Ghrelin Directly Interacts With Neuropeptide-Y–Containing Neurons in the Rat Arcuate Nucleus

Ca\(^{2+}\) Signaling via Protein Kinase A and N-Type Channel-Dependent Mechanisms and Cross-Talk With Leptin and Orexin

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Ghrelin, an endogenous ligand for the growth hormone secretagogue (GHS) receptor (GHSR), is synthesized abundantly in the stomach and to a much lesser extent in the hypothalamic arcuate nucleus (ARC) (1). Peripheral or intracerebroventricular (ICV) injection of ghrelin releases growth hormone, stimulates food intake, and increases body weight in mice, rats, and humans (1–8). ICV injection of antiglucagon IgG suppresses starvation-induced feeding (3). The daily pattern of plasma ghrelin levels in normal humans is characterized by a preprandial rise and postprandial fall (9). These findings have suggested that ghrelin plays a physiological role in the meal initiation.

The neuropeptide-Y (NPY)-containing neurons localized in the ARC have been implicated in the stimulation of food intake—intake of NPY into the hypothalamus of rats potently stimulates food intake (10), and NPY secretion in the hypothalamus is increased during fasting (11). Regarding a possible link between ghrelin and the NPY neurons in the ARC, it has been shown that GHSR mRNA is expressed in 94% of the NPY neurons in the ARC by double-labeling in situ hybridization histochemistry (12). Systemic or ICV administration of ghrelin causes the ARC neurons to express Fos and Egr-1 (3,13–15) and ∼90% of these Fos-positive neurons express NPY mRNA (13). Moreover, ghrelin increases the expression of NPY mRNA (3–5), and ICV administration of a NPY Y1 antagonist suppresses the ghrelin-stimulated food intake (3–5,15). These findings suggest that the NPY neurons in the ARC could be an important effector for the orexigenic action of ghrelin.

The second messenger systems for GHSR have not been fully elucidated. Signaling pathways for artificial GHS that act through GHSR in pituitary cells have been investigated. GHRP-2 (a GHS) increases intracellular cAMP levels in ovine but not rat pituitary somatotropes (16,17). GHRP-6 stimulates GH release from rat pituitary cells by activating protein kinase C (PKC) (17,18). Thus, the post-GHSR pathway could involve Gs or Gq proteins. However, the intracelular signaling for ghrelin's orexigenic action in the effector neurons is not well understood. Therefore, the first aim of the present study was to examine whether ghrelin directly interacts with NPY neurons in the ARC.
and, if so, to explore the signal transduction mechanisms, with special attention toward Ca^{2+} signaling.

Leptin is an adipocyte-derived, potent anorectic peptide. The leptin receptor is present in NPY neurons of the ARC, and leptin exerts its anorectic effect partly through suppression of NPY neurons (19). Coinjection of leptin decreases the stimulatory effect of ghrelin on feeding in free-fed rats (3). Orexin-A and -B are orexigenic peptides produced in cell bodies located in the lateral hypothalamus (20). The orexin-1 receptor is localized in the NPY-containing neurons in the ARC (21) and orexin-induced feeding is inhibited by NPY Y1 and/or Y5 receptor antagonists (22–24), suggesting that orexin could elicit feeding via these NPY neurons. Therefore, the second aim of the present study was to examine whether a subset of the NPY neurons in the ARC could serve as the common target for ghrelin, leptin, and orexins.

We found that ghrelin increases [Ca^{2+}] in 80% of NPY neurons via mechanisms involving protein kinase A (PKA) and N-type Ca^{2+} channels, and that the majority of the ghrelin-activated NPY neurons are also regulated by orexin-A and leptin.

**RESEARCH DESIGN AND METHODS**

**Animals and preparation of single neurons from the ARC.** Adult male Sprague-Dawley (SD) rats were maintained on a 12-h light/dark cycle and given conventional food and water ad libitum. The ARC was isolated from the brain of the 5- to 7-week-old SD rats and then single neurons were prepared according to the procedures reported previously (25,26) with slight modifications. Briefly, rats were anesthetized with an intraperitoneal injection of ketamine (40 mg/kg) and xylazine (10 mg/kg), and their brain was removed. Brain slices containing the ARC were prepared, and the whole ARC was quickly excised and transferred to ice-cold 0.32 M sucrose solution composed of 129 mmol/l NaCl, 5.0 mmol/l NaHCO_3, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.8 mmol/l CaCl_2, 1.2 mmol/l MgSO_4, and 10 mmol/l HEPES at pH 7.4. Ghrelin was provided by Dr. Kangawa and later obtained from Peptide Institute (Osaka, Japan), and calphostin-C and bisindolylmaleimide-I were from Calbiochem (La Jolla, CA). All other chemicals were from Sigma.

**Measurements of [Ca^{2+}] in single ARC neurons.** At 2–12 h after cell preparation, [Ca^{2+}] was measured by radiometric fura-2 microfluorometry in combination with digital imaging as previously reported (25,26). Briefly, following incubation with 2 mmol/l fura-2/AM (Dojin Chemical, Kumamoto, Japan), the cells were kept at 18°C in moisture-saturated dishes for up to 10 h. The animal protocols were approved by the Jichi Medical School Institute of Animal Care and Use Committee and were in accord with the Japanese Physiological Society’s guidelines for animal care.

**RESULTS**

Ghrelin increases [Ca^{2+}] in the ARC neurons, the major populations of which are NPY neurons and...
glucose-sensitive neurons. Single neurons isolated from the ARC were superfused with HKRB and subjected to [Ca\textsuperscript{2+}], measurements with fura-2 fluorescence imaging. The addition of ghrelin at 10\textsuperscript{-10} mol/l for 4–5 min to the superfusion solution increased [Ca\textsuperscript{2+}], in 114 of 330 neurons examined (35%) (Fig. 1A). In randomly selected responsive neurons, the peak [Ca\textsuperscript{2+}], during responses was significantly higher than the basal [Ca\textsuperscript{2+}], (peak [Ca\textsuperscript{2+}]: 485.3 ± 26.2 nmol/l [14] vs. basal [Ca\textsuperscript{2+}]: 90.7 ± 8.8 nmol/l [14], P < 0.001). The ghrelin-induced [Ca\textsuperscript{2+}], increase took place in a long-lasting (7–30 min) manner in the majority of neurons (Fig. 1A). Among 50 neurons that had responded to ghrelin, 48 neurons (96%) were proved to be NPY-containing by subsequent immunocytochemical staining using an anti-NPY antibody (Fig. 1A and C). On the other hand, among 14 neurons that had responded to ghrelin, 8 neurons (58%) exhibited [Ca\textsuperscript{2+}], increases in response to lowering the glucose concentration from 10 to 2.8 mmol/l, thus showing that they were glucose-sensitive neurons (Fig. 1A and B). These results indicate that ghrelin directly targets the NPY neuron and glucose-sensitive neurons in the ARC, and that the former constitutes a dominant population (81%) among the ghrelin-responsive neurons.

Concentration-dependent effects of ghrelin to increase [Ca\textsuperscript{2+}], Ghrerin in a concentration range from 10\textsuperscript{-14} to 10\textsuperscript{-8} mol/l was administered to single ARC neurons. Ghrelin at 10\textsuperscript{-14} mol/l increased [Ca\textsuperscript{2+}], in none of 32 neurons (0%), at 10\textsuperscript{-12} mol/l in 8 of 38 neurons (21%), at 10\textsuperscript{-10} mol/l in 114 of 330 neurons (35%), and at 10\textsuperscript{-8} mol/l in 16 of 39 neurons (41%), showing a concentration-dependent effect (Fig. 3A and B). In a neuron exemplified in Fig. 3A, 10\textsuperscript{-10} mol/l ghrelin induced an oscillatory [Ca\textsuperscript{2+}], increase, and 10\textsuperscript{-8} mol/l ghrelin a sustained [Ca\textsuperscript{2+}], increase with a larger amplitude.

Inhibition of ghrelin-induced [Ca\textsuperscript{2+}], increases under a Ca\textsuperscript{2+}-free condition and by an N-type Ca\textsuperscript{2+} channel blocker. The second addition of ghrelin in each neuron was carried out in the absence of Ca\textsuperscript{2+} or presence of drugs. In a Ca\textsuperscript{2+}-free condition (added with no Ca\textsuperscript{2+} and 0.1 mmol/l EGTA), the [Ca\textsuperscript{2+}], increase in response to ghrelin was abolished in all of seven neurons (Fig. 4A, E, and F), and after bringing Ca\textsuperscript{2+} back to the HKRB, the [Ca\textsuperscript{2+}], response to ghrelin was restored, showing that the inhibition was reversible. The ghrelin-induced [Ca\textsuperscript{2+}], increase was also inhibited by an N-type Ca\textsuperscript{2+} channel blocker, ω-conotoxin GIVA (500 nmol/l), in a reversible manner in 12 of 13 neurons (Fig. 4B and E): the mean amplitude of [Ca\textsuperscript{2+}], increase ([Ca\textsuperscript{2+}], Amp) was significantly reduced compared with that for the response to the second addition of ghrelin without the blocker (control) (51.1 ± 27.7 nmol/l [13] with blocker vs. 357.5 ± 73.3 nmol/l [9] for control, P < 0.005) (Fig. 4F). In contrast, an L-type Ca\textsuperscript{2+} channel blocker, nitrendipine (2 μmol/l), failed to affect the ghrelin-induced [Ca\textsuperscript{2+}], increase in the majority of the neurons (n = 12), while it suppressed the response in a minor fraction of the neurons (n = 5) ([Ca\textsuperscript{2+}], Amp: 259.0 ± 43.2 nmol/l [17]) (Fig. 4C and E). A blocker of endoplasmic reticulum (ER) Ca\textsuperscript{2+} pump, thapsigargin (1 μmol/l), had no effect on the ghrelin-induced [Ca\textsuperscript{2+}], increase ([Ca\textsuperscript{2+}], Amp: 418.5 ± 82.8 nmol/l [9]), while it moderately elevated the basal [Ca\textsuperscript{2+}], (Fig. 4D and E).

Ghrelin-induced [Ca\textsuperscript{2+}], increase is inhibited by an inhibitor of PKA but not PKC. After pretreatment of the neurons with a PKA inhibitor H89 (10 μmol/l), the [Ca\textsuperscript{2+}], response to ghrelin was markedly and significantly inhibited ([Ca\textsuperscript{2+}], Amp: 143.7 ± 34.5 nmol/l [11] after H89 vs. 357.5 ± 73.3 [9] for control, P < 0.05) (Fig. 5A, E, and F). In contrast, H85 (10 μmol/l), a related compound to H89
but without an inhibitory action on PKA, had no significant effect on the ghrelin-induced \([Ca^{2+}]_i\) increase (\([Ca^{2+}]_i\) Amp: 312.2 ± 52.7 nmol/l [12]) (Fig. 5B, E, and F). Neither calphostin-C (100 nmol/l) nor bisindolylmaleimide-I (2 μmol/l), specific PKC inhibitors, affected the ghrelin-induced \([Ca^{2+}]_i\) increase (\([Ca^{2+}]_i\) Amp: 348.5 ± 76.3 nmol/l [10] with calphostin-C; 426.0 ± 148.1 [4] with bisindolylmaleimide-I) (Fig. 5C–E).

**Forskolin and TPA increase \([Ca^{2+}]_i\) in ghrelin-responsive neurons.** An adenylate cyclase activator forskolin (10 μmol/l) increased \([Ca^{2+}]_i\) in 14 of 16 (88%) ghrelin-responsive neurons (\([Ca^{2+}]_i\) Amp: 377.5 ± 36.7 nmol/l [16]) (Fig. 6A and C). A PKC activator TPA (100 nmol/l) also increased \([Ca^{2+}]_i\) in 11 of 17 (65%) ghrelin-responsive neurons (\([Ca^{2+}]_i\) Amp: 337.6 ± 64.8 nmol/l [17]) (Fig. 6B and C). Thus, the ghrelin-responsive neurons highly overlapped with the neurons responding to forskolin and, to a lesser extent, with those responding to TPA. Furthermore, forskolin and TPA increased \([Ca^{2+}]_i\) in a similar pattern to ghrelin.

**Ghrelin and orexin increase \([Ca^{2+}]_i\) in the same neurons.** Ghrelin and orexin at 10^{-10} mol/l, sequentially administered, increased \([Ca^{2+}]_i\) in the same neurons of the ARC in a similar pattern and with comparable amplitudes (Fig. 7A). Among 17 neurons examined, 12 neurons including 8 NPY-containing neurons responded to both ghrelin and orexin-A, 3 neurons responded to ghrelin only, and 2 neurons responded to orexin-A only (Fig. 7B). Thus, ghrelin-responsive neurons extensively overlapped with orexin-responsive neurons (80%) (Fig. 7B).

**Ghrelin-induced \([Ca^{2+}]_i\) increase was counteracted by leptin.** When 10^{-10} mol/l ghrelin was administered for 20 min, \([Ca^{2+}]_i\) increased in a continuous manner in all of eight responsive neurons of the ARC examined (Fig. 8A). The continuous \([Ca^{2+}]_i\) increase was markedly reduced by subsequent administration of 10^{-10} mol/l leptin in 11 of 14
(79%) ghrelin-responsive neurons (Fig. 8B and C). The ghrelin- and leptin-responsive neurons included seven NPY-containing neurons. When 10^{-10} mol/l leptin was administered first and then ghrelin was added, the ghrelin-induced [Ca^{2+}]_i increase was also reduced in three of nine responding neurons, while leptin by itself had little effect on the basal [Ca^{2+}]_i at 10 mmol/l glucose (Fig. 8D).

DISCUSSION
This study presents novel findings on the neuronal signaling mechanisms for the orexigenic action of ghrelin in the hypothalamus. First, we demonstrated that ghrelin concentration-dependently increases [Ca^{2+}]_i in the NPY-containing neurons and the glucose-sensitive neurons of the ARC. The [Ca^{2+}]_i increase often results from depolarization of the plasma membrane and is the key signal for triggering the release of neurotransmitters/hormones and gene expression (27,28). Therefore, the [Ca^{2+}]_i increase is a good indicator of neuronal activation. Therefore, our findings strongly suggest that ghrelin directly activates NPY neurons and glucose-sensitive neurons in the ARC, the neuronal systems implicated in the stimulation of feeding. We previously showed that the majority of the glucose-sensitive neurons in the ARC were NPY neurons (25). Second, the ghrelin-induced [Ca^{2+}]_i increase depends on the Ca^{2+} influx through N-type Ca^{2+} channels and the cAMP-PKA pathway. Third, the target neurons for ghrelin in the ARC extensively overlap with those for orexin and those for leptin.

It has been reported that the concentration of leptin in
the cerebrospinal fluid, measured by radioimmunoassay, is $1.6 \times 10^{-11}$ mol/l and that of orexin is $8 \times 10^{-11}$ mol/l (29). Although the concentration of ghrelin in the cerebrospinal fluid is not available, if ghrelin is transported through the blood-brain barrier at the rate similar to other peptides including leptin (30), the estimated ghrelin concentration in the brain is $\sim 10^{-12}$ mol/l. These values may reflect the physiological concentrations of these peptides in the brain. The concentration of ghrelin, leptin, and orexin used in the present study ($10^{-10}$ mol/l) is close to these values and therefore the effects observed could be physiological.

It was previously shown that GHSR, the ghrelin receptor, was abundant in NPY neurons of the ARC (12) and that the ghrelin-induced food intake was inhibited by an antagonist of the NPY Y1 receptor (3–5,15). These reports suggested an involvement of NPY neurons in the ghrelin signaling pathway and the orexigenic effect. The present study clearly demonstrated that ghrelin directly activates a substantial population (35%) of the ARC neurons and that as high as 80% of these ghrelin-responsive neurons are NPY-containing neurons.

In the pharmacological experiments to study signaling mechanisms for ghrelin, immunocytochemical identification of NPY neurons following [Ca$^{2+}$]i measurements was carried out only in experiments with H89 and ω-conotoxin. However, because we examined 7–17 ghrelin-responsive neurons for each pharmacological experiment and the major population (>80%) of the ghrelin-responsive neurons was proved to be NPY-Containing (Fig. 1), the result obtained may largely, if not solely, reflect that of NPY neurons. Ghrelin-induced [Ca$^{2+}$]i increases were inhibited under a Ca$^{2+}$-free condition and by a blocker of N-type Ca$^{2+}$ channels, while it was slightly suppressed, if at all, by a blocker of L-type Ca$^{2+}$ channels. Thapsigargin, an inhibitor of the Ca$^{2+}$ pump in ER and consequently the Ca$^{2+}$ release from ER, had no effect on ghrelin-induced [Ca$^{2+}$]i increases. The results with N-type Ca$^{2+}$ channel blocker were obtained mainly from immunocytochemically identified NPY neurons. These data indicate that ghrelin increases [Ca$^{2+}$], mainly via Ca$^{2+}$ influx through N-type Ca$^{2+}$ channels. It has been shown that N-type Ca$^{2+}$ channels are involved in the release of NPY stimulated by high potassium (31). It was reported that the NPY Y2 receptor in NPY neurons is linked to a decrease of Ca$^{2+}$ currents due to inhibition of N-type Ca$^{2+}$ channels (32). Thus, N-type Ca$^{2+}$

FIG. 6. Forskolin and TPA increase [Ca$^{2+}$], in ghrelin-responsive neurons: 10 μmol/l forskolin, an adenylate cyclase activator (A), and 100 nmol/l TPA, a PKC activator (B), increased [Ca$^{2+}$], in ghrelin-responsive neurons. C: The incidence of [Ca$^{2+}$], responses to forskolin and TPA in ghrelin-responsive neurons is expressed as the percentage.

FIG. 7. Ghrelin and orexin-A increase [Ca$^{2+}$] in the same NPY neurons. A: Ghrelin-responsive neurons also exhibited [Ca$^{2+}$], responses to 10–10 mol/l orexin-A. B: Twelve neurons responded to both ghrelin and orexin, 3 neurons to ghrelin only, and 2 neurons to orexin only among the 17 neurons that responded to either of the two peptides. The ghrelin- and orexin-responsive neurons included eight NPY-containing neurons.
channels could play a pivotal role in the regulation of Ca\(^{2+}\) influx and [Ca\(^{2+}\)]\(_i\) in NPY neurons. The second messenger systems for ghrelin in the NPY neurons have not been adequately elucidated. In the present study, an adenylate cyclase activator forskolin and a PKC activator TPA increased [Ca\(^{2+}\)]\(_i\), indicating that both cAMP-PKA and PKC pathways are capable of activating Ca\(^{2+}\) signaling. Furthermore, a specific PKA inhibitor, H89, but not a nonspecific inhibitor, H85, and PKC inhibitors significantly suppressed the ghrelin-induced [Ca\(^{2+}\)]\(_i\) increase. The results with the PKA inhibitor were obtained mainly from immunocytochemically identified NPY neurons. These results reveal that PKA is required for ghrelin to activate Ca\(^{2+}\) signaling in the NPY neurons of the ARC. There are two possible explanations for the role of PKA. First, the basal activity of PKA may be required for ghrelin to produce Ca\(^{2+}\) signaling. Second, the ghrelin-GHSR system may activate the Gs-adenylate cyclase-cAMP-PKA cascade, which in turn leads to the Ca\(^{2+}\) influx and [Ca\(^{2+}\)]\(_i\) increase. The PKA-mediated facilitation of the Ca\(^{2+}\) influx and [Ca\(^{2+}\)]\(_i\) increase have been indicated in the cardiac muscle and in pancreatic β-cells (28,33). It has been shown that PKA is indispensable for CREB phosphorylation and cAMP response element-mediated gene expression in the NPY neurons in the fasted state (34). Ghrelin could couple fasting to the activation of PKA because the release of this peptide is greatly stimulated by fasting (9). These findings in the present study and by others indicate that PKA is important for the ghrelin-induced activation of NPY neurons. Whether the ghrelin-induced Ca\(^{2+}\) signaling in NPY neurons depends on the basal PKA activity or requires activation of PKA by this peptide remains to be clarified. In the present study, ghrelin and orexin increased [Ca\(^{2+}\)]\(_i\) in the same neurons of the ARC. The majority of the ghrelin-responsive neurons (80%) also responded to orexin, and vice versa (86%), showing that the two populations of responders highly overlapped. The ghrelin- and orexin-responsive neurons included eight NPY-containing neurons. The orexin-1 receptor is reportedly expressed on orexin-responsive neurons included eight NPY-containing neurons. The ghrelin- and orexin-responsive neurons included seven NPY-containing neurons. D: Leptin (10\(^{-10}\) mol/l), added in advance, suppressed [Ca\(^{2+}\)]\(_i\) responses to subsequent administration of ghrelin (n = 3).
interacts with NPY neurons in the ARC to elicit Ca\(^{2+}\) signaling via PKA and N-type Ca\(^{2+}\) channel-dependent mechanisms. This could serve as the major neuronal signaling mechanism by which ghrelin stimulates NPY release and thereby feeding. The ghrelin-responsive neurons are highly overlapped with orexin- and leptin-responsive neurons. The integration of stimulatory effects of ghrelin and orexin and inhibitory effect of leptin may play an important role in the regulation of the activity of NPY neurons and thereby feeding.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (12470231) and grants from the Japan Diabetes Foundation, the National Cardiovascular Research Institute, and the Asahi Life Foundation Adult Diseases Institute (to T.Y.).

The authors thank Drs. K. Kangawa and M. Nakazato for providing ghrelin and for helpful discussions.

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