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 is a newly discovered peptide that is released from the stomach and from neurons in the hypothalamic arcuate nucleus (ARC) and potently stimulates growth hormone release and food intake. Neuropeptide-Y (NPY) neurons in the ARC play an important role in the stimulation of food intake. The present study aimed to determine whether ghrelin directly activates NPY neurons and, if so, to explore its signaling mechanisms. Whether the neurons that respond to ghrelin could be regulated by orexin and leptin was also examined. We isolated single neurons from the ARC of rats and measured the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) with fura-2 fluorescence imaging. Ghrelin (10^{-12} to 10^{-8} mol/l) concentration-dependently increased $[Ca^{2+}]_i$, which occurred in 35% of the ARC neurons. Approximately 80% of these ghrelin-responsive neurons were proved to be NPY-containing by immunocytochemical staining, and 58% of them were glucose-sensitive neurons as judged by their responses to lowering glucose concentrations. The $[Ca^{2+}]_i$ responses to ghrelin were markedly attenuated by inhibitors of protein kinase A (PKA) but not protein kinase C and by a blocker of N-type but not L-type Ca^{2+} channels. Orexin increased $[Ca^{2+}]_i$ and leptin attenuated ghrelin-induced $[Ca^{2+}]_i$ increases in the majority (80%) of ghrelin-responsive NPY neurons. These results demonstrate that ghrelin directly interacts with NPY neurons in the ARC to induce Ca^{2+} signaling via PKA and N-type Ca^{2+} channel-dependent mechanisms. 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. *Diabetes* 52:948–956, 2003

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ARC, arcuate nucleus; ER, endoplasmic reticulum; GHS, growth hormone secretagogue; GHSR, GHS receptor; HKRB, HEPES-buffered Krebs-Ringer bicarbonate buffer; ICV, intracerebroventricular; NPY, neuropeptide-Y; PKA, protein kinase A; PKC, protein kinase C; TPA, 12-0-tetradecanoylphorbol-13-acetate.

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 antighrelin 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—injection 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 and/or Gq proteins. However, the intracellular 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

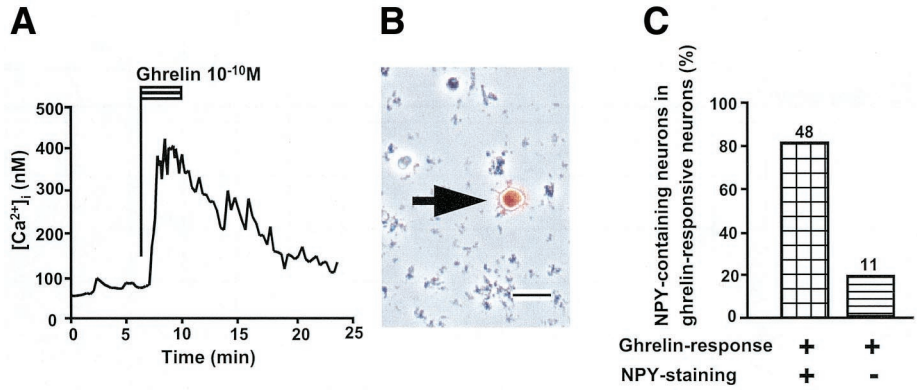

FIG. 1. Ghrelin increases $[Ca^{2+}]_i$ in NPY neurons of the ARC. *A* and *B*: Ghrelin at 10^{-10} mol/l increased $[Ca^{2+}]_i$ in a single ARC neuron (*A*), which was proved to be NPY-containing by subsequent immunocytochemical staining with an anti-NPY antibody (arrow) (*B*). Scale bar, 20 μm. The bar above the tracing specifies the period of exposure to ghrelin. Glucose concentration was 10 mmol/l throughout the measurement. *C*: Among 59 neurons that exhibited $[Ca^{2+}]_i$ increases in response to ghrelin, 48 neurons (81%) were NPY-containing neurons.

and, if so, to explore the signal transduction mechanisms, with special attention toward Ca^{2+} signaling.

Leptin is an adipocyte-derived, potent anorexic peptide. The leptin receptor is present in NPY neurons of the ARC, and leptin exerts its anorexic 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 $[Ca^{2+}]_i$ 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 carbamic acid ethyl ester (900 mg/kg) and decapitated, and their brain was removed. Brain slices containing the ARC were prepared, and the whole ARC of the left and right sides was punched out. The dissected tissues were washed with 10 mmol/l HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) containing 10 mmol/l glucose. Then they were incubated in HKRB supplemented with 20 units/ml papain (Sigma Chemical, St. Louis, MO), 0.015 mg/ml deoxyribonuclease, and 0.75 mg/ml BSA for 15 min at 36°C in a shaking water bath, followed by gentle mechanical trituration for 5–10 min. After trituration, the cell suspension was centrifuged at 100*g* for 5 min. The pellet was resuspended in HKRB and distributed onto coverslips. 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.

Measurements of $[Ca^{2+}]_i$ in single ARC neurons. At 2–12 h after cell preparation, $[Ca^{2+}]_i$ was measured by radiometric fura-2 microfluorometry in combination with digital imaging as previously reported (25,26). Briefly, following incubation with 2 μmol/l fura-2/AM (Dojin Chemical, Kumamoto, Japan) for 1 h at 18°C, the cells were mounted in a chamber and superfused with HKRB at 1 ml/min at 33°C. Fluorescence images due to excitation at 340 and 380 nm were detected every 8.0 s with an intensified charge-coupled device (ICCD) camera, and the ratio image was produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to $[Ca^{2+}]_i$ according to calibration curves (27). All agents were dissolved in DMSO or distilled water in stock and diluted in the superfusate (HKRB) at ≤1:1,000. In control experiments, 0.1% DMSO administered alone had no effect on $[Ca^{2+}]_i$ in the ARC neurons.

Data were taken from the cells that were identified as neurons by the procedures reported previously (25,26). Briefly, the single cells that were immunoreactive for microtubule-associated protein-2, a neuron-specific marker, showed a relatively large diameter (≥10 μm) with clear and round nuclei under a phase-contrast microscope. Data were taken from the cells that fulfilled these criteria for neurons.

Immunocytochemistry and identification of NPY neurons. After the $[Ca^{2+}]_i$ measurement, the cells were fixed with 4% paraformaldehyde overnight. They were incubated first with a rabbit antiserum against NPY (DiaSorin, Stillwater, MN) at a dilution of 1:10,000 for 24 h at 4°C followed by biotinylated antibodies raised against rabbit IgG (Vector Laboratories, Burlingame, CA; diluted 200-fold) for 1 h, and the avidin-peroxidase complex (Vector) for 1 h. The sections were developed with 3,3′-diaminobenzidine. Control experiments were carried out by omitting the primary antiserum.

Correlation of $[Ca^{2+}]_i$ and immunocytochemical data were performed by the procedure reported previously (25–27). In brief, at the end of $[Ca^{2+}]_i$ imaging, we took phase-contrast photographs of all the cells in the microscopic field subjected to $[Ca^{2+}]_i$ measurements. Based on these photographs, the cells in which $[Ca^{2+}]_i$ was recorded were correlated with their corresponding immunocytochemical results.

Criteria for $[Ca^{2+}]_i$ responses and their inhibition and determination of the response amplitude. Ghrelin, forskolin, 12-0-tetradecanoylphorbol-13-acetate (TPA), and orexin were administered for 4–5 min or 20 min to the superfusion solution. Amplitudes of $[Ca^{2+}]_i$ increases in response to agents were calculated by subtracting prestimulatory basal $[Ca^{2+}]_i$ levels from peak $[Ca^{2+}]_i$ levels. When increases in $[Ca^{2+}]_i$ took place within 5 min after addition of agents and their amplitudes were 150 nmol/l or larger, they were considered responses. Repetitive additions of ghrelin twice induced repeated $[Ca^{2+}]_i$ increases. Therefore, to test the effects of drugs, the second challenge to ghrelin was performed in the presence of drugs. Alternatively, pretreatment of neurons with drugs was performed before the second challenge to ghrelin. When the amplitude of the $[Ca^{2+}]_i$ response to the second addition of ghrelin was reduced to ≤40% compared with the control $[Ca^{2+}]_i$ response to the first addition of ghrelin without drugs, it was considered inhibition.

Solutions and chemicals. The measurements were carried out in HKRB 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). ω-Conotoxin GVIA and orexin-A were obtained from Peptide Institute. Fura 2-acetoxymethylester was from Dojin Chemical (Kumamoto, Japan), nitrendipine was from Yoshitomi Pharmaceutical Industries (Osaka, Japan), H89 and H85 were from Seikagaku (Tokyo, Japan), and calphostin-C and bisindolylmaleimide-I were from Calbiochem (La Jolla, CA). All other chemicals were from Sigma.

Data presentation and statistical analysis. The data are presented as the mean ± SE (n = number of neurons). Each study was based on at least seven neurons prepared from at least three rats. A total 535 neurons were examined. Student's paired or unpaired t test was used to evaluate differences, and values of $P < 0.05$ were considered significant.

RESULTS

Ghrelin increases $[Ca^{2+}]_i$ in the ARC neurons, the major populations of which are NPY neurons and

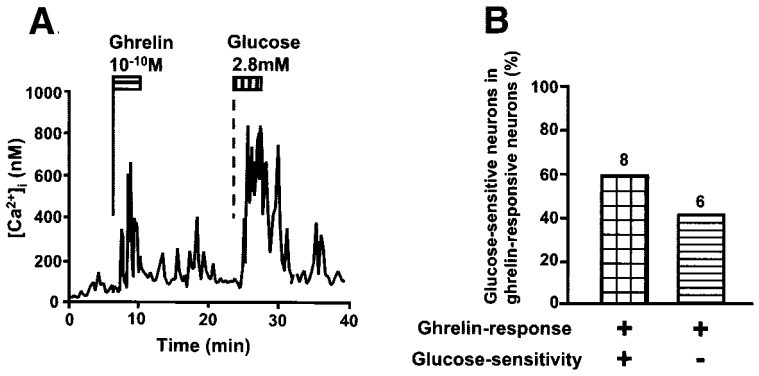

FIG. 2. Ghrelin increases $[Ca^{2+}]_i$ in glucose-sensitive neurons of the ARC. *A*: Ghrelin-responsive neurons exhibited $[Ca^{2+}]_i$ increases in response to lowering the glucose concentration from 10 to 2.8 mmol/l, showing that they were glucose-sensitive neurons. The result shown is representative of eight neurons of the ARC (n = 8). The bars above the tracing specify the periods of exposure to the agents indicated. Unless otherwise specified, the glucose concentration was 10 mmol/l. *B*: Among 14 neurons that had responded to ghrelin, 8 neurons (57%) were glucose-sensitive neurons.

glucose-sensitive neurons. Single neurons isolated from the ARC were superfused with HKRB and subjected to $[Ca^{2+}]_i$ measurements with fura-2 fluorescence imaging. The addition of ghrelin at 10^{-10} mol/l for 4–5 min to the superfusion solution increased $[Ca^{2+}]_i$ in 114 of 330 neurons examined (35%) (Fig. 1*A*). In randomly selected responsive neurons, the peak $[Ca^{2+}]_i$ during responses was significantly higher than the basal $[Ca^{2+}]_i$ (peak $[Ca^{2+}]_i$: 485.3 ± 26.2 nmol/l [14]) vs. basal $[Ca^{2+}]_i$: 90.7 ± 8.8 nmol/l [14]), $P < 0.001$). The ghrelin-induced $[Ca^{2+}]_i$ increase took place in a long-lasting (7–30 min) manner in the majority of neurons (Fig. 1*A*). Among 59 neurons that had responded to ghrelin, 48 neurons (81%) were proved to be NPY-containing by subsequent immunocytochemical staining using an anti-NPY antibody (Fig. 1*A* and *C*). On the other hand, among 14 neurons that had responded to ghrelin, 8 neurons (58%) exhibited $[Ca^{2+}]_i$ increases in response to lowering the glucose concentration from 10 to 2.8 mmol/l, thus showing that they were glucose-sensitive neurons (Fig. 2*A* and *B*). These results indicate that ghrelin directly targets the NPY neuron and glucose-sensitive neuron in the ARC, and that the former constitutes a dominant population (81%) among the ghrelin-responsive neurons.

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

Inhibition of ghrelin-induced $[Ca^{2+}]_i$ increases under a Ca^{2+}-free condition and by an N-type Ca^{2+} channel blocker. The second addition of ghrelin in each neuron was carried out in the absence of Ca^{2+} or presence of drugs. In a Ca^{2+}-free condition (added with no Ca^{2+} and 0.1 mmol/l EGTA), the $[Ca^{2+}]_i$ increase in response to ghrelin was abolished in all of seven neurons (Fig. 4*A*, *E*, and *F*), and after bringing Ca^{2+} back to the HKRB, the $[Ca^{2+}]_i$ response to ghrelin was restored, showing that the inhibition was reversible. The ghrelin-induced $[Ca^{2+}]_i$ increase was also inhibited by an N-type Ca^{2+} channel blocker, ω-conotoxin GIVA (500 nmol/l), in a reversible manner in 12 of 13 neurons (Fig. 4*B* and *E*): the mean amplitude of $[Ca^{2+}]_i$ increase ($[Ca^{2+}]_i$ 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. 4*F*). In contrast, an L-type Ca^{2+} channel blocker, nitrendipine (2 μmol/l), failed to affect the ghrelin-induced $[Ca^{2+}]_i$ increase in the majority of the neurons (n = 12), while it suppressed the response in a minor fraction of the neurons (n = 5) ($[Ca^{2+}]_i$ Amp: 259.0 ± 43.2 nmol/l [17]) (Fig. 4*C* and *E*). A blocker of endoplasmic reticulum (ER) Ca^{2+} pump, thapsigargin (1 μmol/l), had no effect on the ghrelin-induced $[Ca^{2+}]_i$ increase ($[Ca^{2+}]_i$ Amp: 418.5 ± 82.8 nmol/l [9]), while it moderately elevated the basal $[Ca^{2+}]_i$ (Fig. 4*D* and *E*).

Ghrelin-induced $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ response to ghrelin was markedly and significantly inhibited ($[Ca^{2+}]_i$ Amp: 143.7 ± 34.5 nmol/l [11] after H89 vs. 357.5 ± 73.3 [9] for control, $P < 0.05$) (Fig. 5*A*, *E*, and *F*). In contrast, H85 (10 μmol/l), a related compound to H89

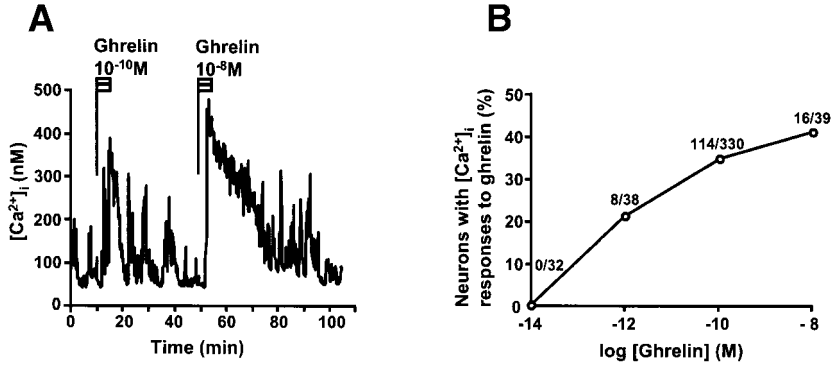

FIG. 3. Concentration-dependent effects of ghrelin to increase $[Ca^{2+}]_i$ in the ARC neurons. *A*: Sequential addition of ghrelin at 10^{-10} mol/l and 10^{-8} mol/l increased $[Ca^{2+}]_i$ in an ARC neuron. *B*: Concentration-response relationship. Response is expressed as the percentage of cells that exhibited $[Ca^{2+}]_i$ increases in response to ghrelin. The numbers above each point indicate the number of neurons that responded over the number of neurons examined.

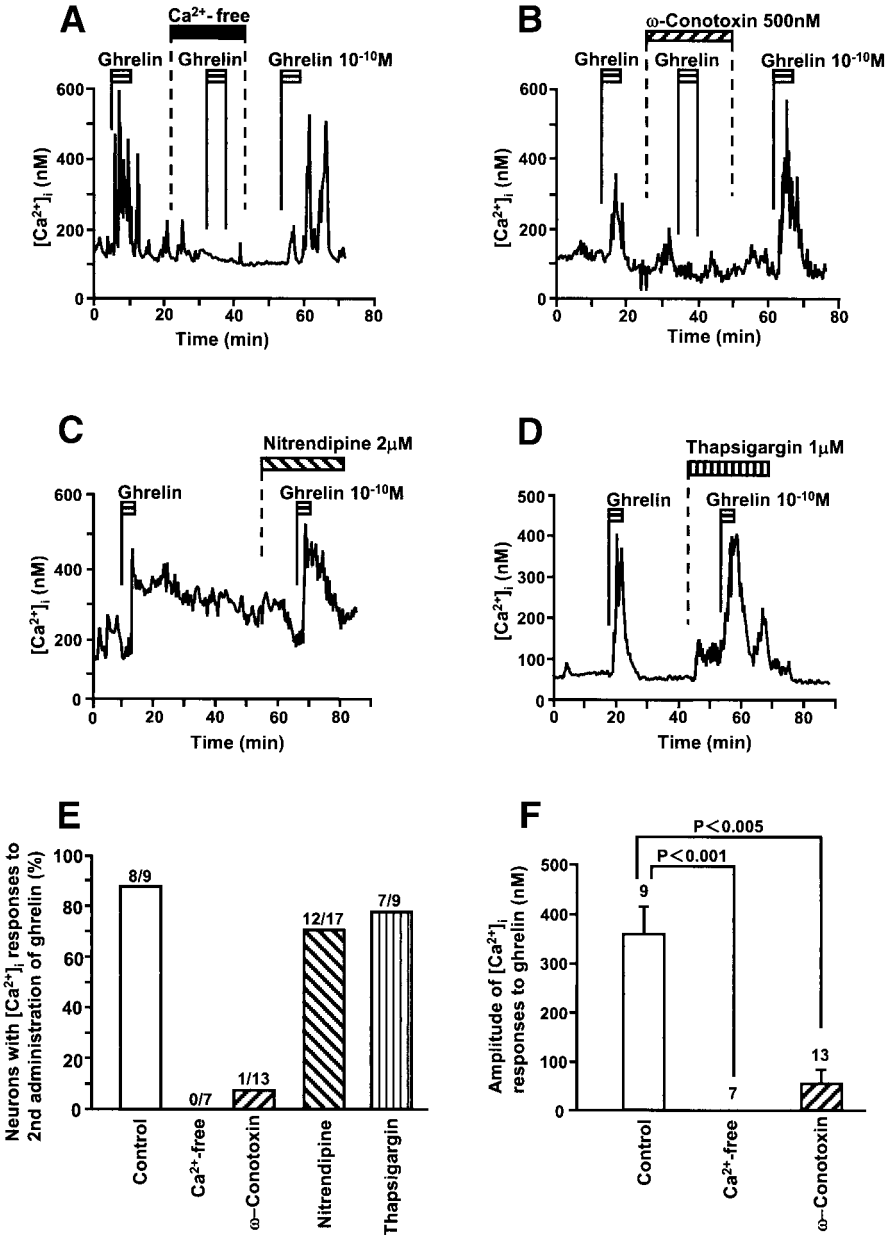

FIG. 4. Ghrelin-induced $[Ca^{2+}]_i$ increases are inhibited under a Ca^{2+}-free condition and by a blocker of N-type Ca^{2+} channels. *A–D*: Ghrelin-induced $[Ca^{2+}]_i$ increases were abolished under a Ca^{2+}-free condition (*A*) ($n = 7$). They were markedly inhibited by ω-conotoxin GVIA (*B*), a blocker of N-type Ca^{2+} channels ($n = 12$ including 8 NPY-containig neurons), while they were only slightly suppressed or unaltered by nitrendipine (*C*), a blocker of L-type Ca^{2+} channels. Thapsigargin (*D*), an inhibitor of the Ca^{2+} pump in the ER, failed to significantly affect ghrelin-induced $[Ca^{2+}]_i$ increases. *E*: Incidence of response under control and test conditions is expressed as the percentage of cells that exhibited $[Ca^{2+}]_i$ responses to the second addition of ghrelin in each cell. The numbers above each bar indicate the number of neurons that responded over the number of neurons examined. *F*: The amplitude of ghrelin-induced $[Ca^{2+}]_i$ increases under control conditions, Ca^{2+}-free conditions, and those with ω-conotoxin. The number above each bar indicates the number of neurons examined. $P < 0.001$ between control and Ca^{2+}-free groups, and $P < 0.005$ between control and ω-conotoxin groups.

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. 5*B*, *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. 5*C–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. 6*A* 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. 6*B* 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. 7*A*). 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. 7*B*). Thus, ghrelin-responsive neurons extensively overlapped with orexin-responsive neurons (80%) (Fig. 7*B*).

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. 8*A*). The continuous $[Ca^{2+}]_i$ increase was markedly reduced by subsequent administration of 10^{-10} mol/l leptin in 11 of 14

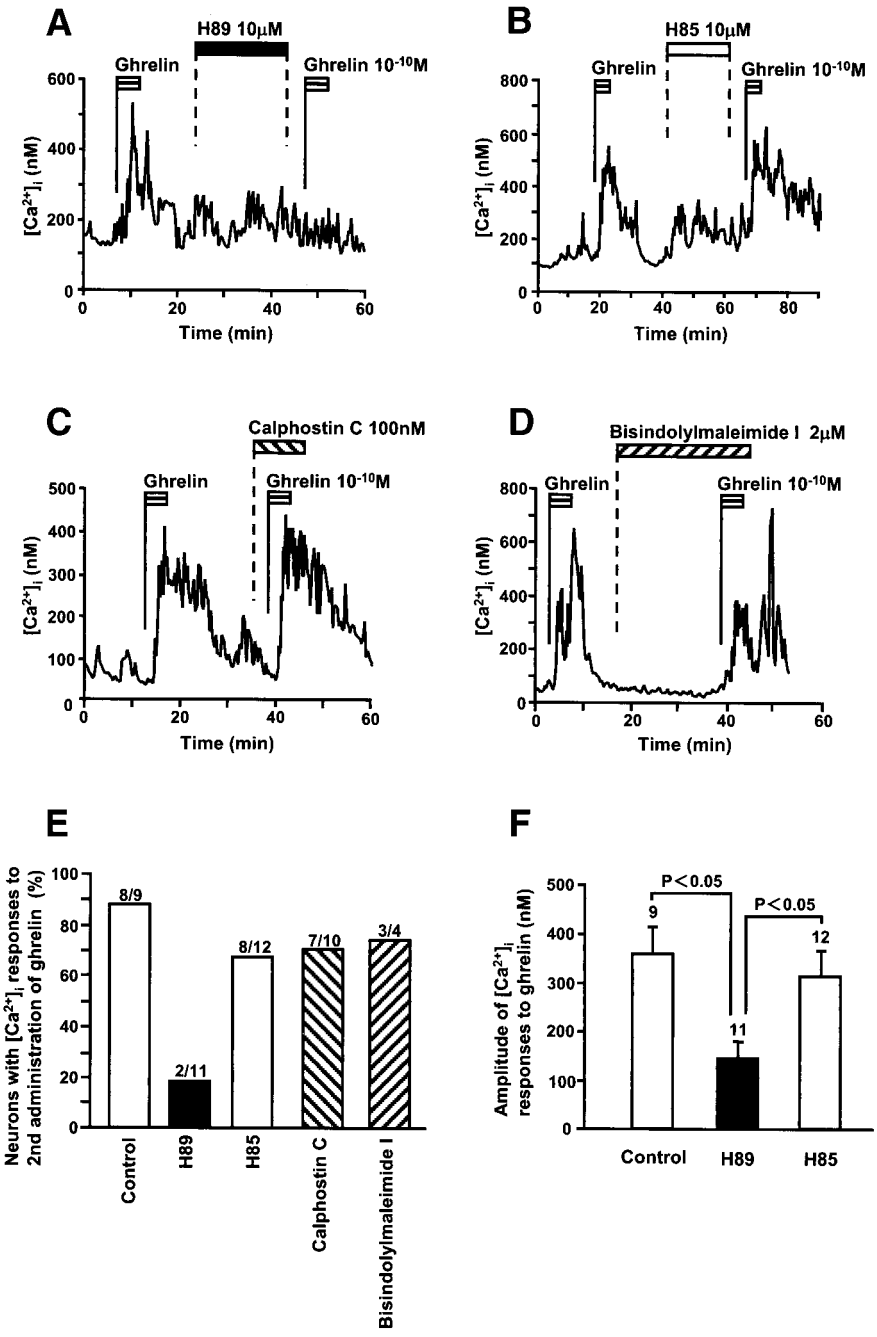

FIG. 5. Ghrelin-induced $[Ca^{2+}]_i$ increases are inhibited by a PKA inhibitor. Pretreatment with 10 μmol/l H89, a PKA inhibitor, inhibited ghrelin-induced $[Ca^{2+}]_i$ increases in the ARC neurons (n = 9 including 7 NPY-containing neurons) (*A*), whereas pretreatment with 10 μmol/l H85, an analog of H89 without an inhibitory action on PKA, had little effect (*B*). Neither 100 nmol/l calphostin-C (*C*) nor 2 μmol/l bisindolylmaleimide-I (*D*), specific PKC inhibitors, affected ghrelin-induced $[Ca^{2+}]_i$ increases. *E*: The incidence of responses under control conditions and those with inhibitors is expressed as a percentage of the cells that exhibited $[Ca^{2+}]_i$ responses to the second addition of ghrelin in each cell. *F*: The amplitude of ghrelin-induced $[Ca^{2+}]_i$ increases under control conditions and those pretreated with H89 and H85. $P < 0.05$ between control and H89 groups and between H89 and H85 groups. No significant difference between control and H85 groups.

(79%) ghrelin-responsive neurons (Fig. 8*B* 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. 8*D*).

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

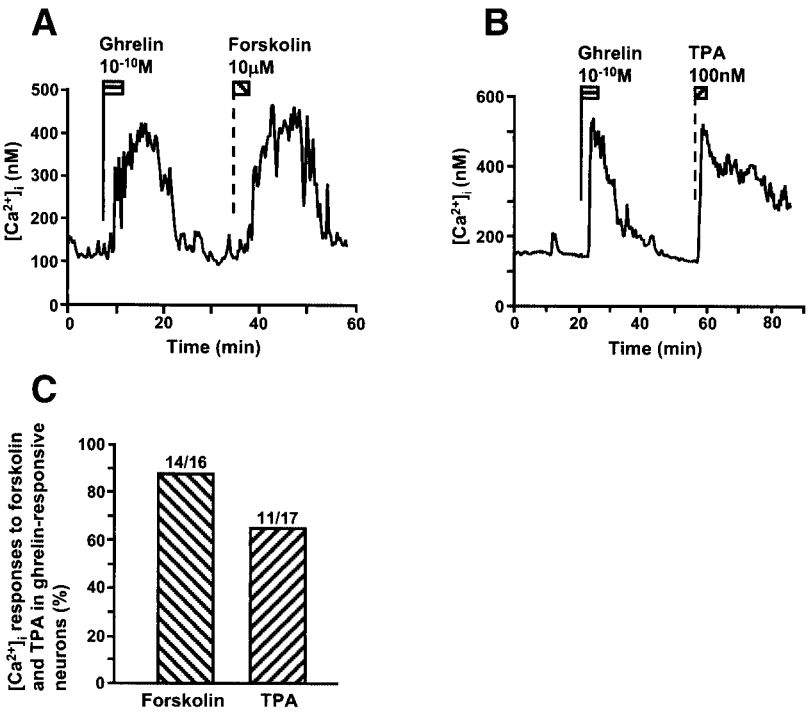

FIG. 6. Forskolin and TPA increase $[Ca^{2+}]_i$ in ghrelin-responsive neurons: 10 μmol/l forskolin, an adenylate cyclase activator (*A*), and 100 nmol/l TPA, a PKC activator (*B*), increased $[Ca^{2+}]_i$ in ghrelin-responsive neurons. *C*: The incidence of $[Ca^{2+}]_i$ responses to forskolin and TPA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

the cerebrospinal fluid, measured by radioimmunoassay, is 1.6×10^{-11} mol/l and that of orexin is 8×10^{-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 $[Ca^{2+}]_i$ 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+}

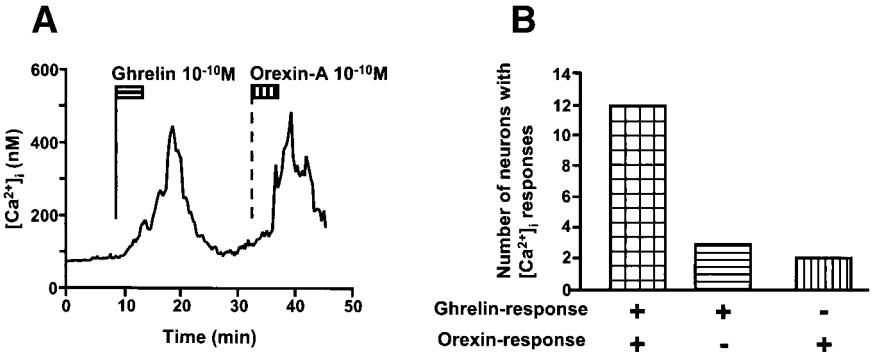

FIG. 7. Ghrelin and orexin-A increase $[Ca^{2+}]_i$ in the same NPY neurons. *A*: Ghrelin-responsive neurons also exhibited $[Ca^{2+}]_i$ responses to 10^{-10} mol/l orexin-A. *B*: Twelve neurons responded to both ghrelin and orexin, 3 neurons to ghrelin only, and 2 neurons to orexin only among the 17 neurons that responded to either of the two peptides. The ghrelin- and orexin-responsive neurons included eight NPY-containing neurons.

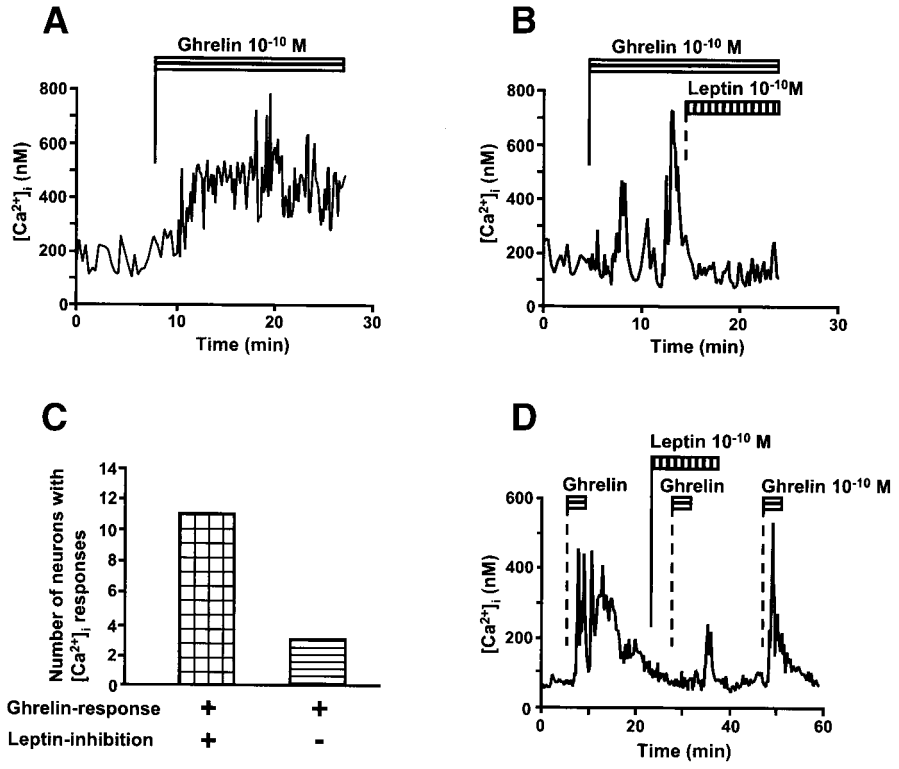

FIG. 8. Ghrelin increases $[Ca^{2+}]_i$ and leptin suppresses it in the same NPY neurons. *A*: Ghrelin, administered alone for 20 min, increased $[Ca^{2+}]_i$ continuously in ARC neurons (n = 8). *B* and *C*: Leptin (10^{-10} mol/l) suppressed ghrelin-induced $[Ca^{2+}]_i$ increases in 11 of 14 ghrelin-responsive neurons. The ghrelin- and leptin-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).

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 vise 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 >90% of NPY neurons (35). Our results clearly demonstrate the presence of a subset of NPY neurons that serves as the common target for ghrelin and orexin. It has been shown that fasting and lowering glucose concentrations increase the level of ghrelin (9) and activate orexin neurons (26,36), suggesting that the release of the two peptides are regulated in parallel. Our finding indicates that ghrelin and orexin may also cooperate at their common effector site, the NPY neurons in the ARC, which could result in an efficient stimulation of the NPY-mediated neural signaling and feeding.

We found that leptin suppresses the ghrelin-induced $[Ca^{2+}]_i$ increase in the majority of the ghrelin-responsive neurons (79%). The ghrelin- and leptin-responsive neurons included seven NPY-containing neurons. Thus, the NPY neurons in the ARC are regulated reciprocally by ghrelin and leptin. This is in accord with the report that 57% of the Fos-positive cells in the ventromedial ARC due to intraperitoneal injection of ghrelin were also immunoreactive for the leptin receptor (37). The recently reported action of leptin to antagonize cAMP elevation (38–40) is a possible mechanism by which leptin counteracts the effect of ghrelin that is dependent on cAMP-PKA as demonstrated in the present study. The action of leptin could also involve phosphoinositide 3-kinase (38–41). Furthermore, we found that the orexin-induced $[Ca^{2+}]_i$ increase in NPY neurons of the ARC is inhibited by leptin (T.Y., personal communications). Thus, NPY neurons are regulated directly and cooperatively by ghrelin, orexin, and leptin, the important regulators of feeding.

In conclusion, our data demonstrate that ghrelin directly

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.

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