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Electrophysiological effects of ghrelin on laterodorsal tegmental neurons in rats: An in vitro study

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ABSTRACT

Ghrelin, a gut and brain peptide, is a potent stimulant for growth hormone (GH) secretion and feeding. Recent studies further show a critical role of ghrelin in the regulation of sleep–wakefulness. Laterodorsal tegmental nucleus (LDT), that regulates waking and rapid eye movement (REM) sleep, expresses GH secretagogue receptors (GHS-Rs). Thus, the present study was carried out to examine electrophysiological effects of ghrelin on LDT neurons using rat brainstem slices, and to determine the ionic mechanism involved. Whole cell recording revealed that ghrelin depolarizes LDT neurons dose-dependently in normal artificial cerebrospinal fluid (ACSF). The depolarization persisted in tetrodotoxin-containing ACSF (TTX ACSF), and is partially blocked by the application of [D-Lys^3]-GHRP-6, a selective antagonist for GHS-Rs. Membrane resistance during the ghrelin-induced depolarization increased by about 18% than that before the depolarization. In addition, the ghrelin-induced depolarization was drastically reduced in high-K^+ TTX ACSF with a K^+ concentration of 13.25 mM. Reversal potentials obtained from *I–V* curves before and during the depolarization were about −83 mV, close to the equilibrium potential of the K^+ channel. Most of the LDT neurons recorded were characterized by an A-current or both the A-current and a low threshold Ca^{2+} spike, and they were predominantly cholinergic. These results indicate that ghrelin depolarizes LDT neurons postsynaptically and dose-dependently via GHS-Rs, and that the ionic mechanisms underlying the ghrelin-induced depolarization include a decrease of K^+ conductance. The results also suggest that LDT neurons are implicated in the cellular processes through which ghrelin participates in the regulation of sleep–wakefulness.

1. Introduction

Ghrelin, a natural endogenous ligand of the orphan growth hormone (GH) secretagogue receptor (GHS-R), is a gut and brain peptide [19]. Like synthetic GH secretagogues, ghrelin displays not only strong GH-releasing activity but also orexigenic activity in animals and humans [7,37]. In the peripheral organs, the stomach is the main source of ghrelin [19]; whereas in the brain, ghrelin is produced by neurons in the arcuate nucleus of the hypothalamus (ARC) [19] and by neurons adjacent to the third ventricle between several medial hypothalamic nuclei [4,22]. GHS-R is distributed in the peripheral organs, such as the heart, lung, liver, kidney, pancreas, stomach, intestine, etc. [8,9,12]. Centrally, GHS-R is highly expressed in the pituitary and hypothalamus such as the ARC and ventromedial nucleus of the hypothalamus (VMH) [9,13,23,24,30]. GHS-R is also found in other brain regions, such as the brainstem, hippocampus and area postrema [9,23,44]. These broad distributions of GHS-R in the brain and peripheral organs suggest that ghrelin has multiple physiological functions beyond the control of GH secretion and food intake. Indeed, it has been demonstrated that ghrelin modulates energy homeostasis, stimulates gastric acid secretion and gastric motility, affects cardiovascular functions such as blood pressure and heart rate, and activates or inhibits the release of many hormones such as insulin [7,12,37]. In addition, recent studies further demonstrate that ghrelin may participate in the regulation of sleep–wakefulness. For example, repeated intravenous administration of ghrelin decreased the duration of rapid eye movement (REM) sleep and modified sleep–wake patterns in rats [35]. In humans, systemic administration of ghrelin promoted [40], whereas hexarelin, a more potent ghrelin analogue [6], suppressed non-REM (NREM) sleep.

Cholinergic neurons in the mesopontine tegmentum including the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT) have been involved in not only the maintenance of wakefulness but also the generation of REM sleep

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[3,10,39]. They project into the thalamus through the ascending activating system, and lead to cortical activation; whereas, they project into the pontine reticular formation, and promote muscle atonia by which REM sleep is characterized. Interestingly, GHS-R is expressed in the LDT and PPT [9,23,44], and ghrelin may participate in the regulation of sleep–wakefulness via an action to LDT and PPT neurons. Indeed, we have shown that ghrelin depolarizes PPT neurons postsynaptically and dose-dependently via a dual ionic mechanism including an inhibition of K^+ channel and an activation of non-selective cationic channel [18]. However, the electrophysiological effects of ghrelin on LDT neurons have not been described. Therefore, the present study was carried out to examine the electrophysiological effects on putative LDT cholinergic neurons with a soma size of more than 30 μm in rat brainstem slice preparations, and to determine the ionic mechanism involved. Some LDT neurons that were responsive or non-responsive to ghrelin were also characterized by their electrophysiological membrane properties, and labeled with biocytin and then stained with nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), a reliable marker for mesopontine cholinergic neurons [38].

2. Materials and methods

2.1. Animals

Male Wistar rats at 1–2 weeks of age were used (Sankyo Lab., Shizuoka, Japan). They were housed with their mother rat in a light-controlled room (light on: 06:00–18:00) at a temperature of 23 ± 2 °C for several days before the experiments. Food and water were available ad libitum for mother rats. The experimental procedures used were approved by the Institutional Animal Care and Use Committee of the University of Toyama.

2.2. Slice preparation

Rats were decapitated after sevoflurane anesthesia and the brain was rapidly removed from the skull. The brain was then submerged in ice cold, oxygenated (95% O_2–5% CO_2) artificial cerebrospinal fluid (ACSF), composition in mM: NaCl 126, KCl 3, $CaCl_2$ 2.4, $MgSO_4$ 1.3, KH_2PO_4 1.25, $NaHCO_3$ 26 and glucose 10 at pH 7.4. Frontal brainstem slices 300 μm thick were cut by a microslicer (ZERO 1, Dosaka EM, Kyoto, Japan). The LDT was identified on the basis of its anatomical location relative to the fourth ventricle. One or two slices including the LDT were selected from each animal and cut with a scalpel at the midline to separate the left and right LDT. Finally, two to four slice preparations including LDT were obtained. The slices containing the LDT were then pre-incubated in a chamber with oxygenated ACSF for about 1 h at room temperature.

2.3. Whole cell patch clamp recording

After pre-incubation, slices were transferred into a whole cell patch clamp recording chamber fixed to the stage of an upright microscope (BX-50WL, Olympus, Tokyo, Japan). The recording chamber was perfused with oxygenated ACSF at 1 ml/min and at 32 °C. LDT neurons were monitored on a television screen through an infrared charge coupled device (CCD) camera (C2741-79, Hamamatsu Photonics, Hamamatsu, Japan) and a real-time digital video microscopy processor (XL-20, Olympus, Tokyo, Japan). LDT neurons with a soma size of more than 30 μm were selected for whole cell patch clamp recording. Electrodes were filled with an internal solution containing in mM: K-gluconate 120, KCl 20, HEPES 10, $MgCl_2$ 2.0, $CaCl_2$ 0.5, EGTA 1.0, Na-ATP 4.6, and Na-GTP 0.4, pH adjusted to 7.3 with KOH, and electrode resistances were 5–9 MΩ. Membrane potentials and currents recorded via the electrodes in "I-clamp normal" current clamp and voltage clamp modes, respectively, were fed into a patch clamp amplifier (Axopatch 200B, Axon Instruments, Union City, CA, USA). An Ag/AgCl reference electrode was placed near the intermediate position between the inlet and outlet of the chamber. Series resistance compensation was performed as much as possible by the amplifier. The output of the amplifier was digitized using an A/D converter board (Digidata 1200, Axon Instruments, Union City, USA) with a sampling rate of 20 kHz, and recorded on a hard disk by data acquisition and analysis software (pCLAMP 8, Axon Instruments, Union City, USA). Membrane potentials were low-pass filtered at 5 kHz. Furthermore, membrane potentials less than −60 mV were set to about −60 mV by current injections. Whole cell liquid junction potentials were calculated to be about −13 mV for our internal solutions, and membrane potentials were not corrected.

2.4. Histochemistry

Each slice with a biocytin-filled cell was fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 2 h, and immersed in a solution containing 15% sucrose in 0.1 M PBS for overnight at 4 °C. The sections were rinsed three times for 5 min in 0.1 M PBS containing 0.3% Triton X-100 (PBST, pH 7.4). According to biocytin histochemistry described previously [11], the sections were incubated with avidin-conjugated Texas Red (25 μg/ml) in 0.3% PBST for 3 h at 27 °C, and again rinsed three times for 5 min in 0.3% PBST. Cholinergic neurons were then stained using NADPH-d histochemistry, a reliable marker of mesopontine cholinergic neurons [38]. The sections were incubated in a solution containing β-NADPH (1 mg/ml) and nitroblue tetrazolium (0.1 mg/ml) in 0.3% PBST for 1 h at 37 °C. After the sections were rinsed in 0.3% PBST, they were mounted on glass slides, dehydrated and coverslipped. Finally, Texas Red-positive and NADPH-d-positive neurons were identified under a microscope equipped with both epifluorescence and bright-field optics (BX-51, Olympus, Tokyo, Japan).

2.5. Drugs

Ghrelin was purchased from Peptide Institute (Osaka, Japan). Tetrodotoxin (TTX) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Biocytin and β-NADPH were purchased from Sigma (Tokyo, Japan). All agents were applied extracellularly.

2.6. Statistics

All data were expressed as means ± SEMs. For statistical analysis, two-way analysis of variance (ANOVA) with repeated measures was used. Paired Student's *t*-test, Wilcoxon signed-rank test and Fisher's exact test were also used. $p < 0.05$ was taken as the level of statistical significance.

3. Results

To examine the effect of ghrelin on LDT neurons, whole cell patch clamp recordings were made on acute slice preparations of rats. In total, 103 LDT neurons with a soma size more than 30 μm in the long axis were recorded. This criterion was used because an average diameter in the long axis of LDT cholinergic neurons is 23–30 μm in rats [21,25]. The mean input resistance and mean resting potential in these neurons were 130.4 ± 6.4 MΩ (n = 103) and −48.6 ± 1.3 mV (n = 103), respectively.

3.1. Ghrelin depolarizes LDT neurons postsynaptically and dose-dependently

Ghrelin at 100 nM was applied to 6 LDT neurons in TTX-containing ACSF as well as normal ACSF to test the postsynaptic