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Arg-Phe-amide-related peptides influence gonadotropin-releasing hormone neurons

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Abstract

The hypothalamic Arg-Phe-amide-related peptides, gonadotropin-inhibitory hormone and orthologous mammalian peptides of Arg-Phe-amide, may be important regulators of the hypothalamus-pituitary-gonadal reproductive axis. These peptides may modulate the effects of kisspeptins because they are presently recognized as the most potent activators of the hypothalamus-pituitary-gonadal axis. However, their effects on gonadotropin-releasing hormone neurons have not been investigated. In the current study, the GT1–7 cell line-expressing gonadotropin-releasing hormone was used as a model to explore the effects of Arg-Phe-amide-related peptides on kisspeptin activation. Intracellular calcium concentration was quantified using the calcium-sensitive dye, fura-2 acetoxymethyl ester. Gonadotropin-releasing hormone released into the medium was detected *via* enzyme-linked immunosorbent assay. Results showed that 100 nmol/L kisspeptin-10 significantly increased gonadotropin-releasing hormone levels (at 120 minutes of exposure) and intracellular calcium concentrations. Co-treatment of kisspeptin with 1 μmol/L gonadotropin-inhibitory hormone or 1 μmol/L Arg-Phe-amide-related peptide-1 significantly attenuated levels of kisspeptin-induced gonadotropin-releasing hormone but did not affect kisspeptin-induced elevations of intracellular calcium concentration. Overall, the results suggest that gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 may have inhibitory effects on kisspeptin-activated gonadotropin-releasing hormone neurons independent of the calcium signaling pathway.

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Key Words

neural regeneration; hypothalamus; gonadotropin-inhibitory hormone; Arg-Phe-amide-related peptide-1; kisspeptin; gonadotropin-releasing hormone; calcium signaling; GT1–7 cells; neuroregeneration

Research Highlights

(1) Kisspeptin-10 caused a significant increase in the level of gonadotropin-releasing hormone in GT1–7 cells.
(2) Co-treatment of kisspeptin with gonadotropin-inhibitory hormone or Arg-Phe-amide-related peptide-1 attenuated kisspeptin-induced levels of gonadotropin-releasing hormone.
(3) Arg-Phe-amide-related peptides may have inhibitory effects on kisspeptin-activated gonadotropin-releasing hormone neurons.

INTRODUCTION

Gonadotropin-releasing hormone is a hypothalamic neuropeptide that regulates the release of follicular-stimulating hormone and luteinizing hormone from the anterior pituitary[1]. These gonadotropic hormones lead to gonadal growth and production of gonadal sex steroids, eliciting the development of secondary sexual characteristics[2]. Serum luteinizing hormone and follicular-stimulating hormone levels increase during puberty and show night-day rhythms with pulsatile secretions[3-6]. The pulsatile pattern of gonadotropin-releasing hormone release has a critical importance in the intermittent release of these gonadotropic hormones from the pituitary[7-9]. Unlike the other hypothalamic cell groups, gonadotropin-releasing hormone neurons originate from the olfactory placode region to the hypothalamus during embryogenesis[10-12]. The activation of these neurons occurs during juvenile development, yet initiates puberty *via* their reactivation. However, the mechanisms underlying this reactivation are not well understood[13-20].

During the last decade, our understanding of this hypothalamus-pituitary-gonadal axis has quickly expanded. Two novel hypothalamic Arg-Phe-amide-related peptides, kisspeptin[21-22] and gonadotropin-inhibitory hormone[23-24], may be important regulators of the reproductive axis.

Kisspeptins are currently recognized as the most potent activators of the hypothalamus-pituitary-gonadal axis[25]. Kisspeptin and its receptor, G protein-coupled receptor 54 (GPR54), are expressed on gonadotropin-releasing hormone neurons, thus regulating the reproductive axis[26-28]. Kisspeptins strongly release gonadotropin-releasing hormone and luteinizing hormone, even at pre-puberty[29]. Central or peripheral administration of kisspeptin stimulates the gonadotropic axis[30-32]. Chronic central administration of kisspeptin to immature female rats was shown to induce a premature activation of the gonadotrophic axis[31], and peripheral injection of kisspeptin significantly increased plasma levels of luteinizing hormone[32]. *In vitro* studies also provide evidence that kisspeptins directly affect gonadotropin-releasing hormone neurons[33-34]. Kisspeptin was demonstrated to increase the intracellular calcium concentration $[Ca^{2+}]_i$ in isolated gonadotropin-releasing hormone neurons[34], as well as in hypothalamic GT1–7 immortalized cell lines[33]. Furthermore, gonadotropin-releasing hormone secretion was increased in kisspeptin-activated GT1–7 cells[35-37]. Taken together, these findings implicate an important role for kisspeptin/GPR54 for the regulation of sexual maturation and the development of the reproductive system.

Gonadotropin-inhibitory hormone was first discovered in birds[23-24]. Orthologous peptides belonging to the Arg-Phe-amide peptide superfamily were then found in mammals[38]. The function of Arg-Phe-amide-related peptides 1, 2 and 3 of mammals (including humans) is similar to that of the avian gonadotropin-inhibitory hormone[38]. Gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-3 inhibit gonadotropin secretion in mammals[23, 39]. Furthermore, Arg-Phe-amide-related peptide functions as gonadotropin-inhibitory hormone, inhibiting gonadotropin-releasing hormone-stimulated gonadotropin mRNA subunits and luteinizing hormone release[40]. Initially, only gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-3 were considered to be functional homologs[41] because Arg-Phe-amide-related peptide-1 was found to only affect prolactin secretion[42]. More recently, however, Arg-Phe-amide-related-peptide-1 was shown to affect luteinizing hormone secretion[43]. Gonadotropin-inhibitory hormone may also directly modulate gonadotropin-releasing hormone because gonadotropin-releasing hormone-immunoreactive neurons have been shown to form close appositions with gonadotropin-releasing hormone in rodents and humans[39, 44]. Electrophysiological recordings of mouse brains reveal that gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-3 have inhibitory actions on both gonadotropin-releasing hormone neurons and kisspeptin-activated gonadotropin-releasing hormone neurons[45-46].

These studies suggest that gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide may regulate the hypothalamus-pituitary-gonadal axis and modulate the effects of kisspeptin on gonadotropin-releasing hormone neurons. Although further investigation of this relationship is required, gonadotropin-releasing hormone-producing neurons are sparsely distributed within the hypothalamus. Therefore, GT1–7 cell lines are widely used as a substitute to model gonadotropin-releasing hormone neurons[47]. However, the effects of gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide on this cell line have not been investigated thus far. Therefore, the aim of the present study was to explore, for the first time, the effects of gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 on calcium signaling and gonadotropin-

releasing hormone secretion in kisspeptin-stimulated GT1–7 cells.

RESULTS

Effects of gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 on $[Ca^{2+}]_i$ in kisspeptin-stimulated GT1–7 cells

$[Ca^{2+}]_i$ was significantly ($P < 0.001$) increased (128.3 ± 3.2% from baseline control) by Kisspeptin-10 (Figure1), and thus generated a peak $[Ca^{2+}]_i$ response. However, no changes (99.8 ± 3.6% or 99.9 ± 2.1%) of this response were detected in kisspeptin co-treated with gonadotropin-inhibitory hormone or Arg-Phe-amide-related peptide-1(Figure 1). No effects were seen with either drug alone (Figure 1).

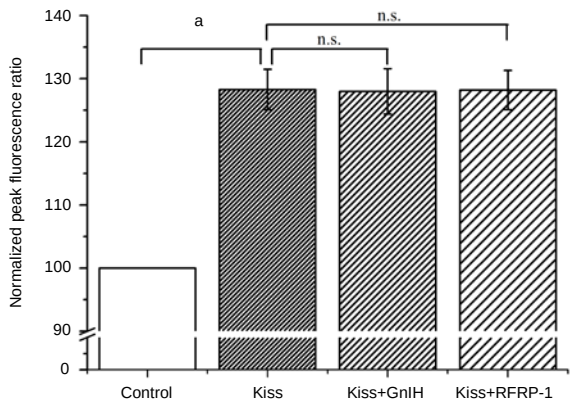

Figure 1 $[Ca^{2+}]_i$ in GT1–7 cells.

The effects of 100 nmol/L kisspeptin-10 (Kiss; $n = 58$) with or without 1 μmol/L gonadotropin-inhibitory hormone (GnIH; $n = 47$) or 1 μmol/L Arg-Phe-amide-related peptide-1 (RFRP-1; $n = 72$) on normalized peak fluorescence ratio. $^aP < 0.001$. n.s: Not significant.

Effects of gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 on gonadotropin-releasing hormone secretion by kisspeptin-stimulated GT1–7 cells

Gonadotropin-releasing hormone levels were not significantly affected by a 60-minute (22.8 ± 3.6 pg/mL) nor 90-minute (25.5 ± 2.3 pg/mL) exposure of kisspeptin-10 compared with their respective control (21.8 ± 1.8 pg/mL or 20.6 ± 3.2 pg/mL) (Figure 2A, B). Gonadotropin-releasing hormone levels were significantly ($P < 0.01$) increased by a 120-minute exposure of kisspeptin-10 (27.7 ± 2.4 pg/mL) compared with control (0.6 ± 1.6 pg/mL) (Figure 2C). However, gonadotropin-releasing hormone secretion was significantly ($P < 0.05$) decreased in a 120 minute exposure of kisspeptin-10 co-treated with gonadotropin-inhibitory hormone or Arg-Phe-amide-related peptide-1 (Figure 2C).

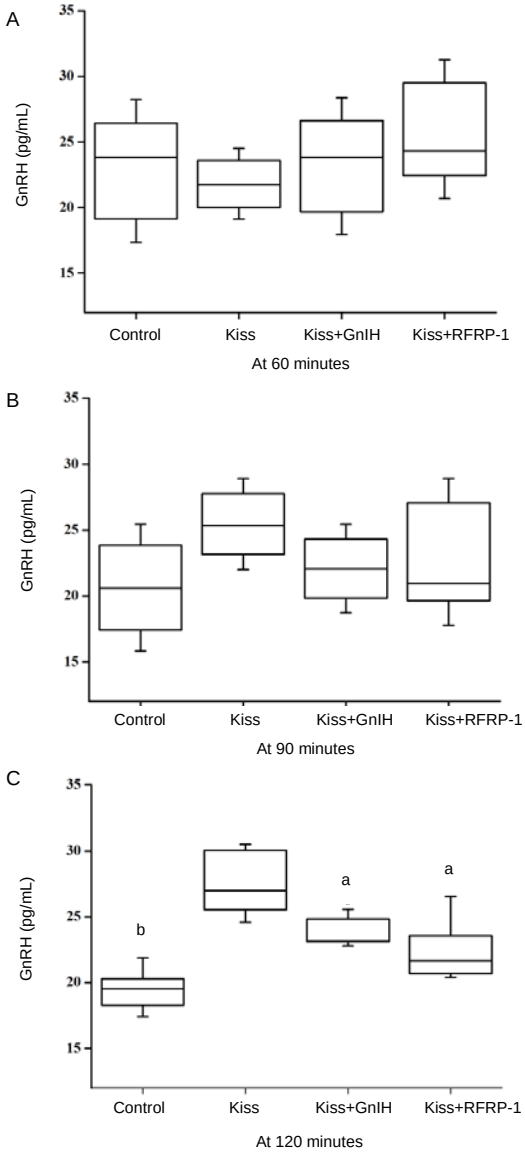

Figure 2 Gonadotropin-releasing hormone (GnRH) levels in GT1–7 cells.

The effects of 100 nmol/L kisspeptin-10 (Kiss; $n = 6$) with or without 1 μmol/L gonadotropin-inhibitory hormone (GnIH; $n = 6$) or 1 μmol/L Arg-Phe-amide-related peptide-1 (RFRP-1; $n = 6$) on GnRH levels at 60 minutes (A), 90 minutes (B), and 120 minutes (C). $^aP < 0.05$, $^bP < 0.01$, *vs*. Kiss.

DISCUSSION

Electrophysiological experiments have demonstrated that gonadotropin-inhibitory hormone and

Arg-Phe-amide-related peptide-3 has inhibitory effects on kisspeptin-stimulated gonadotropin-releasing hormone neurons[46]. Although the interactions of these novel inhibitory peptides with kisspeptin may be important in the regulation of gonadotropin-releasing hormone release, they have not been studied. Therefore, the present study shows, for the first time, the effects of gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 on calcium signaling and gonadotropin-releasing hormone secretion in kisspeptin-stimulated immortalized gonadotropin-releasing hormone neurons (GT1–7 cell line).

Treatment of GT1–7 cells with kisspeptin-10 significantly increased gonadotropin-releasing hormone levels at 120 minutes, but not at 60 or 90 minutes. Co-treatment of kisspeptin-10 with either gonadotropin-inhibitory hormone or Arg-Phe-amide-related-peptide-1 significantly attenuated gonadotropin-releasing hormone levels at 120 minutes. These results show that kisspeptin and gonadotropin-inhibitory hormone only exert their effects completely after a long exposure. As a result, we provide evidence that gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 reduces kisspeptin-stimulated release of gonadotropin-releasing hormone in GT1–7 cells in a time-dependent manner.

Our findings that GT1–7 cells release more gonadotropin-releasing hormone into culture medium in response to kisspeptin-10 treatment are consistent with the results of previous studies[28, 39], despite other studies showing otherwise[38]. In the present study, GT1–7 cells were exposed to kisspeptin for three different incubation times (60, 90 and 120 minutes; static incubation) with 100 nmol/L kisspeptin. However, significant levels of gonadotropin-releasing hormone levels were detected at only 120-minute exposure. The dose of kisspeptin-10 was chosen as 100 nmol/L because the maximal increase in $[Ca^{2+}]_i$ in response to kisspeptin-10 was found at this concentration in our previous study[40]. Static incubation studies involving Kisspeptin-10 treatment of GT1–7 cells have revealed increased secretion of gonadotropin-releasing hormone [35-37]; however, this effect may be disputed[48].

Numerous studies have indicated that kisspeptin stimulates gonadotropin-releasing hormone neurons *via* calcium-dependent regulatory mechanisms[49-50]. However, our findings do not agree with this putative mechanism of action because gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide were found to attenuate gonadotropin-releasing hormone levels without affecting $[Ca^{2+}]_i$, thus rendering this inhibition as possibly calcium-independent. Although gonadotropin-releasing hormone release requires elevations of $[Ca^{2+}]_i$ [29], other signaling mechanisms may play a part in gonadotropin-inhibitory hormone modulation of kisspeptin-stimulated gonadotropin-releasing hormone. Mitogen-activated protein kinase signaling cascade may be involved in gonadotropin-inhibitory hormone modulation because inhibitors of ERK1/2 and p38 kinases have been shown to completely prevent gonadotropin-releasing hormone secretory responses to kisspeptin-10 by hypothalamic fragments[29]. Hypothalamic fragments are horizontal excisions of about 2 mm depth with the following tissue limits: 1 mm anterior from the optic chiasm, and delimited by the posterior border of the mammillary bodies and the hypothalamic fissures. Gonadotropin-inhibitory hormone attenuates kisspeptin-10-mediated increases of $[Ca^{2+}]_i$ in the rat hypothalamal neuronal cell line, rHypoE-8[51]. Therefore, gonadotropin-inhibitory hormone may have different mechanisms of action in the secretion of gonadotropin-releasing hormone and kisspeptin in GT1–7 cells and Hypo8 cells, respectively, thereby implicating direct and indirect effects of gonadotropin-inhibitory hormone on gonadotropin-releasing hormone neurons.

In conclusion, this study demonstrates that gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide reverse kisspeptin-induced activation of gonadotropin-releasing hormone neurons. This inhibitory effect may occur independently of the elevation in $[Ca^{2+}]_i$. Furthermore, we provide further evidence that Arg-Phe-amide-related-peptide-1, in addition to Arg-Phe-amide-related-peptide-3, may be involved in the modulation of the hypothalamus-pituitary-gonadal axis. The interactions of both gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide with kisspeptin may be important in the regulation of reproductive functions, including the onset of puberty. Therefore, further work is warranted for the investigation of the mechanisms underlying gonadotropin-inhibitory hormone inhibition of gonadotropin-releasing hormone release in gonadotropin-releasing hormone neurons.

MATERIALS AND METHODS

Design

An *in vitro* cell study.

Time and setting

Experiments were performed from May 2011 to November 2012 in the Department of Physiology, Medical School, Firat University, Elazig, Turkey.

Materials

GT1–7 cells were a gift from University of California, San Francisco, CA, USA. Kisspeptin-10 was obtained from Sigma (St. Louis, MO, USA), and gonadotropin-inhibitory hormone and Arg-Phe-amide-related peptide-1 were obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Fura-2 acetoxymethyl ester (fura 2-AM) (Invitrogen, Eugene, Oregon, USA) was dissolved in dimethyl sulfoxide. Stock solutions of kisspeptin-10, gonadotropin-inhibitory hormone, and Arg-Phe-amide-related peptide-1 were prepared in imaging bath solution (130 mmol/L NaCl, 3 mmol/L KCl, 0.6 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 1 mmol/L $NaHCO_3$, 5 mmol/L glucose, and 10 mmol/L HEPES [pH 7.4]).

Methods

GT1–7 cell culture

GT1–7 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Life Technologies Inc., Gaithersburg, MD, USA) with 10% fetal bovine serum (JRH, Lenexa, KS, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies Inc). Cells were incubated under normal conditions (95% O_2, 5% CO_2, at 37°C) until plates were 80–90% confluent. The medium was then removed, and plates were rinsed three times with serum-free media, and replaced with serum-free DMEM 24 hours prior to experiments.

Gonadotropin-releasing hormone secretion studies

Cells were challenged with DMEM (vehicle control), 100 nmol/L kisspeptin-10, 1 μmol/L gonadotropin-inhibitory hormone or 1 μmol/L Arg-Phe-amide-related peptide-1 for 60, 90 and 120 minutes. Media (50 μL) was collected at each time point to monitor changes in gonadotropin-releasing hormone secretion. The gonadotropin-releasing hormone ELISA kit (Phoenix Pharmaceuticals Inc, Burlingame, CA, USA) was used to determine gonadotropin-releasing hormone concentrations from the media, according to the manufacturer's protocol.

Measurement of $[Ca^{2+}]_i$

Cells in imaging bath solution were loaded with the Ca^{2+}-sensitive dye, 1 μmol/L fura 2-AM, and incubated for approximately 60 minutes at 37°C (5% CO_2 humidified incubator). All imaging experiments were performed in the dark, at room temperature. Glass coverslips with fura-2-loaded cells were mounted in an imaging/ perfusion chamber equipped with a perfusion valve system (Warner Instruments, Hamden, CT, USA), which was mounted and viewed through an inverted microscope (Nikon TE2000S, Tokyo, Japan). The bath volume of the chamber was 600 μL. The fura-2-loaded GT1–7 cells were alternately illuminated with 340 nm and 380 nm wavelengths from a 175-W Xenon ozone-free lamp source (Sutter Instruments Co., Novato, CA, USA) optically coupled to the microscope (Nikon TE2000S inverted) and cooled charge-coupled device camera (C4742-95; Hamamatsu Photonics, Japan). Fluorescence intensity values as an indication of $[Ca^{2+}]_i$ were detected from each wavelength. After subtraction of background fluorescence, the values were expressed as a ratio (F_{340}/F_{380}). Kisspeptin-10 was used as the positive control (*i.e.* 100% response) to normalize the responses by gonadotropin-inhibitory hormone or Arg-Phe-amide- related peptide-1 for each cell. Values from control and drug treatments were obtained from the same cells for equal comparison.

After we began to measure the basal intracellular Ca^{2+} levels, we then exposed the cells to the drugs for 10 minutes. The final concentration of dimethyl sulfoxide in the bathing solution did not exceed 0.2 % (v/v). Dimethyl sulfoxide alone did not elicit any change in $[Ca^{2+}]_i$ (data not shown).

Statistical analysis

All values are expressed as mean ± SD. The values obtained from $[Ca^{2+}]_i$ imaging and gonadotropin-releasing hormone secretion studies were analyzed by one-way analysis of variance followed by the Tukey's honest significance test. Significance was reached at values of $P < 0.05$.

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Author contributions: Haluk Kelestimur participated in the study design, manuscript writing and providing the funds. Emine Kacar was responsible for data collection and integration. Aysegul Uzun performed gonadotropin-releasing hormone analysis. Mete Ozcan and Selim Kutlu performed calcium imaging analysis and statistical processing.

Conflicts of Interest: None declared.

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