERK activation mediated by AC/cAMP/PKA pathway in LβT2 cells. Inhibitors for AC, PKA, PKC, and activator for AC are *shaded*. B, Phosphorylated and total ERK were detected by Western blot analysis. LβT2 cells were pretreated (15 min) with 10^{-6} M mRFRP (1, 3L, and 3S), 10^{-6} M GF, 10^{-4} M MDL, and 10^{-6} M H89 before 10^{-7} M GnRH stimulation for 30 min. Clarified cell lysates were separated by SDS-PAGE and immunoblotted with an antibody for phosphorylated ERK (Pho-ERK) (*top panel*). The blots were then stripped and reblotted with an antibody to total ERK to determine total ERK protein loading (Total-ERK) (*bottom panel*). The phosphorylated ERK were quantified with Scion imaging software (Scion Corp., Frederick, MD). Data are expressed as a fold activation over its basal (Pho-ERK normalized to Total-ERK). Data shown are mean ± SEM of repeated experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* cells only stimulated by GnRH; n = 3–6.

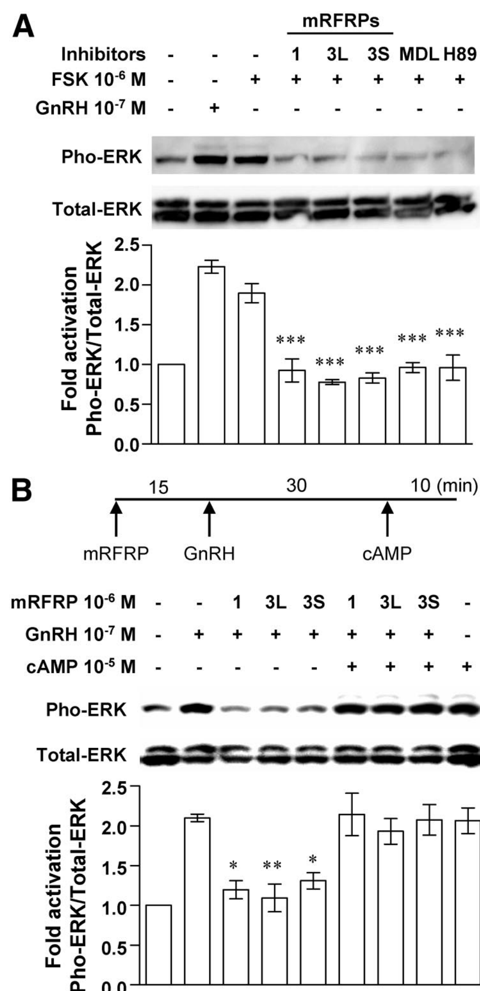

FIG. 6. Inhibitory effect of mRFRP on GnRH-induced ERK activation mainly depends on AC/cAMP/PKA-mediated pathway. A, Inhibitory effect of mRFRP on FSK-induced ERK activation determined by Western blot analysis as described in Fig. 5B. LβT2 cells were pretreated for 15 min with 10^{-6} M mRFRP (1, 3L, and 3S), 10^{-4} M MDL, and 10^{-6} M H89 before 10^{-6} M FSK stimulation for 30 min. The phosphorylated ERK were quantified, and data are expressed as a fold. Data shown are mean ± SEM of repeated experiments. ***, $P < 0.001$ *vs.* cells only stimulated by FSK; n = 3. B, No inhibitory effect of mRFRP on GnRH-induced ERK activation in the presence of exogenous cAMP. LβT2 cells were pretreated for 15 min with 10^{-6} M mRFRP (1, 3L, and 3S) before GnRH stimulation for 30 min, and the cells were treated with 10^{-5} M 8-CPT-cAMP (a cell-permeable cAMP analog) for 10 min. Western blot analysis was performed as described in Fig. 5B. The phosphorylated ERK were quantified, and data are expressed as a fold. *, $P < 0.05$; **, $P < 0.01$ *vs.* cells only stimulated by GnRH; n = 3.

AC/cAMP/PKA-dependent pathway to activate ERK but not PKC-dependent ERK activation.

To further demonstrate that mRFRP inhibit GnRH-induced ERK activation by the inhibiting AC/cAMP/PKA pathway, we used the cell-permeable cAMP analog, 8-CPT-cAMP, which directly activates PKA. Treatment of 8-CPT-cAMP completely canceled the inhibitory effect of mRFRP on GnRH-induced ERK activation (Fig. 6B), indicating that mRFRP inhibit ERK activation by interfering GnRH action on AC/cAMP/PKA pathway in LβT2 cells. We also observed that 8-CPT-cAMP alone can effectively activate ERK (Fig. 6B).

Inhibitory effects of mRFRP on gonadotropin subunits gene transcriptions

In gonadotropes, a substantial fraction of activated ERK undergoes rapid translocation to the nucleus and induces the gene expressions of gonadotropin subunits, FSHβ, LHβ, and common α (for a review, see Ref. 44). If mRFRP act as GnIH in mouse, inhibitory effects of mRFRP on the expressions of gonadotropin subunit genes are expected. Because LβT2 cells express gonadotropin subunits, we examined the effects of mRFRP on the expressions of these genes (Fig. 7, A–C). Treatment with GnRH increased the expressions of LHβ, FSHβ, and common α-subunit mRNA by 2.0- to 2.5-fold in LβT2 cells. Although the stimulatory effect of GnRH alone on FSHβ gene expression in LβT2 cells had been conflictive and dependent on the experimental condition, resent studies showed GnRH-induced FSHβ transcription in LβT2 cells (46–48), consistent with our result (Fig. 7B). The substantial expression of FSHβ mRNA supports that LβT2 cell line is a good model of mature, fully differentiated gonadotropes.

Next, we observed that all mRFRP totally abolished the increases in LHβ, FSHβ, and common α-subunit mRNA expressions stimulated by GnRH (Fig. 7, A–C), confirming that mRFRP-1, mRFRP-3L, and mRFRP-3S are inhibitors of GnRH-stimulated gonadotropin subunit gene transcriptions in LβT2 cells. On the other hand, mRFRP did not affect basal mRNA levels of gonadotropin subunits in absence of GnRH stimulation (data not shown). Both of the inhibitors of AC/cAMP/PKA pathway, MDL (inhibitor of AC) and H89 (inhibitor of PKA), effectively inhibited GnRH-stimulated gonadotropin expressions (Fig. 7, A–C). On the contrary, the inhibitor of PKC, GF, did not significantly inhibit GnRH-stimulated gonadotropin expressions (Fig. 7, A–C). These results indicate that the AC/cAMP/PKA pathway is more important than the PKC pathway for GnRH-induced gonadotropin gene expressions in LβT2 cells.

Inhibitory effect of mRFRP on LH release

Finally, we examined whether mRFRP inhibit GnRH-induced gonadotropin release. LβT2 cells were treated with increasing doses of GnRH (10^{-9} to 10^{-5} M). We observed a clear dose-response curve (data not shown), with maximal stimulation at 10^{-8} M (Fig. 8), consistent with previous reports (49). We then examined the inhibitory effect of mRFRP at concentrations of 10^{-7} to 10^{-6} M. As shown in Fig. 8, mRFRP-1 significantly inhibited

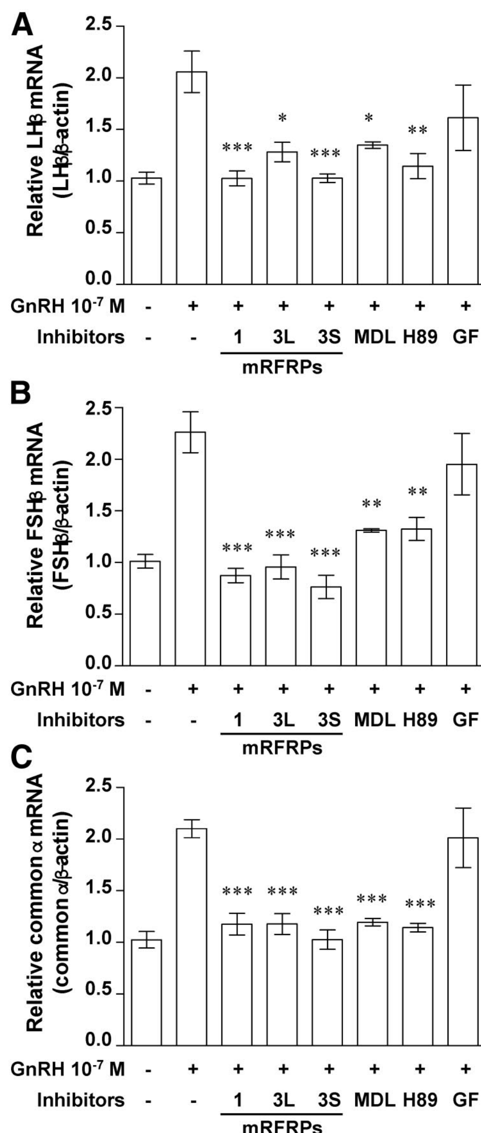

FIG. 7. Inhibitory effects of mRFRP on GnRH-induced gonadotropin subunit gene expressions in LβT2 cells. The mRNA expression levels of LHβ- (A), FSHβ- (B), and common α- (C) subunits were measured by real-time PCR analysis after treatments with 10^{-7} M GnRH and 10^{-6} M mRFRP (1, 3L, and 3S) and indicated inhibitors (MDL, 10^{-4} M; H89, 10^{-6} M; and GF, 10^{-6} M) for 60 min. The relative gonadotropin gene expressions were normalized by the expression of β-actin and represented as fold activation with respect to basal. Data shown are mean ± SEM of repeated experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* cells only stimulated by GnRH; n = 4–6.

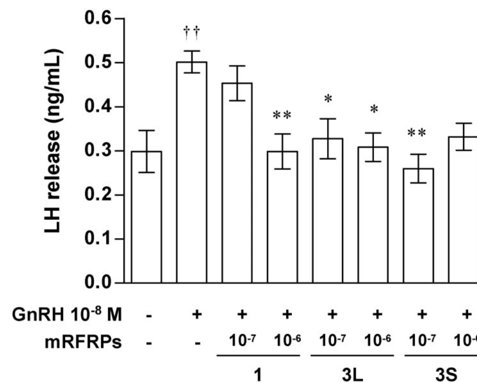

FIG. 8. Inhibitory effect of mRFRP on GnRH-induced LH release. LβT2 cells were pretreated with the indicated concentrations of mRFRP for 30 min before 10^{-8} M GnRH stimulation for 1.5 h. Culture media were collected, and LH release was measured by ELISA. Data shown are mean ± SEM of repeated experiments. ††, $P < 0.01$ *vs.* basal (−); *, $P < 0.05$; **, $P < 0.01$ *vs.* cells only stimulated by GnRH; n = 3.

GnRH-induced LH release at 10^{-6} M, and mRFRP-3L exhibited significant inhibitory effects at both concentrations of 10^{-7} and 10^{-6} M. mRFRP-3S also significantly inhibited GnRH-induced LH release at 10^{-7} M.

Discussion

GnIH has the potential to directly inhibit gonadotropin synthesis and release in gonadotropes via its receptor, GnIH-R (for reviews, see Refs. 6, 7). Despite the physiological importance of GnIH, to date, limited information regarding the GnIH cell signaling pathway through GnIH-R is available. Based on the character of GnIH-R (GPR147 and GPR74) that is coupled with Gα_i to inhibit AC, suppression of cAMP production by GnIH was shown only in few studies (25, 27, 29). Furthermore, the detailed signaling pathway mediating the inhibitory action of GnIH on gonadotropin secretion was still unclear. Therefore, we wanted to understand in depth the signaling pathway of GnIH action on gonadotropes that express GnIH-R. We investigated the effects of mouse GnIH orthologs, mRFRP, on the cell signaling pathways in a mouse gonadotrope cell line, LβT2. We began our study by demonstrating the expression of GnIH-R in LβT2 cells and found its expression in this cell line. We then revealed that all of the predicted mouse mature RFRP, mRFRP-1, mRFRP-3L, and mRFRP-3S, effectively inhibit GnRH-induced AC/cAMP/PKA activation in LβT2 cells, using CRE-reporter system. We further confirmed the inhibitory effect of mRFRP on GnRH-induced cAMP production by measuring cAMP levels. Next, by treating with specific inhibitors for the signaling cascade, we demonstrated the importance of AC/cAMP/PKA pathway in GnRH-stimulated ERK activation in LβT2 cells. We found that mRFRP eliminate GnRH-stimulated ERK activation in a similar manner to the inhibitors for AC and PKA. Finally, we found that mRFRP abolished GnRH-stimulated gonadotropin subunit gene transcriptions (LHβ, FSHβ, and common α-subunit), and also GnRH-induced LH release. All these results suggest that mRFRP function as GnIH by inhibiting AC/cAMP/PKA-dependent ERK activation pathway in LβT2 cells.

The existence of mammalian RFRP that are structurally similar to GnIH (4) was initially predicted by gene data-

base search (25). The cDNA of human and bovine GnIH precursor polypeptide were initially thought to encode three mature peptides, RFRP-1, RFRP-2, and RFRP-3. After attempts to isolate these three putative peptides, up until now, only two RFRP, RFRP-1 and mRFRP-3, were identified as mature peptides in mammals (for reviews, see Refs. 6, 7). RFRP-1 and mRFRP-3 were purified from bovine (8, 12), rat (11), rhesus macaque (9), and human (10). As far as we know, RFRP have not been isolated from mouse. In this study, we synthesized mRFRP-1, mRFRP-3L, and mRFRP-3S, based on the analysis of the mRFRP precursor sequence (25), and examined their functions. Although there is some evidence that RFRP-3 is a functional ortholog of avian GnIH (for reviews, see Refs. 6, 7), we observed apparent inhibitory effects of both mRFRP-1 and mRFRP-3 on cAMP/PKA activation, ERK activation, and gonadotropin subunit gene transcriptions in LβT2 cells. These results suggest that both mRFRP-1 and mRFRP-3 may function as GnIH.

It is noteworthy that no inhibitory effect by mRFRP alone without GnRH or FSK stimulation was observed in this study. Also in other studies, a significant reduction in CRE-luciferase activity by chicken GnIH was only observed in cells that were stimulated by FSK (30). Similarly, RFRP-3 treatment did not affect basal ERK levels, and LHβ and FSHβ mRNA expressions in the absence of GnRH stimulation in ovine pituitary cells (21). These results indicate that GnIH/RFRP exert their effects through Gα_i by blocking the activity of AC, thereby inhibiting the downstream effects, such as cAMP production, ERK phosphorylation, and gonadotropin gene expressions, rather than reducing its basal expression.

Although it is thought that most of the biological actions of GnRH are mediated by G$\alpha_{q/11}$-coupled pathway (for a review, see Ref. 50), it has been reported that GnRH signaling may not be exclusively linked to G$\alpha_{q/11}$-coupled pathway but also may involve other pathways and may be cell context dependent (51). In fact, GnRH-R was also reported to be coupled to Gα_s to stimulate the AC/cAMP/PKA pathway (38, 52). In primary pituitary culture, G-GH3, and LβT2 cells, GnRH-R couples to Gα_s as well as G$\alpha_{q/11}$, whereas in αT3-1 pituitary precursor cells as well as in CHO-K1 and COS-7 cells, GnRH-R seems to couple exclusively to G$\alpha_{q/11}$ (37, 42, 53). Several studies have also suggested a physiological role of cAMP as a mediator of GnRH actions via Gα_s-coupled pathway in the pituitary gland. A cell-permeable peptide that uncouples Gα_s from receptors is able to inhibit ERK and c-Fos activation, and LHβ expression in LβT2 cells, indicating that Gα_s is involved in GnRH-R signaling (38). Our data also suggest that the Gα_s-AC/cAMP/PKA pathway is involved in GnRH-R signaling in LβT2 cells.

It has been reported that PKCδ and PKCϵ are the two endogenous isoforms mediating GnRH action on the activation of AC/cAMP pathway in LβT2 cells (36). To date, AC2, AC5, and AC7 have been shown to be directly phosphorylated by PKC (54–56), and two PKC-sensitive AC isoforms, AC5 and AC7, seem to be the potential targets for GnRH stimulation in LβT2 cells (36). These reports indicate that the AC/cAMP/PKA pathway is also activated via G$\alpha_{q/11}$-mediated PKC pathway. In contrast, we could not observe any significant inhibition by a broad-spectrum PKC inhibitor, GF, on GnRH-induced condition, indicating that GnRH-induced AC activation was not mediated by PKC in our experimental condition. In addition to PKC, GnRH-dependent Ca^{2+} elevation via G$\alpha_{q/11}$-mediated pathway may activate the Ca^{2+}/calmodulin-sensitive AC isoforms (34). Because we did not examine the effect of Ca^{2+} inhibitors, we cannot rule out the possibility that G$\alpha_{q/11}$-mediated pathway is involved in AC activation by Ca^{2+} elevation.

Although previous studies have shown that GnRH-stimulated ERK phosphorylation is PKC mediated and sensitive to GF inhibition, GF did not significantly inhibit GnRH-stimulated ERK phosphorylation in this study. This discrepancy may be due to different experimental conditions used in the studies, such as the concentration of GnRH and the duration of stimulation. It was observed that long-term exposure of LβT2 cells to high concentrations of GnRH treatment resulted in selective decrease in G$\alpha_{q/11}$ protein level and has no effect on the levels of other G protein subunits (57). Reduced G$\alpha_{q/11}$ proteins would lead to impairments in G$\alpha_{q/11}$-mediated downstream signaling. The PKC-dependent ERK activation by GnRH peaked at 5 min in LβT2 cells (58). However, we treated 10^{-7} M GnRH for 30 min to show ERK phosphorylation, and we could not observe a significant inhibition by GF on GnRH-induced ERK activation in this condition (Fig. 5B). We tested various concentrations of GnRH to stimulate LβT2 cells in each experiment to find the appropriate concentration as shown in Fig. 3A; 10^{-7} or 10^{-8} M was proved to be an effective concentration to activate cells in our culture condition (Figs. 3–8). Various mRFRP were also effective at the concentrations between 10^{-8} and 10^{-6} M (Fig. 3). These concentrations may be rather high. However, we consider that this may be due to the responsiveness of the LβT2 cells and culture condition that we used in this study.

Studies on the mechanism of GnRH-induced ERK activation in gonadotropes have yielded conflicting results depending on the model systems employed. The requirement for extracellular Ca^{2+} influx and activation of PKC isoforms for ERK activation has been widely demonstrated (45, 59). On the other hand, there is a report show-

ing that GnRH induction of both FSHβ and common α-subunit genes is sensitive to PKA inhibition in tilapia pituitary cells (60), similar to our results suggesting the involvement AC/cAMP/PKA signaling in GnRH-induced gonadotropin gene expressions in LβT2 cells. There are further evidences for PKA-mediated ERK activation. Although direct activation of Raf serine/threonin-protein kinase (Raf)-1 by PKC was often cited as a predominant ERK activation pathway by GnRH, recent studies suggest that the alternative isoform B-Raf is the upstream activator through a pathway involving PKA activation (61). Indeed, within the pituitary, activation of ERK pathway in B-Raf-expressing cells, such as corticotrope and somatotrope cells, does not involve Raf-1 but proceeds through a pathway involving $G\alpha_s$, PKA, and B-Raf (62, 63), indicating that B-Raf expression can contribute to cell type-specific differences in the regulation of ERK pathway. Similar studies of the role of Raf kinase isoforms regulating ERK activation after GnRH stimulation have not been reported in gonadotropes (for a review, see Ref. 44). In this study, we found that in the gonadotrope LβT2 cells, GnRH-induced ERK phosphorylation was not significantly inhibited by GF, a PKC inhibitor, but abolished by MDL, an AC inhibitor, and H89, a PKA inhibitor (Fig. 5B). We also confirmed the expression of B-Raf in LβT2 cells by quantitative RT-PCR (data not shown). Although future experiments investigating the involvement of B-Raf in GnRH-induced ERK activation are necessary, these results suggest the possibility that ERK is activated by a PKA-dependent B-Raf pathway rather than a PKC-dependent Raf-1 pathway in LβT2 cells.

In summary, the present study demonstrates the inhibitory mechanism of GnIH on GnRH-induced signaling cascade using the LβT2 cell line, an excellent gonadotrope-derived cellular model. We found that mRFRP inhibit GnRH-stimulated gonadotropin subunit gene transcriptions by inhibiting the AC/cAMP/PKA-mediated ERK activation in gonadotrope cells. Thus, our study provides new findings that contribute to our understanding of GnIH signaling in pituitary gonadotropes. Although we did not examine the inhibitory effect of mRFRP on GnRH-induced gonadotropin synthesis in primary cultures in this study, we expect that similar results may be obtained in primary cultures, based on the report showing that human RFRP-3 eliminated GnRH-stimulated ERK activation in cultured sheep primary pituitary cells (21).

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