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Kisspeptin leads to calcium signaling in cultured rat dorsal root ganglion neurons

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Abstract. Although kisspeptin and GPR54 have been reported to be expressed in the neurons of the dorsal root ganglion (DRG) of rats, and kisspeptin has been suggested to be involved in pain modulation in rodents, there is no study on the effects and mechanisms of kisspeptin on sensory neurons. Therefore, the aim of this study was to investigate the effects and mechanism of kisspeptin on intracellular free calcium levels in cultured rat DRG neurons. Bath application of kisspeptin-10 increased intracellular free calcium levels ($[Ca^{2+}]_i$). In the absence of extracellular calcium, the kisspeptin induced an attenuated but still significant increase in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ responses persisted in the presence of protein kinase C (PKC) inhibitor. Data from this study revealed that kisspeptin-10 activates $[Ca^{2+}]_i$ signaling independent of PKC in cultured rat sensory neurons suggesting that peripheral site is also involved in the pain modulating effect of kisspeptin.

Key words: Kisspeptin — Sensory neurons — Calcium signaling — Protein kinase C

Introduction

Kisspeptins are the natural ligands for kisspeptins receptor KISS1R, a G protein-coupled receptor GPR54, which is a $G\alpha_{q/11}$-coupled seven-transmembrane receptor (Kotani et al. 2001; Muir et al. 2001). Since the first identification of kisspeptin as an antimetastatic peptide in 1996 (Lee et al. 1996), several studies including our previous ones have implicated that this neuropeptide possess important reproductive effects. KISS1/KISS1R-inactivating mutations result in a phenotype of delayed puberty and idiopathic hypogonadotropic hypogonadism. Conversely, activating mutations in KISS1R cause central precocious puberty. These observations established the essential role which kisspeptin plays in the timing of puberty and regulation of the hypothalamus-pituitary-gonadal (HPG) axis (de Roux et al. 2003; Seminara et al. 2003; Teles et al. 2008; Topaloglu et al. 2012; Sahin et al. 2015). Kisspeptins are reported to be the most potent activators of HPG axis known to date (Han et al. 2005). They potently elicit GnRH release and luteinizing hormone (LH) secretion even at the pre-pubertal periods (Castellano et al. 2006).

It has been recently reported that in addition to reproduction, kisspeptin signaling influences energy metabolism including body weight, energy expenditure, and glucose homeostasis in a sexually dimorphic and partially sex steroid-independent manner. Therefore, alterations in kisspeptin signaling have been suggested to contribute, directly or indirectly, to some aspects of human obesity, diabetes, or metabolic dysfunction (Tolson et al. 2014).

Moreover, kisspeptin and GPR54 have been reported to be expressed in the neurons of the dorsal root ganglion (DRG) of rats (Mi et al. 2009), and kisspeptin has been suggested to be involved in pain modulation in rodents (Ayachi and Simonin 2014). But, there is no study on the effects and mechanisms of kisspeptin on sensory neurons. Hence, the aim of this study was to investigate the effects of kisspeptin on intracellular free calcium levels ($[Ca^{2+}]_i$) in cultured rat DRG neurons. DRG neurons are primary sensory neurons that transmit afferent signals about potentially damaging stimuli that results in perception of pain. Following tissue injury, DRG cells fire action potentials that convey their message to the brain. Therefore, they present a suitable cellular model for peripheral nociception and are considered to be key targets

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for research of analgesia (Vyklický and Knotková-Urbancovä 1996; Chung and Chung 2002). Since intracellular calcium is one of the most diverse and widespread intracellular second messengers, which are involved in nociceptive events, investigation of the effects of kisspeptin on $[Ca^{2+}]_i$ in DRG neurons may contribute to understanding of its modulatory role in peripheral pain signaling mechanism.

Materials and Methods

Preparation of primary culture of rat DRG neurons

In this study DRG neurons were obtained from 1-day-old to 2-day-old Wistar rats. The animals were sacrificed by decapitation and their spinal cords were removed, and primary cultures of dissociated DRG neurons from the cervical, thoracic, lumbar and sacral levels were prepared (~45–50/pup). Afterward, they were treated enzymatically and mechanically by trituration. DRG neurons were plated onto round, poly-d-lysine/laminin-coated glass coverslips (BD BioCoat, Bedford, MA) and bathed in the culture medium supplemented with nerve growth factor (NGF 2.5 S, Sigma-Aldrich, Germany) at 37°C in a humidified incubator with 5% CO_2/95% air (Heracell, Kendro Lab GmbH, Germany). Coverslips with cells were used 6 hours after plating.

Ratiometric intracellular calcium imaging

DRG neurons were loaded with dye fura-2/AM ester (1 μM, Molecular probes Inc., 1 mM stock in dimethylsulphoxide (DMSO)) in imaging bath solution for 60 min, then washed 3–4 times for 20 min with imaging bath solution in dark room. The imaging bath solution contained (in mM): 130.0 NaCl, 3.0 KCl, 0.6 $MgCl_2$, 2.0 $CaCl_2$, 1.0 $NaHCO_3$, 5.0 glucose and 10.0 HEPES. The pH was adjusted with NaOH to 7.4, and the osmolarity was adjusted to 310–320 mOsm by sucrose.

DRG neurons were mounted in an imaging/perfusion chamber (bath volume of 600 μl) equipped with perfusion valve system (Warner Instruments, Hamden, CT) which was viewed under an inverted Nikon TE 2000 S microscope (S-fluor, 40X oil, 1.3 NA) attached with a CCD camera (ORCA 285, Hamamatsu Photonics, Hamamatsu, Japan). 340–380 nM excitation filter and 510 nM emission filter were used in fluorescence ratio recording. 340–380 nM excitation filter switched using a computer-controlled filter wheel (Lambda-10; Sutter Instruments, Novato, CA). The calculations and analysis of $[Ca^{2+}]_i$ were performed using a computer with data-analysis software and image processor sPCI (Hamamatsu Photonics, Herrsching, Germany; Hamamatsu Photonics, Japan).

Fluorescence intensity of individual cells was determined over time by selecting a region of interest using the imaging software. An estimate of $[Ca^{2+}]_i$ was calculated from the ratio of 340/380 nM fluorescence intensity values (after correction by subtraction of background fluorescence) and expressed as a ratio (F340/F380) for unselected DRG neurons. Results are given in the form of normalized mean peak amplitude. The baseline fura-2 emission ratio was used as 100% to normalize the effects of kisspeptin for each cell. Each cells treated with protein kinase-C (PKC) inhibitor chelerythrine chloride (ChCl) after application of 300 nM kisspeptin.

All agents were delivered directly to the imaging bath solution for a total application time as indicated in the results.

Chemicals

Kisspeptin-10 was obtained from Sigma (St. Louis, MA) and dissolved in the imaging bath solution, and aliquots were frozen. Each stock solution was diluted to the appropriate concentration in the imaging bath solution minutes before bath application. Fura-2 AM was obtained from Invitrogen (Invitrogen, Karlsruhe, Germany) and dissolved in DMSO. The final concentration of DMSO in the bathing solution did not exceed 0.2% (v/v), which did not elicit any change in $[Ca^{2+}]_i$ by itself in control experiments.

Statistical analysis

Differences between the $[Ca^{2+}]_i$ levels at baseline and after the doses of 100, 300 and 1000 nM kisspeptin and effects of PKC inhibitor ChCl on kisspeptin-induced $[Ca^{2+}]_i$ were calculated by means of one-way analysis of variance (ANOVA) followed by a *post-hoc* Tukey HSD test. The effects of kisspeptin in the calcium-free condition on $[Ca^{2+}]_i$ transients were evaluated using unpaired Student's *t*-test. For all analyses, $p < 0.05$ was accepted as evidence of significance.

Results

Kisspeptin-10 produced significant change in $[Ca^{2+}]_i$ transients of DRG neurons in a dose-dependent manner. Effects of kisspeptin-10 on the fura-2AM 340/380 nM fluorescence ratio due to changes in $[Ca^{2+}]_i$ were tested for 100, 300 and 1000 nM (Fig. 1). The increases in $[Ca^{2+}]_i$ as % of preceding control (baseline) levels were significantly and dose-dependently to 118.4 ± 4.6 ($n = 41$, $p < 0.01$), 126.6 ± 4.1 ($n = 36$, $p < 0.001$) and 140.2 ± 4.9 ($n = 44$, $p < 0.001$) from baseline level (100%) after application of 100, 300 and 1000 nM kisspeptin-10, respectively.

Significant increases in $[Ca^{2+}]_i$ response to kisspeptin (300 nM) also occurred in calcium-free conditions but with lower potency (112.1 ± 5.1%, $n = 33$) compared to the kiss-

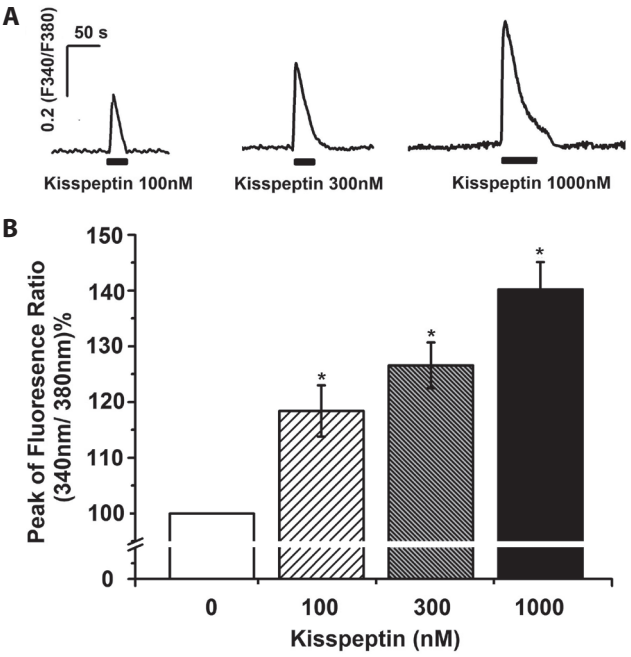

Figure 1. Dose-dependent effects of kisspeptin on intracellular calcium levels in dorsal root ganglion (DRG) neurons. **A.** Original responses trace of fluorescence ratio to kisspeptin treatment from one DRG neuron. 100 nM (n = 41), 300 nM (n = 36) and 1000 nM (n = 44) kisspeptin lead to Ca^{2+} responses in different DRG cells, in the presence of extracellular Ca^{2+}. **B.** Bar graph comparing the effects of different concentrations of kisspeptin on percentage of total fluorescence ratio. * $p < 0.01$ *vs.* baseline level (0 nM kisspeptin) (ANOVA followed by a *post-hoc* Tukey HSD test).

peptin-10 (300 nM)-induced responses (126.6 ± 4.1, n = 36) in the presence of extracellular calcium (Fig. 2).

To explore whether PKC were associated with the one of the possibilities of the mechanism of kisspeptin induced $[Ca^{2+}]_i$ rise, the PKC inhibitor, ChCl (10 μM), was applied. When the DRG cells were tested with 300 nM kisspeptin-10 in the presence of 10 μM PKC inhibitor (126.1 ± 5.7, n = 27), a similar increase in the peak $[Ca^{2+}]_i$ response was observed with alone 300 nM kisspeptin-10 (Fig. 3).

Discussion

Previous studies have found that some appetite-regulating peptides have effects on peripheral pain modulation. Intraperitoneal injection of leptin, an appetite-inhibiting peptide, was reported to increase pain sensitivity in mice (Kutlu et al. 2003). Contrarily, central or peripheral administration of ghrelin, an appetite-stimulating hormone, was shown to exert an inhibitory role on the development of inflammation and hyperalgesia (Sibilia et al. 2006). Another appetite peptide, orexin has antinociceptive effect at both spinal and supraspinal levels (Chiou et al. 2010) and in our previous study, we have observed that orexin activates calcium signaling in DRG neurons (Ozcan et al. 2010).

As a novel peptide, studies about pain modulation by kisspeptin are limited and mostly based on indirect experiments. The aim of this study was to explore the possible role of kisspeptin in the pain modulation by investigating the effects of kisspeptin on $[Ca^{2+}]_i$ levels in cultured neonatal rat DRG neurons.

The present results showed that kisspeptin-10 increases $[Ca^{2+}]_i$ concentration for the first time in cultured rat DRG neurons. The effect on $[Ca^{2+}]_i$ was in a dose-dependent manner. The response was not abolished by PKC inhibitor ChCl, suggesting that this activation is independent of PKC. Kisspeptin-10-induced calcium increases were maintained with attenuated but still significant increase level in the DRG neurons preincubated in the absence of extracellular calcium, indicating that the peptide activates cytosolic Ca^{2+} increases mainly by Ca^{2+} influx from intracellular sources. In our

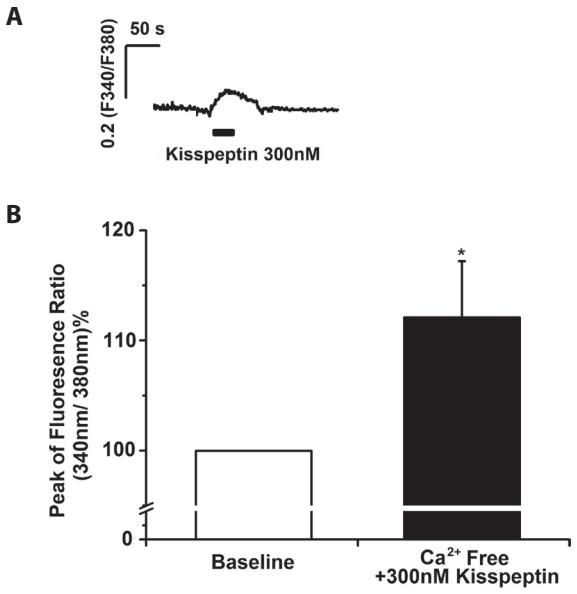

Figure 2. Effects of kisspeptin (300 nM) on intracellular calcium levels of dorsal root ganglion (DRG) neurons in calcium-free bathing conditions. Original representative trace (**A**) and bar graph (**B**) showing the intracellular calcium response of a DRG cell to acute perfusion of 300 nM kisspeptin after 5 min in standard bath solution lacking calcium chloride (with added 0.5 mM EGTA). Data are calculated as peak ratio of fluorescence intensity (340/380 known to reflect intracellular calcium, $[Ca^{2+}]_i$), and the results are shown as peak ratios normalized to the percent of respective baseline $[Ca^{2+}]_i$, (n = 33). * $p < 0.01$ *vs.* baseline level (Unpaired t-test).

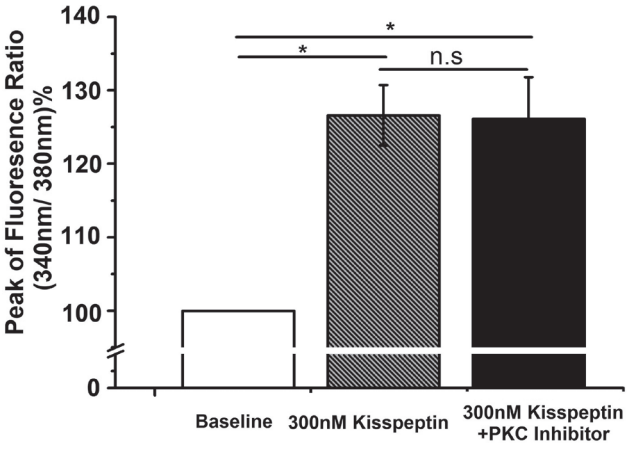

Figure 3. Effects of PKC inhibitor chelerythrine chloride (ChCl) on kisspeptin-induced intracellular calcium responses in dorsal root ganglion (DRG) neurons. Values are given as a percentage of the induced fluorescence (340/380 nM) ratio ($n = 27$). * $p < 0.01$ *vs*. baseline level (ANOVA followed by a *post-hoc* Tukey HSD test). n.s., non significant.

previous study, orexin, which is known to have orexigenic effects, was shown to activate intracellular calcium signaling in cultured rat sensory neurons through PKC-dependent pathway (Ozcan et al. 2010). Thus, kisspeptin, which is also suggested to be an anorexigenic peptide (Dong et al. 2020), and orexins show the effects on $[Ca^{2+}]_i$ signaling by different mechanisms.

While the neuronal activity of kisspeptin was assessed in central neurons regarding reproduction and energy metabolism, there is a paucity of studies investigating its roles in peripheral nervous system. In our previous study (Ozcan et al. 2011), the pattern of kisspeptin-induced intracellular signaling was investigated by fluorescence calcium imaging using the immortalized GnRH-secreting GT1-7 hypothalamic neurons. Kisspeptin-10 caused a triphasic change characterized by an initial small increase followed by a significant decrease and increase in intracellular free calcium concentrations. The changes in $[Ca^{2+}]_i$ were significantly attenuated by pre-treatment with PKC inhibitor. The compatibility of appeared mirrored-patterns of kisspeptin-10-induced changes in $[Ca^{2+}]_i$ concentrations in these neurons and GnRH secretion confirm the importance of $[Ca^{2+}]_i$ flux downstream from GPR54 through PKC signaling pathway. In our previous study which has been carried out in cultured hippocampal neurons from rats (Serhatlioglu et al. 2018), kisspeptin-10 caused $[Ca^{2+}]_i$ transients in hippocampal neurons. In that study, the change in $[Ca^{2+}]_i$ by kisspeptin was prevented by pre-treating the cells in PKC inhibitor ChCl, suggesting that kisspeptin activates $[Ca^{2+}]_i$ signaling in hippocampal neurons *via* the pathway that depends on PKC. According to these results, the effects of kisspeptin-10 on $[Ca^{2+}]_i$ involves different downstream effectors in central and peripheral neurons.

Consistent with the treatments used in this study, elevated $[Ca^{2+}]_i$ following kisspeptin activation of cell lines expressing KiSS1R has been demonstrated in several studies (Liu et al. 2008; Babwah et al. 2012; Magee et al. 2020). In the present experiment, the rise in $[Ca^{2+}]_i$ in a Ca^{2+}-free media is the first direct evidence of kisspeptin-10 stimulation of the kisspeptin receptor. The kisspeptin receptor, GPR54, which is a $G\alpha_{q/11}$-coupled receptor, seems to activate calcium signaling with phospholipase C-mediated generation of inositol 1,4,5 trisphosphate (IP3) for mobilization of $[Ca^{2+}]_i$ stores in rat primary DRG cells. The fact that the kisspeptin-10 induced attenuated but still significant increase in intracellular free calcium levels in the absence of extracellular calcium shows the possible contribution of extracellular calcium. The RYR channel is usually activated by a small Ca^{2+} influx from the extracellular space and mediates Ca^{2+}-induced Ca^{2+} release, therefore, when extracellular Ca^{2+} was absent, this channel cannot be activated and cannot contribute to Ca^{2+} signaling in the cell, resulting an attenuation in $[Ca^{2+}]_i$.

It has been reported that central administration of kisspeptin-13 to rodents causes nociceptive effects (Spampinato et al. 2011; Elhabazi et al. 2013; Csabafi et al. 2018). The activation of KISS1R has been shown to result in calcium mobilization and recruitment of mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated kinases (ERK) and p38 (Castano et al. 2009). It has been demonstrated that both ERK and p38 contribute to central pain sensitization (Ji et al. 2009). Therefore, kisspeptin seems to exert its nociceptive effects by stimulating ERK1/2 and p38 in the spinal cord dorsal horn neurons or upper brain regions implicated in nociceptive regulation.

Intracellular calcium is one of the most diverse and widespread intracellular second messengers. Increases in $[Ca^{2+}]_i$ play key roles in a variety of neuronal functions such as release of neurotransmitters, neuroplasticity, gene expression, activation of certain types of enzymes and Ca^{2+}-dependent channels and control of neuronal excitability (Berridge 1998), almost all of which are involved in nociceptive events. Thus, the effects of kisspeptin on $[Ca^{2+}]_i$ in DRG neurons may contribute to understanding of its modulatory role in peripheral pain perception.

In summary, our results show for the first time that kisspeptin-10 stimulates calcium influx in cultured DRG neurons using PKC independent mechanism differently from hypothalamic and hippocampal neurons. Although the physiological significance of the data obtained from the present experiment remains to be determined, our results suggest that kisspeptin could modulate somatosensory transmission including nociceptive stimuli *via* non-PKC-mediated mechanisms in the cultured rat sensory neurons.

Conflict of interest. The authors declare no conflict of interest.

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