

ORIGINAL

Expression of gonadotropin-inhibitory hormone receptors in mouse pituitary gonadotroph L β T2 cells and hypothalamic gonadotropin-releasing hormone-producing GT1-7 cells

Unurjargal Sukhbaatar, Haruhiko Kanasaki, Tselmeg Mijiddorj, Aki Oride and Kohji Miyazaki

Department of Obstetrics and Gynecology, Shimane University School of Medicine, Izumo 693-8501, Japan

Abstract. Gonadotropin-inhibitory hormone (GnIH) was first identified in quail as a novel neurohormone that acts directly on the anterior pituitary to inhibit gonadotropin release. GnIH inhibits not only gonadotropin release from the pituitary gland but also inhibits the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. In this study, we examined how GnIH receptors were regulated in pituitary gonadotroph cells and GnRH-producing neurons in the hypothalamus. In the mouse pituitary gonadotroph cell line L β T2, GnRH increased expression of the GnIH receptor, G-protein coupled receptor 74 (GPR74). GnRH also stimulated the expression of GPR74 and GPR147 in primary cultures of rat anterior pituitary cells. In addition, when GnRH was administered to L β T2 cells in a pulsatile manner, low frequency GnRH pulse stimulation stimulated GPR74 and GPR147 expression more than did high frequency GnRH pulses. In the mouse hypothalamic GnRH-producing cell line GT1-7, hypothalamic kisspeptin did not significantly increase the expression of GnIH receptors. However, the intermittent administration of kisspeptin to GT1-7 cells significantly increased GPR74 and GPR147 mRNA expression. The overexpression of either constitutively active MEK kinase (MEKK) or protein kinase A (PKA) in L β T2 cells increased the expression of GPR74 mRNA. Conversely, in GT1-7 cells, although the overexpression of either MEKK or PKA failed to stimulate GnIH receptor expression, the combined overexpression of both kinases together increased GPR74 and GPR147 mRNA levels. Our current observations suggest that two central controllers of reproductive function, GnRH and kisspeptin, stimulate the expression of GnIH receptors in pituitary gonadotroph cells and hypothalamic GnRH neurons.

Key words: GnIH, GnRH, Kisspeptin

GONADOTROPINS, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) regulate reproductive function by stimulating follicular growth, oocyte maturation, and the synthesis of sex steroids. Gonadotropin secretion is under the control of gonadotropin-releasing hormone (GnRH) [1]. GnRH is released in a pulsatile manner from the hypothalamus, and changes in the pulse frequency and amplitude of GnRH alter the secretion of LH and FSH [2].

Kisspeptins, which are alternatively called metastatins, were originally identified as products of the metastasis suppressor gene KiSS-1 [3]. After kisspeptins were identified as the natural ligands for G protein-coupled

receptor 54 (GPR54) [4, 5] and a GPR54 mutation was discovered in a patient with hypogonadotropic hypogonadism who failed to undergo puberty [6, 7], kisspeptins released from kisspeptin neurons in the hypothalamus have been shown to activate GnRH neurons [8, 9].

In a search for novel neuropeptides regulating the release of pituitary hormones, Tsutsui and co-workers identified a novel hypothalamic decapeptide that acts directly on the pituitary to inhibit gonadotropin release in quail and termed this molecule gonadotropin-inhibitory hormone (GnIH) [10]. GnIH has been found in several other avian species, and GnIH homologs (RF-amide-related peptides) have been identified in other vertebrates, including mammals [11]. GnIH and its homologs appear to act similarly across vertebrate species to regulate reproductive function by inhibiting GnRH-stimulated gonadotropin synthesis and secretion [12]. GnIH acts not only at the anterior pituitary to inhibit gonadotropin release by GnRH, but also acts

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Correspondence to: Haruhiko Kanasaki, M.D., Ph.D., Shimane University, School of Medicine, Dept. of Obstetrics and Gynecology, 89-1 Enya Cho, Izumo City 693-8501, Shimane Prefecture, Japan.
E-mail: kanasaki@med.shimane-u.ac.jp

at the hypothalamus to regulate GnRH release. GnIH-positive fibers were observed in extremely close proximity to GnRH-positive neurons in the preoptic area [13]; contact was also observed between GnIH and GnRH fibers in the median eminence [14].

Two receptors, GPR74 and GPR147, for GnIH have been identified [15, 16]. The expression patterns of GPR74 and GPR147 in the central nervous system of rat are reportedly distinct, with GPR147 expression much more broadly distributed, with the highest expression levels in the lateral septum, thalamic nuclei, hypothalamus, and amygdala. In contrast, GPR74 was expressed mainly in the spinal cord and some regions of the thalamus [17]. GnIH also shows higher affinity for GPR147, whereas it has a potent agonistic activity for GPR74 [17, 18]. Expression of GnIH receptor (GnIHR) mRNAs has been also observed in the pituitary gland in quail [13, 19], and in gonadotroph cells in the human pituitary [20]. GnIHR expression has also been observed in GnRH neurons [13]. These observations suggest that, in addition to acting on the pituitary, GnIH acts on GnRH neurons to inhibit gonadotropin release and synthesis.

The development of the immortalized murine pituitary gonadotroph cell-derived cell line L β T2 has facilitated the study of the signal transduction pathways activated by the GnRH receptor in pituitary gonadotroph cells [21]. This cell line expresses the gonadotropin α , LH β , and FSH β subunits as well as the GnRH receptor, and also synthesizes and releases LH and FSH in response to GnRH stimulation. In addition, immortalized GT1-7 cells [21] serve as an *in vitro* model for hypothalamic GnRH-producing neurons.

In this study, we examined the regulation of GnIHR expression in gonadotroph L β T2 cells by GnRH and in GnRH-producing GT1-7 cells by kisspeptin.

Materials and Methods

Materials

The following chemicals and reagents were obtained from the indicated sources: fetal bovine serum (FBS) and trypsin (GIBCO, Invitrogen, Carlsbad, CA); GnRH, Dulbecco's Modified Eagle's medium (DMEM), and penicillin-streptomycin (Sigma Chemical Co., St. Louis, MO); kisspeptin-10 (ANA SPEC, Fremont, CA), pFC-MEKK and pFC- PKA (Stratagene, La Jolla, CA); and pCI-neo (Promega, Madison, WI).

Cell culture

L β T2 and GT1-7 cells were kindly provided by Dr. P. Mellon (University of California, San Diego, CA). The cells were plated in 35-mm tissue culture dishes and incubated with high-glucose DMEM containing 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere of 5% CO₂ in air. After 24 h, the culture medium was changed to high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin, and the cells were incubated without (control) or with the test reagents for the indicated periods. When the PKA inhibitor, H89 (Biomol, Plymouth, PA), and the MEK inhibitor, U0126 (Calbiochem, La Jolla, CA) were used, cells were pre-incubated with inhibitors for 60 min and then incubated with the test reagent (GnRH) for the indicated periods of time.

RNA preparation, reverse transcription, and real-time quantitative RT-PCR

Total RNA from untreated or treated cells was extracted using TRIzol-S (GIBCO BRL Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. To obtain cDNA, 1.0 μ g total RNA was reverse transcribed in reverse transcription (RT) buffer with an oligo-dT primer (Promega, Madison, WI) using a First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). The preparation was supplemented with 0.01M dithiothreitol, 1 mM each dNTP, and 200 U RNase inhibitor/human placental ribonuclease inhibitor (Ribonuclease Inhibitor, Code No. 2310; Takara, Tokyo, Japan) in a final volume of 10 μ L. The reaction was incubated at 37 °C for 60 min. GPR74 and GPR147 mRNAs were quantified by real-time quantitative PCR (ABI Prism 7000; Perkin Elmer Applied Biosystems, Foster City, CA) following the manufacturer's protocol (User Bulletin No. 2), with a Universal Probe Library Probe and Fast Start Master Mix (Roche Diagnostics, Mannheim, Germany). The PCR primers were designed based on previously reported GPR74 and GPR147 sequences [22]. The sequences of the primers used to amplify the partial mouse glyceraldehyde-3-phosphatase dehydrogenase cDNA (133 bp) were: 5'-GCCCTCCTTTTCATCCTTTC-3' forward (F) and 5'-TGGAAAGCATCTTGGAAC-3' reverse (R), to amplify the partial mouse GPR74 cDNA (209 bp); 5'-CCGAGTCTGAACGAGAGTGA-3' (F) and 5'-CGGTTCTTAAGCACGATGAA-3' (R), to amplify the partial mouse GPR147 cDNA (173 bp);

and 5'-ACAACCTTGGCATTGTGGAA-3' (F) and 5'-GATGCAGGGATGATGTTCTG-3' (R). The simultaneous measurement of the target mRNA and GAPDH mRNA permitted normalization of the amount of cDNA added per sample. For each set of primers, a no-template control was included. The thermal cycling conditions were: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The threshold crossing value (CT) was determined using PRISM 7000 software and post-amplification data were analyzed using the delta-delta CT method with Microsoft Excel.

Primary culture of anterior pituitary cells

Female rats were perfused with Ca²⁺- and Mg²⁺-free Hank's Balanced Salt Solution (CMF-HBSS) under deep sodium pentobarbital anesthesia. The anterior pituitary glands were excised and minced into pieces that were incubated in CMF-HBSS containing 10 mg/mL trypsin and 2 mg/mL collagenase (Nitta Gelatin, Osaka, Japan) for 15 min at 37 °C. The pieces were then incubated in the same solution containing 0.5 µg/mL DNase I (Boehringer-Mannheim, Mannheim, Germany) for 5 min at 37 °C. After incubation in CMF-HBSS containing 5 mM ethylenediaminetetraacetic acid (Wako Pure Chemicals, Osaka, Japan) for 5 min at 37 °C, the digested samples were washed with CMF-HBSS. The dispersed cells were suspended in CMF-HBSS by pipetting, passed through a 70-µm nylon mesh (Becton Dickinson Labware, Franklin Lakes, NJ), and then collected by centrifugation. The pellet was resuspended in DMEM containing 10% FCS and 1% penicillin-streptomycin. After the cells were cultured for 24 h, they were collected and used for experiments. This protocol was approved by the committee of the Experimental Animal Center for Integrated Research at Shimane University.

Perfusion system

The perfusion system used in this study was designed and described previously [23]. Briefly, LβT2 cells were plated in perfusion chambers mounted on glass slides coated with Matrigel (Becton Dickinson and Co. Labware, Bedford, MA) and were incubated for 24 h in the static culture system described above. The chambers were then connected to the perfusion system and continuously perfused with high-glucose DMEM containing 1% heat-inactivated FBS and 1% penicillin-streptomycin at a constant flow rate of 0.25 mL/min. During perfusion, the cells were treated with

medium alone, pulsatile GnRH, or kisspeptin at two different frequencies (1 pulse every 30 min or 1 pulse every 120 min). Up to nine chambers were run simultaneously. Pulse stimulations were delivered using a set of peristaltic pumps controlled by a time controller (ChronTrol XT; ChronTrol Corp., San Diego, CA). GnRH is released from the hypothalamus in a pulsatile manner and determines the production of LH and FSH. LβT2 cells were stimulated with pulsatile GnRH (10 nM, 5 min per pulse), at a frequency of 1 pulse every 30 min (high) or 1 pulse every 2 h (low) for 20 h. These pulse frequencies were chosen based on a previous study indicating that these frequencies were optimal for LHβ and FSHβ gene expression, respectively [23].

Plasmid construct and transfection

The cells were transfected by electroporation with pFC-MEKK (2.0 µg DNA), pFC-PKA (2.0 µg DNA), or both. pCI-neo expression vectors were used as a mock control. After incubation for 48 h, the cells were harvested and assayed for mRNA determination.

Statistical analysis

All experiments were repeated independently at least three times. Each experiment was performed with duplicate samples for each experimental group. The values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance, followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

GnIHR mRNA expression in LβT2 and GT1-7 cells

Both GPR74 and GPR147 have been identified as GnIH receptors [15, 16]. First, we examined whether mRNA for GPR74 and GPR147 were detected in the gonadotroph and GnRH-producing neuron cell models, LβT2 and GT1-7. Using specific primers for GPR74 and GPR147, we confirmed that each type of GnIHR mRNA was amplified in LβT2 and GT1-7 cells (Fig. 1).

Effects of GnRH on GnIHR mRNA expression on LβT2 cells

To examine whether GnRH modulates the gene expression of GnIHRs in pituitary gonadotroph LβT2 cells, GPR74 and GPR147 mRNA levels were determined by quantitative RT-PCR using specific primers for murine GnIH receptors. Treatment of the LβT2

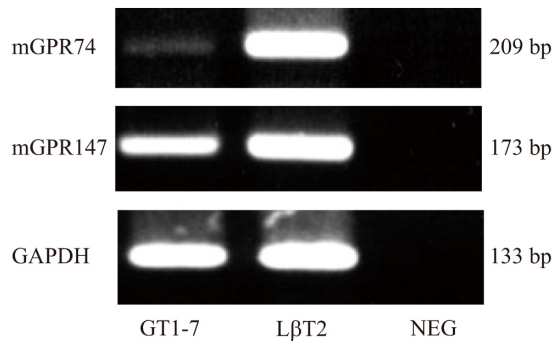


Fig. 1 GnIHR mRNA expression in GT1-7 cells and LβT2 cells. Total RNA (1.0 μg) prepared from GT1-7 cells and LβT2 cells was reverse transcribed and RT-PCR performed using specific primers for GPR74, GPR147, and GAPDH (40 cycles of amplification). In the negative control (NEG), PCR was performed without the reverse-transcribed reaction. PCR products were separated by electrophoresis on a 1.5 % agarose gel and visualized with ethidium bromide staining.

cells with GnRH significantly increased the mRNA levels of GPR74 by 3.97 ± 0.66 -fold after 24 h and 4.10 ± 2.20 -fold after 48 h (Fig. 2A). GPR147 mRNA levels were increased by 1.54 ± 0.32 -fold after 48 h of GnRH stimulation, but the difference was not statistically significant (Fig. 2B).

Effects of GnRH on GnIHR mRNA expression in primary cultures of rat pituitary cells

We confirmed the stimulatory effect of GnRH on GnIHR expression using primary cultures of rat anterior pituitary cells. Similar to the phenomenon observed in LβT2 cells, GPR74 mRNA expression was significantly increased by GnRH stimulation in rat anterior

pituitary cells (Fig. 3A). GPR147 mRNA expression was also increased by GnRH stimulation (Fig. 3B).

Effects of pulsatile GnRH stimulation on GnIHR expression in perfused LβT2 cells

Next, LβT2 cells were stimulated with high (1 pulse/30 min) and low (1 pulse/120 min) frequency GnRH pulses. Under the low GnRH pulse frequency, GPR74 mRNA levels were significantly increased by 2.48 ± 0.45 fold compared to the non-stimulated control. GPR74 mRNA levels were significantly increased by the high-frequency GnRH pulses (1.68 ± 0.08 fold), but the magnitude of GPR74 mRNA increase was lower than the increase observed under low-frequency GnRH pulses (Fig. 4A). Similar to GPR74, GPR147 mRNA expression was increased 2.72 ± 0.42 -fold under low frequency GnRH pulse stimulation (Fig. 4B).

Effect of kisspeptin on GnRH-producing GT1-7 cells

Kisspeptin, which is released from hypothalamic kisspeptin neurons, is a regulator of GnRH release through its receptor GPR54. We examined the effects of kisspeptin on GnIHR expression in hypothalamic GnRH-producing GT1-7 cells. Under static conditions in which kisspeptin was added directly to the culture dishes, 24 h treatment with kisspeptin slightly increased GPR74 mRNA levels by 1.29 ± 0.12 -fold, but failed to increase GPR147 mRNA expression (Fig. 5A and 5B). Interestingly, when GT1-7 cells were treated with kisspeptin intermittently at an interval of 30 min, GPR74 and GPR147 mRNA levels were significantly increased (Fig. 5C and 5D). We also confirmed that

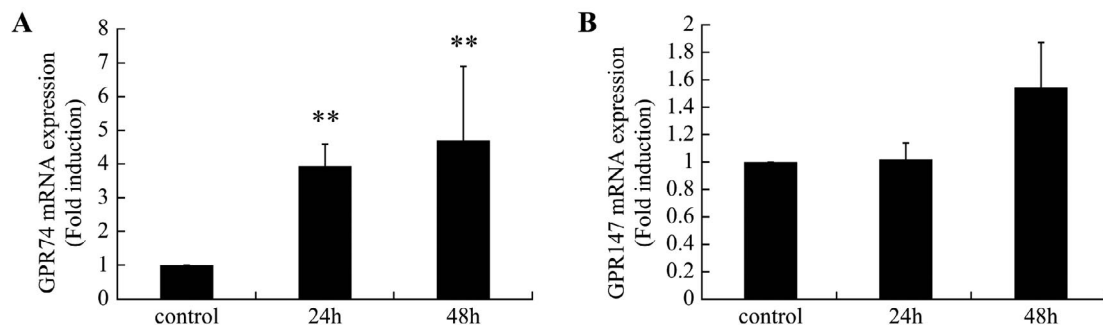


Fig. 2 GnIHR mRNA expression following GnRH stimulation in LβT2 cells. LβT2 cells were treated with 100 nM GnRH for the indicated times. All samples were prepared in duplicate. GPR74 (A) and GPR147 (B) mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and were normalized to mRNA levels of GAPDH as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated control group. Values represent the mean \pm SEM fold stimulation taken from three independent experiments. ** $P < 0.01$ vs. control.

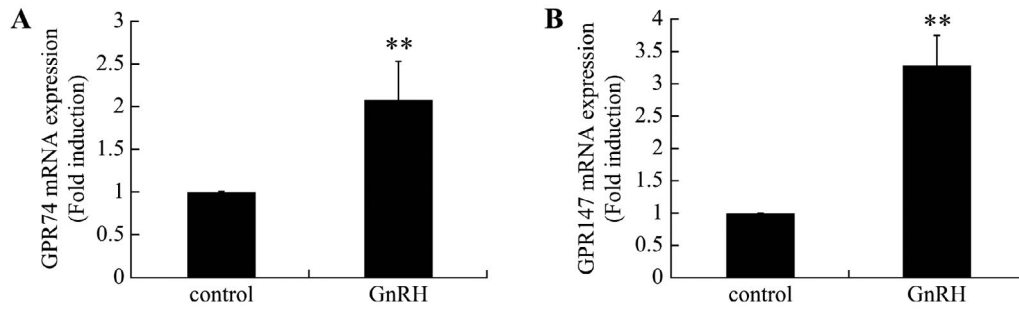


Fig. 3 GnHR mRNA expression following GnRH stimulation in primary cultures of rat anterior pituitary cells

Rat anterior pituitary cells were treated with 100 nM GnRH for 24 h. GPR74 (A) and GPR147 (B) mRNA levels were measured by quantitative real-time PCR after mRNA extraction and reverse transcription. Samples for each experimental group were run in duplicate and were normalized to mRNA levels of GAPDH as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated control group. Values represent the mean \pm SEM of fold stimulation taken from three independent experiments. ** $P < 0.01$ vs. control.

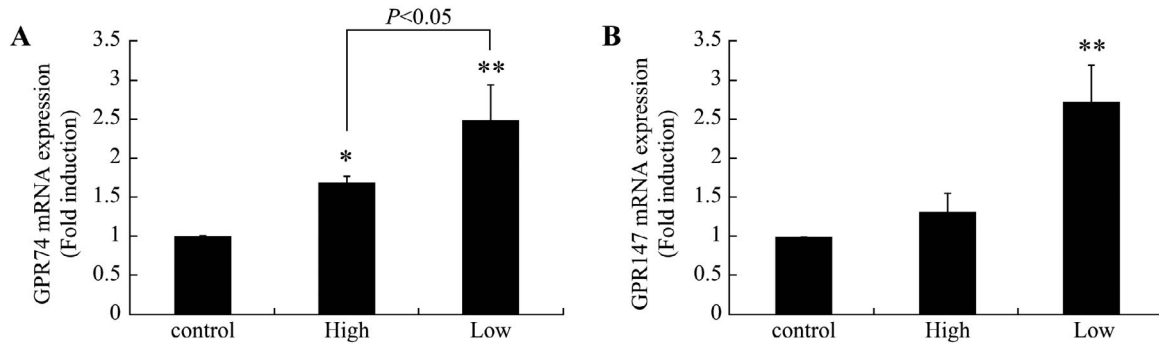


Fig. 4 GnHR mRNA expression following pulsatile GnRH stimulation in LβT2 cells

LβT2 cells were plated in perfusion chambers and perfused with 10 nM GnRH, which was administered at pulse intervals of 30 (high) or 120 min (low) for 20 h. The samples were prepared in triplicate. After the last pulse, the cells were harvested and their mRNA was extracted and reverse transcribed. GPR74 (A) and GPR147 (B) mRNA levels were measured by quantitative real-time PCR. Samples for each experimental group were run in duplicate and were normalized to mRNA levels of GAPDH as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values represent the mean \pm SEM of fold stimulation taken from three independent experiments. * $P < 0.05$; ** $P < 0.01$ vs. control. The difference between the high and low frequency pulse-induced levels of GPR74 mRNA in Fig. 4A was statistically significant ($P < 0.05$).

GnRH failed to modulate the expression of GPR74 under static conditions (data not shown).

Effects of overexpression of MEKK or PKA on GnHR expression in LβT2 cells

We transfected LβT2 cells with the pFC-MEKK or pFC-PKA plasmid vector to induce the expression of constitutively active MEKK (an upstream kinase that activates the extracellular signal-regulated kinase; ERK) or PKA. In LβT2 cells, GPR74 mRNA expression was significantly increased by PKA overexpression compared to that in the pCI-neo-transfected cells (mock transfection). The overexpression of MEKK also increased GPR74 mRNA expression; however, it

did not modulate the positive effect of PKA (Fig. 6A). GPR147 mRNA levels were also increased by PKA overexpression (Fig. 6B), but were not significantly increased by MEKK overexpression. To confirm the importance of PKA and ERK pathways, the effects of U0126 and H89, which inhibit PKA and ERK, respectively, were examined. GnRH-stimulated GPR74 mRNA expression was completely abolished in the presence of these inhibitors (Fig. 6C).

Effects of MEKK and PKA overexpression on GnHR expression in GT1-7 cells

In GT1-7 cells, transfection with pFC-MEKK or pFC-PKA failed to increase GPR74 mRNA expression.

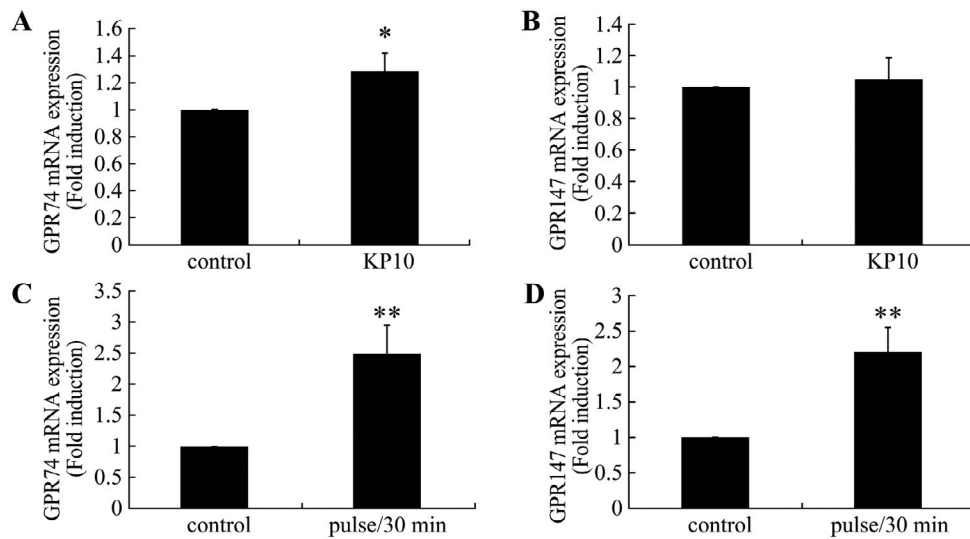


Fig. 5 GnIHR mRNA expression following kisspeptin stimulation in GT1-7 cells

GT1-7 cells were treated with 100 nM kisspeptin (KP10) for 24 h under static conditions (A and B). The cells were plated in perfusion chambers and perfused with 10 nM kisspeptin, which was administered at pulse intervals of 12 h (C and D). After mRNA extraction and reverse transcription, GPR74 (A and C) and GPR147 (B and D) mRNA levels were measured by quantitative real-time PCR. The samples for each experimental group were run in duplicate and normalized to mRNA levels of GAPDH as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated control group. Values represent the mean \pm SEM of fold stimulation taken from three independent experiments. * P < 0.05; ** P < 0.01 vs. control.

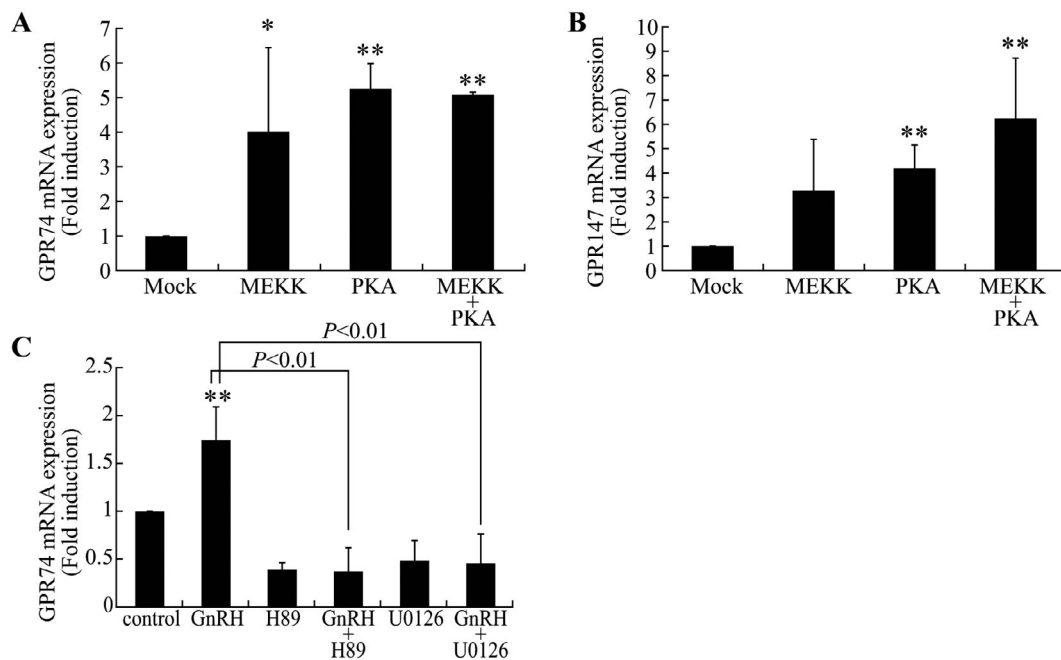


Fig. 6 Effects of the overexpression of pFC-MEKK and pFC-PKA on GnIHR mRNA levels in LβT2 cells

LβT2 cells were transfected with 2.0 μ g of pFC-MEKK, 2.0 μ g of pFC-PKA, or cotransfected with 2.0 μ g of pFC-MEKK and pFC-PKA. As a mock control, the cells were transfected with 2.0 μ g pCI-neo. After 48 h of culture, the cells were harvested and their mRNA extracted and reverse transcribed. GPR74 (A) and GPR147 (B) mRNA levels were measured by quantitative real-time PCR. (C) Effects of PKA and MEK inhibitors on GnRH-increased GPR74 mRNA expression LβT2 cells were pre-incubated with or without 10 μ M of H89 (PKA inhibitor) and 10 μ M of U0126 (MEK inhibitor) for 1 h. GnRH (100nM) was then added directly, and the cells incubated for 24 h before harvesting. The samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values represent the mean \pm SEM of fold stimulation taken from three independent experiments. * P < 0.05; ** P < 0.01 vs. control. The differences between GnRH and GnRH+H89 and between GnRH and GnRH + U0126 were statistically significant (P < 0.01).

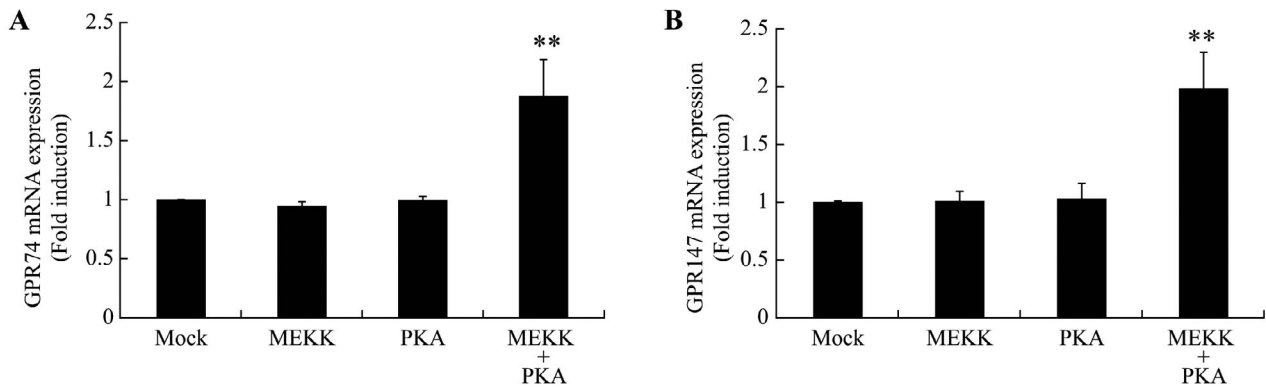


Fig. 7 Effects of the overexpression of pFC-MEKK and pFC-PKA on GnIHR mRNA levels in GT1-7 cells

GT1-7 cells were transfected with 2.0 μ g of pFC-MEKK, 2.0 μ g of pFC-PKA, or cotransfected with 2.0 μ g of pFC-MEKK and pFC-PKA. As a mock control, the cells were transfected with 2.0 μ g pCI-neo. After 48 h of culture, the cells were harvested and their mRNA extracted and reverse transcribed. GPR74 (A) and GPR147 (B) mRNA levels were measured by quantitative real-time PCR. The samples for each experimental group were run in duplicate and normalized to GAPDH mRNA levels as a housekeeping gene. Results are expressed as fold stimulation over the unstimulated group/control. Values represent the mean \pm SEM of fold stimulation taken from three independent experiments. ** $P < 0.01$ vs. control.

Similarly, GPR147 mRNA levels were unchanged by MEKK or PKA overexpression. However, cotransfection of the cells with both pFC-MEKK and pFC-PKA significantly increased GPR74 and GPR147 mRNA levels compared to those observed in mock transfected cells (Fig. 7).

Discussion

GnIH has the potential to inhibit gonadotropin synthesis and release in gonadotroph cells *via* its receptor. GnIH directly inhibits basal release of LH and FSH from cultured quail anterior pituitaries [10] and also inhibits GnRH-stimulated release [22]. This then decreases gonadal steroids synthesis and prevents the development and maintenance of the gonadal organs [24]. The GnIH system also participates in the circadian control of ovulation [25]. GnRH neurons also express GnIHRs and are thereby under the control of GnIH [11, 26]. Conversely, hypothalamic kisspeptin regulates GnRH release from GnRH neurons, and GnRH plays a pivotal role in the regulation of pituitary gonadotropin synthesis and release. In this study, we investigated the effects of these two peptides on the regulation of GnIHRs in pituitary gonadotroph cells and GnRH neurons and revealed that kisspeptin and GnRH are able to induce GnIHR expression.

GnRH regulates gonadotropin gene expression and release from gonadotrophs in the anterior pituitary. In addition, GnRH regulates the expression of its own

receptor, and the surface density of GnRH receptors then determines the differential transcriptional regulation of the LH β and FSH β subunit genes by GnRH [23, 27]. That is, GnRH receptor expression is preferentially increased under high frequency GnRH pulse stimulation, when the expression of the LH β subunit gene is optimally stimulated. However, the FSH β gene is optimally stimulated at a lower cellular concentration of the GnRH receptor, which occurs under low frequency GnRH pulse stimulation. Although the detailed mechanisms of GnRH pulse frequency-dependent gonadotropin subunit gene expression have not been fully elucidated, the pattern of GnRH receptor expression is one of the causes of this phenomenon. GnRH also regulates the expression of the pituitary adenylate cyclase-activating polypeptide type I receptor within pituitary gonadotroph cells and potentiates the effect of GnRH on gonadotropin subunit gene expression [28]; it is also reportedly involved in the specific regulation of gonadotropin subunits [29]. Thus, GnRH regulates the expression of receptors for other peptides that have effects on gonadotroph cells and probably modulates the synthesis and release of gonadotropin. In the present study, we found that the expression of the GnIHRs GPR74 and GPR147 was increased by GnRH stimulation in gonadotroph cell lines and primary cultures of rat pituitary cells. These observations suggested that GnRH can not only positively regulate gonadotropin synthesis and release, but also regulate gonadotropin inhibition by increasing the expression of GnIHRs.

Interestingly, the mRNA levels of GPR74 and GPR147 were preferentially increased by low frequency GnRH pulse stimulation. As the expression of the FSH β subunit is predominantly increased in gonadotroph cells by low frequency GnRH pulse stimulation [30], GnIH might affect LH β expression primarily by changing the expression of its own receptors. However, GnIH inhibited gene expression of all three GnRH-increased gonadotropin subunits, α , LH β , and FSH β [22].

The expression of GnIHRs by GnRH-producing neurons was also increased by kisspeptin, which was recently identified as a GnRH-releasing peptide acting through its receptor GPR54 [8]. Although a significant stimulatory effect was not observed following static kisspeptin stimulation of GT1-7 cells, the pulsatile administration of kisspeptin further increased GnIHR expression. At present, we do not know why the induction of GnIHRs requires pulsatile kisspeptin stimulation; however, one possibility might be receptor desensitization in GT1-7 cells. A previous study in juvenile male rhesus monkey demonstrated that continuous kisspeptin infusion desensitized GPR54 and abolished the LH response to kisspeptin [31]. Recent study by Matsui *et al.* also reported that chronic administration of a kisspeptin analog to rat suppressed hypothalamic pituitary gonadal function with concomitant reduction of GnRH content in the hypothalamus [32]. These observations suggest that chronic administration of kisspeptin disrupts endogenous kisspeptin signals to suppress the intrinsic GnRH pulse, perhaps by attenuating the GnRH-neuronal response and inducing continuous GnRH leakage from the hypothalamus. In this study we used GT1-7 cells overexpressing GPR54 to obtain a clear response to kisspeptin stimulation. We know that the GT1-7 cells used in this experiment did not respond to exogenous kisspeptin without GPR54 overexpression because they lack sufficient GPR54. Although intermittent administration of kisspeptin induced GnIHR in GPR54-overexpressing GT1-7 cells, chronic treatment with kisspeptin failed to induce GnIHR in these cells. This exogenous receptor might become desensitized and fail to induce GnIHR expression upon chronic kisspeptin treatment, as observed in rat. We have already confirmed that after chronic kisspeptin treatment, endogenous levels of GPR54 are unchanged (data not shown). As for GnRH receptor function within the GnRH neuron, a previous study demonstrated that GnRH secretory profiles are influenced by the regulatory action of GnRH itself on GnRH receptors endoge-

nously expressed in GnRH neurons [33]. This suggests the operation of an autocrine feedback mechanism that exerts both positive and negative effects for the integrated control of GnRH secretion from hypothalamic neurons. Kisspeptin stimulates the release of GnRH from GnRH neurons; thus, secreted GnRH might affect its own function in an autocrine fashion. On the other hand, in addition to the GnRH-releasing factors, kisspeptin regulated GnIHR expression only when it was administered intermittently. In this study, we chose only 30-min intervals for intermittent kisspeptin administration. However, at present, we are not sure whether this administration interval represents physiological conditions. In addition, how GnIHRs are regulated by various intervals of kisspeptin administration remains unknown; therefore, the control of GnRH neurons by kisspeptin needs to be understood in greater detail.

The GnRH receptor mainly couples with the Gq protein to activate the phospholipase C (PLC)/inositol (1,4,5)-triphosphate (IP3) pathway, resulting in calcium elevation and the activation of protein kinase C (PKC) and ERK [34]. GnRH also couples with adenylate cyclase-coupled G proteins and activates cAMP/PKA [35]. Conversely, the kisspeptin receptor GPR54 is also believed to activate the Gq protein/PLC/IP3 pathway, resulting in the release of calcium from calcium stores [4, 36]. PKC activation, as well as activation of the ERK and phosphatidylinositol-3 kinase/Akt pathways, has also been observed [37]. In addition, we have shown that kisspeptin increased cAMP accumulation in GPR54-overexpressing GT1-7 cells (submitted). In the experiments using expression vectors for MEKK or PKA, GnIHR expression was induced by PKA overexpression and weakly increased by MEKK overexpression in L β T2 cells. These observations suggested that the cAMP/PKA pathways, which were activated by GnRH, are involved primarily in the expression of GnIHRs in pituitary gonadotroph cells. Conversely, neither MEKK nor PKA overexpression stimulated GnIHR expression in GT1-7 cells; however, the combined expression of MEKK and PKA could increase GnIHR expression. These observations showed that ERK and cAMP/PKA activation is necessary to induce GnIHR expression in GT1-7 cells.

In this study, we have demonstrated that GnRH can stimulate GnIHR expression in pituitary gonadotropin-secreting cells. In addition, we observed that kisspeptin could increase the expression of GnIHRs in GnRH-producing neuronal cells. GnIH is an impor-

tant neurohormone controlling reproduction by inhibiting gonadal development and maintenance through the decreased release of gonadotropin. Conversely, GnRH and kisspeptin are known to stimulate gonadotropin release from gonadotroph cells and GnRH release from GnRH neurons. These peptides might facilitate acceptance of GnIH by gonadotroph cells and GnRH neurons through increased expression of GnIHRs and might also be inactivated by their own function.

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Disclosure Statement

The authors have nothing to disclose.

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