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Original Research

Kisspeptin increases intracellular calcium concentration by protein kinase C-mediated signaling in the primary cultured neurons from rat hippocampus

Ihsan Serhatlioglu[1], Hande Yuksel[1], Emine Kacar[2], Sibel Ozcan[3], Sinan Canpolat[2], Mete Ozcan[1*], Ahmet Ayar[4], Haluk Kelestimur[2]

[1] Department of Biophysics, Faculty of Medicine, Firat University, Elazig, Turkey
[2] Department of Physiology, Faculty of Medicine, Firat University, Elazig, Turkey
[3] Department of Anesthesiology and Reanimation, Faculty of Medicine, Firat University, Elazig, Turkey
[4] Department of Physiology, Faculty of Medicine, Karadeniz Technical University, Trabzon, Turkey

Correspondence to: mozcan@firat.edu.tr, meteozcan@hotmail.com

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Abstract: In addition to the fact that kisspeptin and its receptor GPR54 are well known to be abundantly expressed in the hypothalamus with suggestive roles in the initiation of puberty and similar reproductive system properties, there is also proof showing that kisspeptin might have influences on hippocampal functions. In our previous study, it was shown that kisspeptin increased free intracellular Ca^{2+} values ($[Ca^{2+}]_i$) through protein kinase C (PKC) activation in GT1-7 cells. For this reason, we examined the influences of kisspeptin on $[Ca^{2+}]_i$ in hippocampal neurons to determine if kisspeptin shows its effects on hippocampus through the same mechanism. Hippocampal neurons were excised from the brains of fetuses on 17th embryonic day from maternal rats. The influences of kisspeptin on $[Ca^{2+}]_i$ in hippocampal neurons were examined through *in vitro* calcium imaging system. The responses of $[Ca^{2+}]_i$ to kisspeptin were quantified by the changes in 340nm/380nm ratio. Kisspeptin-10 caused $[Ca^{2+}]_i$ transients in hippocampal neurons. The change in $[Ca^{2+}]_i$ by 100 nM kisspeptin was prevented by pre-treating the cells in PKC inhibitor chelerythrine chloride. According to the results, kisspeptin activates intracellular calcium signaling in hippocampal neurons via the pathway that depends on PKC. The results of this study suggest that kisspeptin may have a role in hippocampal neuron functions.

Key words: Kisspeptin; Calcium; Hippocampus; Protein kinase C; Calcium imaging.

Introduction

G protein-coupled receptor 54 (GPR54) uses kisspeptins as natural ligands (1-3). GPR54 mutations are related to immaturity in sexual terms, and human and rodent infertility (4-6). It was shown that Gonadotropin-releasing hormone (GnRH) neurons express GPR54 receptor (7), and with this receptor, kisspeptins activate GnRH excretion (8). It was shown that kisspeptins are the most potent activators of hypothalamus-pituitary-gonadal (HPG) axis (9). The administration of kisspeptin in a chronic central way to immature female rats induced a premature gonadotrophic axis activation (10), and peripheral kisspeptin injection increased plasma levels of luteinizing hormone (LH) at a significant level (11).

Even though the peptide and peptide receptor GPR54 are plenty in the hypothalamus and are considered to be associated with puberty initiation and similar functions of the reproductive system, there is also proof that kisspeptin might have important influences on hippocampal activities. In the hippocampus, the density of GPR54 is extremely high in the dentate gyrus (DG) granule cell layer. Metastin, also called kisspeptin, is suggested to potentiate excitatory synaptic transmission reversibly in hippocampus (12), which makes us think that the kisspeptin-GPR54 system may have selective functions in the hippocampus. In the previous study of ours (13), we demonstrated that kisspeptin increases free intracellular Ca^{2+} ($[Ca^{2+}]_i$) through protein kinase C (PKC) activation in hypothalamic GT1-7 immortalized cell lines. For this reason, in this study, the influences of kisspeptin on $[Ca^{2+}]_i$ were examined in the neurons in the hippocampal area to determine if kisspeptin shows its influences on hippocampus through the same mechanism. Since much of neural development occurs during fetal stages of life, and neurogenesis is limited in adult brain, we measured $[Ca^{2+}]_i$ in fetal hippocampal neurons, using calcium imaging system.

Materials and Methods

Preparation of culture of rat hippocampal neurons

All animal use procedures were approved by the Firat University Animal Experiments Local Ethics Committee and rats were treated in accordance with the national and international laws and policies on the care and use of laboratory animals. Hippocampal neurons were isolated from 17th embryonic day (E17) from Sprague-Dawley maternal rats. Prenatal pups were removed from the uterus of maternal rats after intraperitoneal anesthesia with 10% chloral hydrate (1 mL/250 g body weight). Then animals were decapitated to remove brain tissue under a dissecting microscope on ice using sterile scissors and forceps. Brain hemispheres were separated, and remove the midbrain using forceps in order to ex-

pose the hippocampus. Hippocampus was dissected from the cortex using finer scissors and cleaned off the meninges. Then the hippocampal tissue was carefully chopped and then mechanically dissociated through trituration by using flame-narrowed Pasteur pipette.

Hippocampal cells were then plated on to 12 mm round, poly-D-lysine/laminin-coated glass coverslips (BD BioCoat, Bedford, MA, USA) and bathed in Neurobasal-A medium (Gibco, Paisley, Scotland), supplemented with 2% B27 (Gibco), 1% Glutamax (Gibco), and penicillin (5000 IU/mL) streptomycin (5000 µg/mL).

Hippocampal neurons were kept in a humidified incubator containing 95% air and 5% CO_2 at 37°C (HeraCell, Heraeus, Germany). The cells were used 6 hours after the plating up to 24 hours in culture.

Ratiometric intracellular calcium imaging

Hippocampal neurons were treated with dye that was sensitive to calcium fura-2/AM ester in recording solution for 60 minutes. After washing out the dye-containing medium for 3-4 times with recording solution, the cells were used for image analysis. The recording solution had (in mM): 5.0 glucose, 0.6 $MgCl_2$, 10.0 HEPES, 3.0 KCl, 1.0 $NaHCO_3$, 2.0 $CaCl_2$ and 130.0 NaCl. The recording system had inverted Nikon TE 2000 S microscope (S-fluor, 40X oil, 1.3 NA) and CCD camera (ORCA 285, Hamamatsu Photonics, Hamamatsu, Japan). 340nm-380nm excitation filters and 510nm emission filter were used during recording. Fluorescence ratio of cells was determined by using the imaging software (simple PCI). Results were computed as mean peak amplitude from at least three independent experiments. Effect of kisspeptin on intracellular calcium values was normalized and expressed as percentage change from the baseline calcium level (100%).

There was no effect on $[Ca^{2+}]_i$ when administered alone in the control experiment of Fura-2AM dissolved in DMSO.

Statistical analysis

The data were given as mean±SEM values. Effects of kisspeptin (10, 30 and 100 nM) on calcium levels were determined analyzed by One-Way Analysis of Variance (ANOVA) and then Post-Hoc Tukey HSD test was also applied. The influences of protein kinase C inhibitor chelerythrine chloride on kisspeptin induced $[Ca^{2+}]_i$ levels were assessed with unpaired Student's t-test. P<0.05 was taken as significant.

Results

Kisspeptin dose-dependently increased intracellular Ca2+ transients in hippocampal neurons. Fig.1 shows that the mean peak amplitude data of $[Ca^{2+}]_i$ increase evoked by kisspeptin was significantly and dose-dependently to 104.4±3.6 (n=31, p<0.01), 111.6±3.9 (n=34, p<0.01) and 141.2±4.1 (n=42, p<0.01) from baseline level after application of 10nM, 30nM and 100 nM kisspeptin, respectively.

The ability of the protein kinase C inhibitor (chelerythrine chloride) to influence the $[Ca^{2+}]_i$ response to kisspeptin (100nM) was also investigated. As shown in Fig2, peak $[Ca^{2+}]_i$ response to kisspeptin (100nM) was strongly attenuated to 107.4±4.6% (n=42, p<0.001) after adding 10 µM PKC inhibitor (Fig. 2).

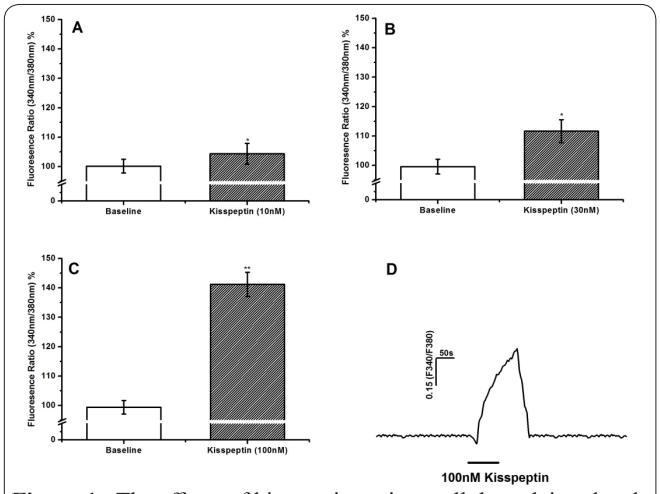

Figure 1. The effects of kisspeptin on intracellular calcium levels in hippocampal neurons. A)10 nM kisspeptin (n=31), B) 30nM kisspeptin (n=34) and C) 100nM kisspeptin (n=42) stimulated Ca^{2+} responses in different hippocampal cells, D) original responses trace of fluorescence ratio to 100 nM kisspeptin application from one hippocampal neuron. Single asterisk (*) $p < 0.01$ and double asterisks (**) $p < 0.001$ vs control (one-way analysis of variance and post-hoc Tukey HSD test) from three independent experiments.

Discussion

We have found that intracellular calcium signaling in hippocampal neurons is activated by kisspeptin. This shows that kisspeptin has new roles in hippocampal functions. Whereas in our previous study (13), it was found that there was a kisspeptin-stimulated intracellular calcium response in a triphasic pattern in GT1-7 cells which are immortalized GnRH neurons, kisspeptin-10-induced [Ca^{2+}]i response in the hippocampal neurons was in a different pattern (Fig. 1), suggesting the presence of functional kisspeptin receptors in these cells as well. Although there are many studies suggesting an important role of kisspeptin in hippocampal functions (14-16), molecular mechanisms are not well known. There is only one study (17) looking at the effects of kisspeptin on intracellular calcium concentration in hippocampal neurons to date. At that study, the brains of the 4-6 day

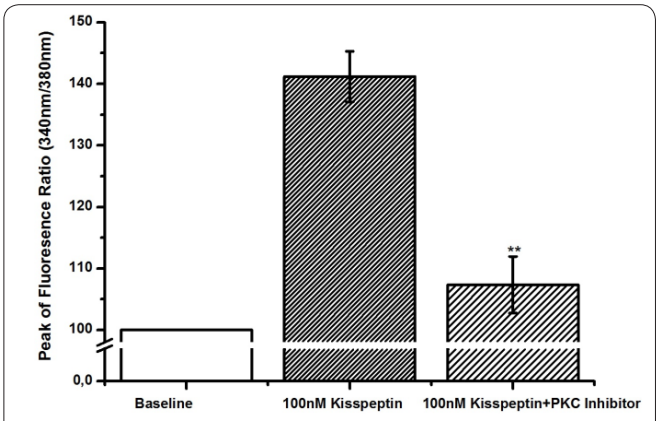

Figure 2. The effect of PKC inhibition on 100nM kisspeptin-stimulated intracellular calcium rise in hippocampal neurons. * P<0.001 vs 100nM kisspeptin (unpaired *t* test) from three independent experiments.

newborn rats were used to culture the hippocampal neurons, and an increase of $[Ca^{2+}]_i$ was seen in only 6 of the 40 cultured hippocampal neurons. In our study, hippocampal neurons were taken from fetal brains removed on E17 from maternal rats, and almost all the neurons responded to kisspeptin. Several factors may underline in the different findings between the study of Brailoiu et al. (17) and ours. One difference is that we used kisspeptin-10 whereas they used metastin or kisspeptin-54. A 145-amino acid polypeptide (Kp-145) is the primary translation product of the KiSS-1 gene (3), but shorter 'kisspeptins' with 10, 13, 14 or 54 amino acid residues have been discovered, and are termed kisspeptin-10, -13, -14 and -54 according to number of amino acids in length. The decapeptide kisspeptin-10 (shared by all the members of kisspeptin family) is necessary for biological activity (1). Kisspeptin-54, also termed 'metastin', is the putatively secreted and biological active form of kisspeptin-145 (18). Our study shows that kisspeptin-10 is more effective compared to metastin at least in terms of effects on $[Ca^{2+}]_i$ in the hippocampal neurons. Interestingly, only kisspeptin-10, but not other kisspeptins increased $[Ca^{2+}]_i$ in isolated first trimester trophoblasts (19), invasion of which is a key process for successful reproduction and embryonic development. Thus, kisspeptin-10 seems to increase $[Ca^{2+}]_i$ more effectively not only in fetal brains but also in fetal peripheral tissues. Secondly, the differences in $[Ca^{2+}]$i response to kisspeptin may result from the use of the hippocampal neurons obtained from the brains at different ages. We used 17 day fetuses whereas Brailoiu et al. (17) used 4-6 day newborn rats. Thus, it can be speculated that kisspeptin may be more effective prenatally compared to its postnatal effects. Whether the different effectiveness of prenatal and postnatal kisspeptin is related to receptor sensitivity remains to be clear.

Although it was found that kisspeptins could increase intracellular calcium ions, there is no knowledge concerning the mechanisms by which kisspeptin exerts its effects on $[Ca^{2+}]_i$. In our study, we determined that calcium-enhancing influence of kisspeptin-10 was blocked by PKC inhibitor, chelerythrine chloride. These outcomes show that enhancement of $[Ca^{2+}]_i$ by kisspeptin-10 involves the PKC pathway in a specifically manner. Our results are consistent with the findings that inhibitors of phospholipase C (PLC) and PKC abolished the regulation of the tested genes transcriptionally-regulated through intracellular calcium release in the human MDA-MB-435S cell line originating from a metastatic ductal breast carcinoma (20).

There were several limitations in this study. First, we used short term culture protocol. The development of axonal and dendritic extensions of hippocampal cells may affect fluorescence calcium imaging calculations. So, neurons were used 6 hours after the plating up to 24 hours in culture. Secondly, it was too difficult to determine the effects of kisspeptin on specific hippocampal regions such as CA1, CA2, CA3 and dentate gyrus because of use of prenatal cells.

Our findings first time demonstrated that kisspeptin increased $[Ca^{2+}]_i$ via PKC mechanism in hippocampal neurons. Kisspeptin may have a role in hippocampal neuron functions. The understanding of the kisspeptin-Ca^{2+} signaling pathway associated with learning and memory processing may establish the novel therapeutic strategies for neurodegenerative disorders.

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Interest conflict
The authors declare there is no conflict of interest with regard to data presented in this article.

Author's contribution
İ.S, HY, E.K, SO; experimentation,
S.C, A.A; Experiment design
M.O; Statistical analysis and manuscript preparation
A.A, H.K; Experiment design and manuscript preparation.

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