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Ghrelin modulates electrical activity of area postrema neurons

Mark Fry and Alastair V. Ferguson

Department of Physiology, Queen's University, Kingston, Ontario, Canada

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Fry M, Ferguson AV. Ghrelin modulates electrical activity of area postrema neurons. *Am J Physiol Regul Integr Comp Physiol* 296: R485–R492, 2009. First published December 31, 2009; doi:10.1152/ajpregu.90555.2008.—Ghrelin, a peptide hormone secreted from the stomach, is known to have a potent appetite-stimulating activity. Recently, it has been shown that area postrema (AP), a caudal brain stem center that lacks a blood-brain barrier, is a key site of activity for ghrelin in stimulating appetite and regulating pancreatic protein secretion. In this study, we have examined the ability of ghrelin to regulate the electrical activity of area postrema neurons using patch-clamp electrophysiology. Using current-clamp configuration, we found that at a concentration of 10 nM, ghrelin caused inhibition in 19% of neurons tested, while a further 19% were excited by similar application of ghrelin. The remaining 62% of AP neurons were insensitive to ghrelin. These effects were concentration dependent, with an apparent EC_{50} of 1.9 nM. Voltage-clamp recordings revealed that ghrelin caused a potentiation of voltage-gated K^+ currents in neurons that exhibited a hyperpolarization and a potentiation of a depolarizing nonspecific cation current (NSCC) in those neurons that exhibited a depolarization of membrane potential. These are the first data showing that ghrelin exerts a direct effect on electrical activity of AP neurons and supports the notion that ghrelin can act via the AP to regulate energy homeostasis.

patch clamp; action potential; sensory circumventricular organ; energy homeostasis

GHRELIN IS AN ACYLATED 28-amino acid peptide hormone, which was discovered on the basis of its activity as an endogenous ligand for the growth hormone secretagogue receptor (GHSR) (35). Originally isolated from the stomach, ghrelin has also been shown to be expressed in various tissues, including the duodenum, adrenal gland, lung, and gonads (22), as well as in neurons in the hypothalamus (11). In addition to its role in the stimulation of growth hormone release in the pituitary, ghrelin is thought to play several roles in the regulation of energy balance. Circulating levels of ghrelin are increased in the fasting state and in anticipation of food (2, 12, 13). Conversely, ghrelin levels are attenuated by feeding and the presence of nutrients in the stomach (58), or by treatment with leptin (2). Administration of ghrelin potently stimulates food intake in rats (58, 64, 65), mice (2), and humans (63). These studies suggest a physiological role in initiation of feeding. Ghrelin is also suggested to play a role in long-term regulation of energy balance, as chronic administration of ghrelin causes weight gain by reducing fat utilization as an energy source (58). Lastly, ghrelin also plays a role in the digestion of food and the stimulation of gastric motility, acid secretion, and pancreatic protein secretion (38, 41).

There is considerable interest as to the mechanism by which ghrelin exerts its appetite-stimulating effects, and, in particular, the site of action of ghrelin in the central nervous system (CNS). For example, the neuropeptide Y/Agouti-related peptide (NPY/AGRP) neurons within the arcuate nucleus of the hypothalamus (ARC) are suggested by many to be the primary location for the orexigenic activity of ghrelin (10). Microinjections into the ARC and intracerebroventricular injections of ghrelin strongly stimulated weight gain and feeding in rats and activated NPY and AGRP neurons within the ARC, as determined by c-Fos immunolabeling (3, 45, 64). Ghrelin receptors are highly localized on NPY neurons, and direct application of the hormone in patch-clamp experiments increased their electrical activity (11). Ablation of the ARC resulted in loss of appetite-stimulating effects (57), as did double knockout of NPY and AGRP (8). Importantly, peripherally injected ghrelin increased c-Fos immunolabeling in the paraventricular nucleus and ARC (28, 37, 61), leading to the suggestion that ghrelin gains access to the hypothalamus via a weak blood-brain barrier (BBB) or specialized transport mechanism at the ARC (3, 30, 33; however, see Ref. 4).

Caudal brain stem has also been proposed as a key site of ghrelin's appetite-stimulating action. The GHSR is expressed in the area postrema (AP), the nucleus of the solitary tract (NTS), and the dorsal motor nucleus of the vagus (70). Injection of ghrelin into the NTS or nearby areas stimulated feeding at doses below the threshold required for forebrain injections (17). Many investigators observe that peripheral administration of ghrelin also caused increases in caudal brain stem c-Fos immunoreactivity (24, 37, 38, 56), although such changes have not been reported in all studies (34). Moreover, recent observations by Gilg and Lutz (21) have convincingly demonstrated a role for AP in ghrelin-mediated feeding. They observed that ablation of AP eliminated ghrelin-induced hyperphagia (although an increase in body weight was still observed, perhaps owing to ghrelin's ability to reduce fat utilization). However, whereas ghrelin was observed to modulate electrical activity of ARC neurons using the patch-clamp technique (11), such direct evidence for modulation of caudal brain stem neurons is lacking.

Address for reprint requests and other correspondence: A. V. Ferguson, Queen's Univ., Dept. of Physiology, Botterell Hall, 4th Fl., Kingston, ON Canada K7L 3N6 (e-mail: avf@queensu.ca).

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Whether ghrelin can directly modulate electrical activity neurons of the AP is of particular interest. The AP is a sensory circumventricular organ and lacks the normal BBB found elsewhere in the CNS, providing an unfettered site of access in the CNS. Indeed, AP neurons are in constant contact with constituents of the circulation, including satiety signals and other homeostasis-related hormones (23, 43). AP is proposed to act as an integration center for homeostatic information and is well known to play a role in regulation of energy homeostasis. In addition to ghrelin receptors, AP neurons possess receptors for numerous satiety signals, including amylin, CCK, glucagon-like peptide-1 (GLP-1), and leptin (for a review, see Refs. 18, 19, 47). Moreover, lesions of AP cause well-defined alterations in feeding behavior (9, 16). AP communicates with feeding-associated areas of the CNS areas, such as ARC and paraventricular nucleus via strong connections to the adjacent NTS and the lateral parabrachial nucleus (49, 53, 60; see also Ref. 43 for a review).

In this study, we investigated potential roles of ghrelin in controlling the excitability of AP neurons and thus contributing to the effects of this peptide in the regulation of feeding. Using the patch-clamp technique, we provide the first direct demonstration that ghrelin modulates electrical activity of AP neurons, thus providing support for the notion that caudal brain stem plays an important role in ghrelin signaling through the gut-brain axis.

MATERIALS AND METHODS

Cell culture. All animal protocols conformed to the standards of the Canadian Council on Animal Care and the Queen's University Animal Care Committee. Cell cultures were prepared according to protocols modified from Fry et al. (20) and Brewer (6). Briefly, three or four Sprague-Dawley (~150 g) rats were decapitated, and the brains were quickly removed and placed into oxygenated, ice-cold artificial cerebral-spinal fluid containing the following (in mM): 124 NaCl, 2 KCl, 1.25 KH_2PO_4, 2.0 $CaCl_2$, 1.3 $MgSO_4$, 20 $NaHCO_3$, and 10 glucose. Brain stem slices were cut (300 μm) and transferred into Hibernate media (Brain Bits, Springfield, IL) supplemented with 1 × B27 supplement (Invitrogen, Burlington, Ontario, Canada). AP was carefully dissected from surrounding NTS, transferred to 5 ml Hibernate media containing 10 mg of papain (Worthington, Lakewood, NJ), and incubated for 30 min at 30°C. Tissue was washed in Hibernate media/B27. Following this, AP tissue was triturated, centrifuged at 100 *g* for 2 min, and resuspended in Neurobasal-A/B27 (Invitrogen). Dissociated neurons were then plated on glass-bottomed 35-mm culture dishes (MatTek Ashland, MA) at a low density to ensure synaptic contacts did not form between cells. The cells were incubated at 37°C in 5% CO_2. Experiments were performed within 1–3 days. Contact between neurons was never observed, nor was any evidence of synaptic activity.

Electrophysiology. Whole cell current-clamp recordings from AP neurons were made using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Current-clamp recordings were carried out using Spike2 ver. 4 software (CED, Cambridge, UK) for stimulation and recording. Data were filtered at 1 kHz and acquired at 10 kHz using a Cambridge Electronics Design Micro1401 interface. The external recording solution contained the following (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.3 with NaOH. Patch electrodes were fabricated from borosilicate glass (World Precision Instruments, Sarasota FL) and had resistances of 2.5–5 MΩ when filled with internal recording solution (in mM): 130 K-gluconate, 10 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 1 EGTA 4 Na_2ATP, and 0.1 GTP. Voltage-clamp recordings were carried out using the same solutions. Signal ver. 4.03 software (CED) was used for stimulation and data acquisition. Data were filtered at 3 kHz and acquired at 10 kHz. Series resistance was less than 20 MΩ and was compensated from 70% to 80%.

In experiments where voltage-clamp experiments were carried out on the same neuron before and after current-clamp recordings and ghrelin treatment, access resistance (R_S) did not vary by more than 10%. There were no significant differences in the mean R_S before and after ghrelin treatment (preghrelin R_S = 15.5 ± 2.1 MΩ, postghrelin R_S = 16.6 ± 2.3 MΩ).

All chemicals were purchased from Sigma (Oakville, Ontario, Canada). Ghrelin was purchased from Phoenix Pharmaceuticals (Burlingame, CA). The ghrelin antagonist [D-lys-3] -GHRP-6 was purchased from Bachem (Torrance, CA).

A puffer pipette was used to focally apply ghrelin to patch-clamped neurons. This arrangement allowed rapid application of defined concentration of ghrelin and efficient washout of the hormone. Using this technique, we found that application of external recording solution alone did not alter membrane potential or action potential firing rates (n = 6). Application of external recording solution containing 10 mM KCl resulted in rapid depolarization of membrane potential followed by recovery to control levels after cessation of the application of KCl.

Data analysis. Responsiveness of AP neurons to 1 μM-1 pM ghrelin was determined by comparing membrane potential of neurons before and after direct application. AP neurons were considered responsive if their mean membrane potential demonstrated a shift of at least 4.5 mV or a change in firing frequency of greater than 1 Hz (over a 60-s period) during the 120 s after application compared with the 100 s before application (20). Outward K^+ currents were elicited by step depolarizations from a holding potential of −100 mV. Peak K^+ current was determined by measuring the peak outward current during the first 25 ms of membrane depolarization, and sustained K^+ current was determined by measuring the mean current from 800–900 ms during a 1-s depolarization. Nonspecific cation currents (NSCC) were evaluated using a voltage ramp protocol (100 mV/s). Changes in NSCC were examined by subtracting the current obtained during the preghrelin treatment (control) from those obtained after ghrelin treatment.

Statistical analyses were performed using Origin 7.5 (Microcal Software, Northampton, MA) and GraphPad Prism 4.0 (San Diego, CA).

RESULTS

Ghrelin directly affects AP neurons. We first obtained current-clamp electrophysiological recordings from neurons maintained in culture for 1–3 days and examined the effect of exogenously applied ghrelin. AP neurons were visually identified as being phase bright, 6–10 μm in diameter, and often having one to several short primary neurites. Neurons in contact with other cells in the culture were not chosen for patch-clamp analysis. We carried out current-clamp recordings from a total of 93 neurons with a mean resting membrane potential of −48.7 ± 1.1 mV (n = 93) and a mean input resistance of 2.1 ± 0.2 GΩ (n = 89). Thirty nine percent of these neurons displayed spontaneous activity with a mean frequency of 1.6 ± 0.2 Hz.

We tested the sensitivity of neurons to application of 10 nM ghrelin (n = 31), a concentration we expected would elicit near maximum response based on previous work (11, 48). At this concentration, 19.4% (6/31) of the neurons tested exhibited a hyperpolarization of membrane potential (mean of 5.3 ± 0.7 mV), 19.4% (6/31) exhibited a depolarization of membrane potential (mean of 9.7 ± 1.8 mV), and the remaining 61.2% (22/31) were insensitive to application of ghrelin, as illustrated in Fig. 1, *A–D*. These changes in membrane potential were

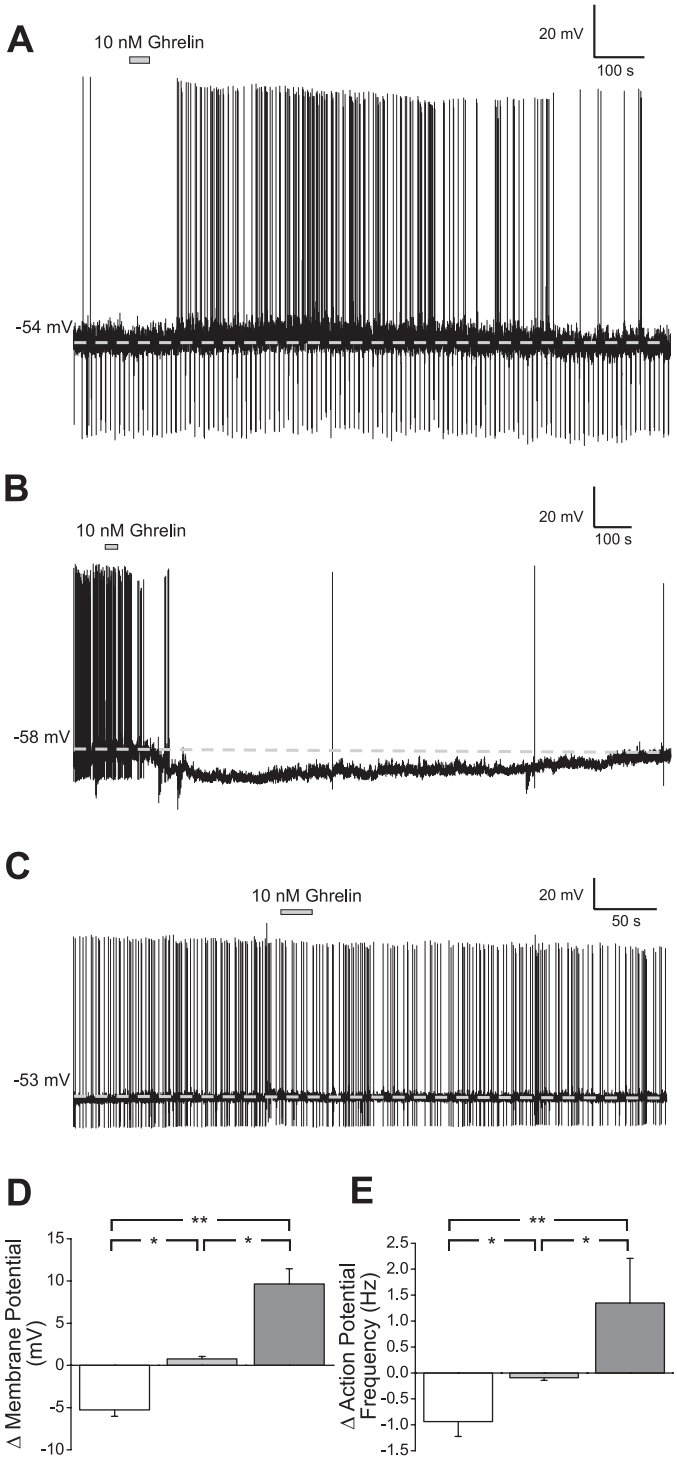

Fig. 1. Ghrelin modulates electrical activity of AP neurons. Representative current-clamp recordings from neurons that were depolarized (*A*), hyperpolarized (*B*), or insensitive to focally applied ghrelin (*C*). Bar graphs representing mean changes in membrane potential (*D*) and action potential frequency (*E*) for cells treated with 10 nM ghrelin. Open bars denote hyperpolarized; light gray denote insensitive; dark gray denote depolarized. The mean changes in action potential frequency were significantly different between all groups using the Kruskal-Wallis nonparametric ANOVA followed by Dunn's multiple-comparison test. Neurons that were quiescent before hyperpolarization were not included in the analysis. $*P < 0.05$; $**P < 0.001$.

usually accompanied by predictable changes in spontaneous action potential frequency: neurons that depolarized exhibited a mean increase of 1.35 ± 0.81 Hz in spontaneous activity and those that hyperpolarized exhibited a mean decrease of 0.94 ± 0.27 Hz. The mean changes in action potential frequency were determined to be significantly different between all groups using the Kruskal-Wallis nonparametric ANOVA ($P = 0.001$), followed by Dunn's multiple-comparison test (Fig. 1*E*). The mean latency of an effect from the time of ghrelin application was 69.6 ± 10.2 s. In many cases, the response to ghrelin was reversible after washout.

To determine whether the effects of ghrelin were concentration dependent, we next treated AP neurons with ghrelin at concentrations ranging from 1 μM to 10 pM. As shown in Fig. 2, *A* and *B*, as the concentration of applied ghrelin decreased from 1 μM to 10 pM, the percentage of the neurons that exhibited a change in membrane potential decreased, as did the mean amplitude of the change in membrane potential. For example, at 1 μM, 50% exhibited a response (depolarization or hyperpolarization), at 100 nM, 48% of cells exhibited a response, whereas this decreased to 38.7% at 10 nM, 20% at 1 nM, and 0% at 100 pM and 10 pM (Fig. 2*A*). Fitting these data to a Hill equation revealed an apparent EC_{50} of 1.9 nM. This value corresponds well with the observed concentration dependence of the change in membrane potential (Fig. 2*B*).

To confirm the specificity of ghrelin's activity on AP neurons, we next treated neurons with 10 nM ghrelin in the presence of 100 μM [D-lys-3]-GHRP-6, a specific GHSR antagonist (Fig. 2*C*). In control neurons treated with only external recording solution, the mean absolute value of the change in membrane potential (considering all changes from baseline as positive) was 1.3 ± 0.4 mV ($n = 12$). This was significantly different from the change in membrane potential of all cells treated with 10 nM ghrelin (absolute value of 3.5 ± 0.7 mV, $n = 31$) and the change in membrane potential of the responding neurons (absolute value of 7.9 ± 1.1 mV, $n = 12$). In the presence of antagonist, no neurons ($n = 10$) exhibited a response to application of 10 nM ghrelin, with the mean change in membrane potential observed to be 1.2 ± 0.4 mV. This change in membrane potential was significantly different than those observed for application of ghrelin without the antagonist and not different from the control neurons (using the Kruskal-Wallis nonparametric ANOVA, followed by Dunn's multiple-comparison test). Further comparison indicated that the proportion of neurons influenced by 10 nM ghrelin (12/31) was significantly different from that under conditions of 10 nM ghrelin with the antagonist (0/10; χ^2-test, $P = 0.02$), supporting the notion that the effect on membrane potential was specific to activation of the GHSR by focally applied ghrelin.

Ghrelin modulates a nonspecific cation conductance and an outward K^+ conductance in AP neurons. It was established above that AP neurons are sensitive to ghrelin, exhibiting both hyperpolarization and depolarization of membrane potential, concurrent with changes in action potential frequency. Previous experiments on subfornical organ (SFO) neurons have revealed that ghrelin modulates membrane potential and electrical activity by modulating activity of a NSCC. To determine the ionic mechanisms by which ghrelin causes depolarization or hyperpolarization of membrane potential in AP neurons, we carried out a set of experiments combining current-clamp and voltage-clamp recording techniques (Fig. 3). First, neurons

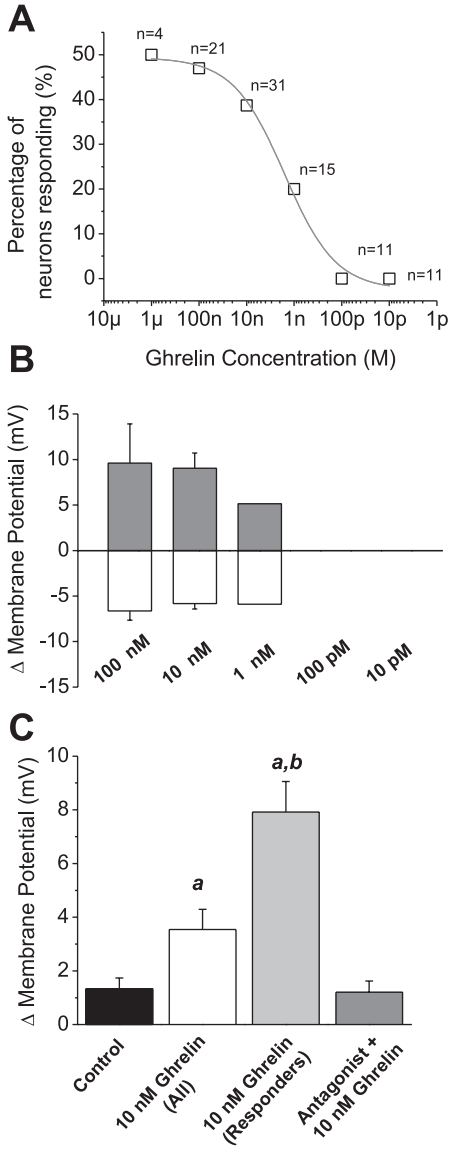

Fig. 2. Effects of ghrelin are concentration dependent and specific to the GHSR. *A*: percentage of neurons that responded to application of ghrelin decreased with decreasing concentrations. The data were fitted with the Hill equation, revealing an apparent EC_{50} of 1.9 nM. *B*: change in membrane potential also decreased with decreasing concentration of ghrelin. Data for 1 μM has been pooled with data for 100 pM. Open bars denote hyperpolarized; gray bars denote depolarized. *C*: absolute change in membrane potential (considering all changes to be positive) caused by 10 nM ghrelin in all of the neurons tested ($n = 31$) and in only the responding neurons ($n = 12$) was significantly greater than that observed for control neurons treated with external recording solution only ($n = 12$) ($P < 0.05$ using Kruskal-Wallis nonparametric ANOVA followed by Dunn's multiple-comparison test). Change in membrane potential of neurons treated with 10 nM ghrelin in the presence of the antagonist [D-lys-3]-GHRP-6 ($n = 10$) was not different from control, indicating that the response was specific to the GHSR. ***a*** indicates value is significantly different from control; ***b*** indicates value is also significantly different from 10 nM ghrelin (All) data.

were patch-clamped and subjected to a series of voltage-clamp protocols, including voltage step and voltage ramp protocols to determine the baseline properties of the ionic currents. Next, recording was switched to current-clamp mode to record membrane potential and spontaneous activity. Current clamped neurons were treated with 10 nM ghrelin, a concentration known to exert near-maximal effects (Fig. 2*B*) in responding neurons. After recording electrical responses in membrane potential after application of ghrelin, we switched back to recording voltage-clamp mode, and the voltage-clamp protocols were repeated to ascertain what, if any, ionic currents had been modulated by ghrelin. This type of experiment was successfully carried out on 19 neurons, where access resistance did not show more than a 10% change between the two sets of voltage-clamp recordings (Figs. 3 and 4). As shown in Fig. 3*A*, 10 of the 19 AP neurons tested in this manner did not respond to ghrelin. They also did not exhibit significant changes in ionic currents elicited during depolarizing voltage steps or depolarizing voltage ramp protocols (Figs. 3*A* and 4). Similar lack of changes were observed in neurons treated with external recording solution ($n = 4$, not shown). In contrast, alterations in ionic currents were observed in neurons that hyperpolarized or depolarized with ghrelin treatment (Figs. 3, *B* and *C*, and 4). Specifically, 4 of the 19 neurons tested exhibited a hyperpolarization of membrane potential. They exhibited a significant 1.38 ± 0.13-fold increase in amplitude of transient K^+ current and 1.29 ± 0.15-fold increase in sustained K^+ current ($P < 0.05$, Kruskal-Wallace test followed by Dunn's multiple-comparison test) during a voltage steps to +20 mV after treatment with ghrelin. No change was observed in the currents elicited during the ramp (Fig. 3*B* and Fig. 4). Furthermore, neurons that exhibited a depolarization of membrane potential exhibited a change in current elicited during the voltage-ramp protocol (Figs. 3*C* and 4). Subtracting the current obtained during the preghrelin treatment (control) from those obtained after ghrelin treatment revealed a linear current with a reversal potential of -37.7 ± 2.4 mV and a mean change in conductance of 0.75 ± 0.14 nS ($n = 5$), indicative of a nonselective cationic conductance (NSCC). This corresponded to a 2.70 ± 0.73-fold change in peak NSCC conductance after treatment with ghrelin. Depolarizing neurons did not exhibit a significant change in the amplitude of voltage-gated K^+ currents. In summary, these data indicate that ghrelin alters electrical excitability of two subpopulations of AP neurons via modulating different populations of ion channels.

DISCUSSION

This study is the first to directly demonstrate that neurons of the caudal brain stem are sensitive to the circulating hormone ghrelin. Specifically, we used patch-clamp electrophysiology to investigate the response of isolated AP neurons to focal application of ghrelin at a concentration of 10 nM. We observed that 38% of neurons tested responded with changes in membrane potential, while the remaining 62% were insensitive to the hormone. About half of those responding exhibited a hyperpolarization of membrane potential, while the other half exhibited depolarization of membrane potential. The observation that ghrelin induced both depolarizing and hyperpolarizing effects on the membrane potential of AP neurons is consistent with previous studies demonstrating the existence of subpopulations of neurons within the AP that respond to specific hormones with either depolarization or hyperpolarization of membrane potential (7, 20, 54, 67, 68). In fact, the present study highlights a previously described (31) shortcoming of measuring changes in expression levels of c-Fos as a technique to identify populations of neurons that are sensitive to modu-

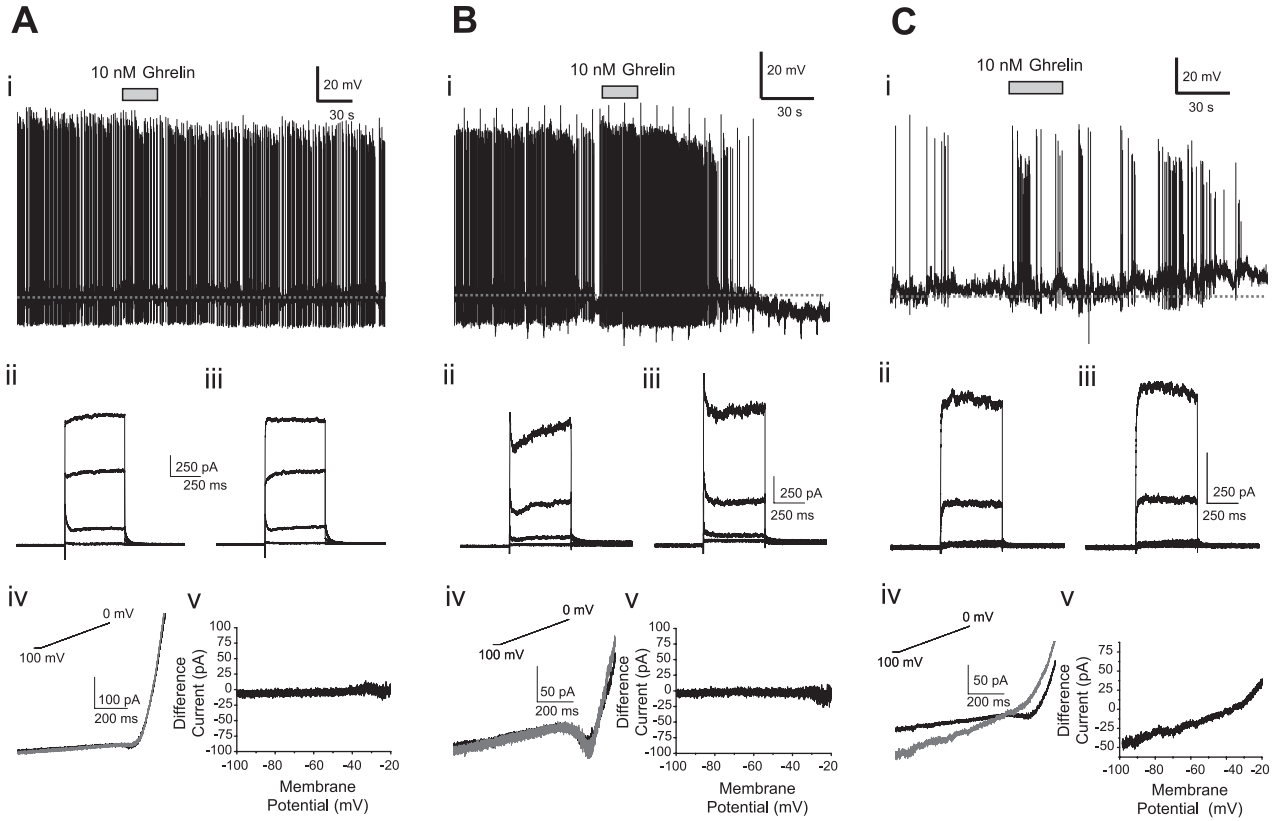

Fig. 3. Ionic mechanisms underlying ghrelin-induced changes in membrane potential. Examples of results from nonresponding neurons (*A*), hyperpolarizing neurons (*B*), and depolarizing neurons (*C*). For all parts: example of a current lamp trace (i). ii and iii: examples voltage-clamp K^+ currents elicited by depolarizing voltage steps from a holding potential of −100 mV to 1-s command potentials of −40 mV, −20 mV, 0 mV, and +20 mV; before (ii) and after (iii) application of ghrelin. iv: example of voltage-clamp currents elicited during a depolarizing ramp from −100 mV to 0 mV. Currents shown are the mean of 3–5 current traces. Black trace represents current before application of ghrelin, gray trace after application. v: difference current obtained by subtraction of ghrelin-induced currents from control current. In *A*, note the absence of change in baseline membrane potential (i), or changes in ionic currents (ii to v). *B*: note the hyperpolarization of membrane potential (i), and the increase in outward K^+ currents (ii and iii). *C*: note the depolarization of membrane potential (i) and the increase in the nonspecific cation current elicited by the voltage ramp (iv and v).

lation satiety signals. While the c-Fos immunolabeling technique can provide information regarding increases in neuronal activity, decreases in activity brought about by membrane hyperpolarization cannot be identified. Indeed, some c-Fos immunolabeling studies have reported that ghrelin does not induce c-Fos in the AP (34) or induces only weak immunolabeling (56) and suggested that AP is not an important site of action within the CNS. The fact that 19% of the neurons modulated by ghrelin in this study were hyperpolarized suggests that previous studies have underestimated the ability of AP to detect circulating ghrelin and communicate this information to other sites involved in regulation of energy homeostasis.

The observed dose-response curve from the present experiments suggests an EC_{50} of 1.9 nM (Fig. 2*A*). This EC_{50} is similar to that previously observed in SFO neurons, between 100 pM and 1 nM (48). These data fall between EC_{50} values observed for heterologous expression of GHSR of 0.19 nM, to 32 nM (5, 29). Specificity of the ghrelin effect on AP neurons was confirmed with experiments demonstrating that preapplication of the GHSR antagonist 100 μM [D-Lys-3]-GHRP-6 abolished ghrelin-induced changes in membrane potential.

Previous work from our laboratory has indicated that ghrelin depolarized SFO neurons via activation of a depolarizing NSCC. The present work indicates that this mechanism for exerting depolarization is conserved in some AP neurons. Currents elicited in response to ramp protocols from cells that depolarized exhibited a ghrelin-activated current with a reversal potential near −38 mV, characteristic of a NSCC (15, 46, 48). Modulation of the NSCC in AP neurons has also been suggested as a mechanism of action for other circulating hormones, including orexin-A and adrenomedulin (67, 68).

In contrast to SFO neurons, which only depolarized in response to ghrelin application, we frequently observed that AP neurons exhibited both depolarizations and hyperpolarizations. We observed that cells that hyperpolarized exhibited an increase in peak and sustained outward K^+ currents. While the specific identity of the ghrelin-modulated K^+ channels is not known, AP neurons are known to exhibit three types of outward K^+ current: the transient current (I_A or I_{TO}), the delayed rectifier (IK_D), and a Ca^{2+}-activated K^+ current (maxi-K) (27, 39). Given the high-input resistance of dissociated AP neurons (about 2 GΩ), enhanced opening of only a few channels at resting membrane potential would be predicted to contribute to the observed hyperpolarization. Importantly, the modulation of K^+ currents, which we report here as a consequence of ghrelin administration, has been previously reported as a mechanism by which other circulating hormones

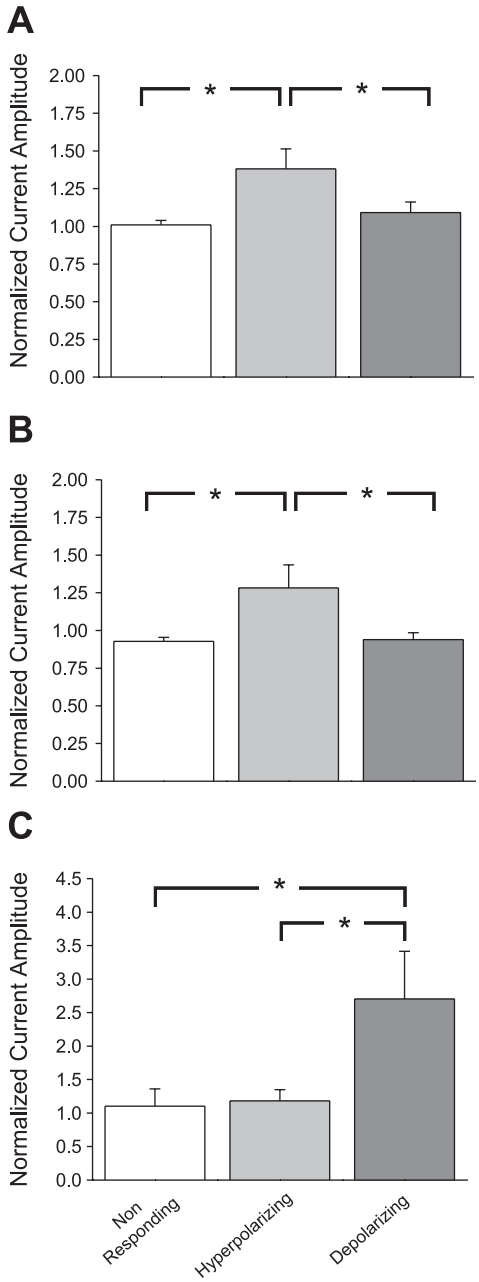

Fig. 4. Summary of changes in normalized amplitudes of the peak K^+ current (*A*), sustained K^+ currents (*B*), and ramp currents (*C*) from nonresponding neurons (open bars), hyperpolarizing neurons (light gray bars), and depolarizing neurons (dark gray bars). Currents were normalized by dividing the amplitude of that current before application of ghrelin by the amplitude after application of ghrelin. Magnitude of changes in normalized currents for nonresponding neurons, hyperpolarizing neurons, and depolarizing neurons were compared using Kruskal-Wallis nonparametric ANOVA followed by Dunn's multiple-comparison test ($*P < 0.05$).

also alter electrical activity of AP neurons. Specifically, AP neurons have been reported to exhibit increases in activity when treated with ANG II via attenuation of both transient and steady-state K^+ current (27). Another study reported 17-β-estradiol caused decreases in spontaneous activity via activation of the maxi-K current (39). Although the present study indicates that both voltage-gated K^+ channels and nonspecific cation channel are modulated by ghrelin, the possibility exists that coordinated modulation of other ion channels may also contribute to electrical regulation of AP neurons by ghrelin. Further experimentation will be required to determine the specific identities of AP neuron ion channels that are modulated by ghrelin.

Several sites in the CNS appear to play key roles in mediating the orexigenic activity of ghrelin. Some studies have suggested a role for the afferent vagus in ghrelin-stimulated appetite (2, 14) because vagotomy or capsaicin treatment abolished ghrelin-stimulated appetite. However, Arnold et al. (1) recently carried out carefully controlled experiments using complete subdiaphragmatic deafferentation of rat and still observed ghrelin-stimulated appetite, thus concluding that ghrelin does not require vagal afferent signaling. The importance of the NPY/AGRP neurons of the ARC has been demonstrated by studies using monosodium glutamate to create lesions of the ARC and showing that feeding in response to ghrelin treatment is abolished (57). Moreover, double knockout of NPY and AGRP clearly abolishes responses to peripheral ghrelin (8). However, recent experiments in caudal brain stem have also demonstrated that ablation of AP eliminates ghrelin-induced feeding (21). These data demonstrating the requirement of AP in mediating ghrelin's orexigenic effect, together with the present experiments showing direct modulation of electrical activity in AP neurons convincingly indicate that ghrelin may act on the AP to stimulate appetite. While the mechanism by which circulating ghrelin accesses hypothalamic neurons to induce electrical modulation in the ARC (11) has yet to be elucidated, recent research showing widespread localization of the ghrelin receptor (70) suggests that multiple complementary pathways within the CNS and periphery are involved in the physiological orexigenic responses to ghrelin.

Of particular importance to the notion of caudal brain stem in sensing ghrelin in the circulation, histological and electron microscopy data show the presence of a continuous monolayer of glial cells joined by tight junctions surrounding the AP (36, 62). This indicates that while AP neurons are in free contact with circulating hormones such as ghrelin, other adjacent caudal brain stem areas, such as the NTS and dorsal motor nucleus are functionally isolated from the circulation. Activation of neurons in the NTS and other brain stem sites by peripheral ghrelin (24, 56) is likely to be secondary to modulation of AP neurons (38). Together with the present results, these data suggest that AP is the primary sensor of ghrelin in the caudal brain stem.

Area postrema is a well-known CNS site of action for satiety signals, including amylin, CCK, peptide YY, and GLP-1 (19, 40). Current evidence suggests that the population of AP neurons that acts to inhibit feeding is a separate subpopulation of neurons from the subpopulation that is modulated by ghrelin. For example, a large proportion of AP neurons that are activated after feeding (as determined by c-Fos immunoreactivity) are catecholamine (CA)-synthesizing neurons (50). Consistent with this, intravenous injection of the anorectic hormones GLP-1 or CCK activates CA-synthesizing neurons in the AP (51). It has also recently been reported that 90% of CA-synthesizing neurons of the AP also express receptors for GLP-1 (66). Importantly, the receptor for ghrelin does not immunolocalize to the CA-synthesizing neurons in the AP (70). Together, these data indicate that ghrelin-sensitive neu-

rons are a separate subpopulation of AP neurons from those that inhibit feeding.

In addition to its role in regulation of energy balance, the AP is known to act as a homeostatic integration center involved in cardiovascular regulation (32, 69). In particular, AP is a key center in regulation of baroreflex sensitivity. A significant body of work has established that the circulating hormone arginine vasopressin acts directly on AP neurons, to inhibit sympathetic output and shift the baroreceptor reflex setpoint to lower arterial pressure (26, 59; for a review, see also Ref. 25). Intriguingly, ghrelin is also reported to have cardiovascular effects by modulating baroreflex sensitivity. For example, intravenous injection of ghrelin elicited a decrease in blood pressure without causing tachycardia in healthy men (44). Moreover, Matsumura et al. (42) demonstrated that intracerebroventricular ghrelin decreases sympathetic nerve activity and arterial blood pressure in rabbits by alteration of baroreflex sensitivity. More recently, ghrelin has shown promise as an emerging clinical treatment to improve survival after an acute myocardial infarction. Specifically, treatment of rats with intravenous ghrelin within 2 h after a myocardial infarction abolishes elevated cardiac sympathetic output and significantly reduces associated mortality (52, 55). The present study supports the notion that cardiovascular effects of circulating ghrelin may potentially occur via direct modulation of the electrical activity of neurons in the AP. Detailed investigation is urgently required to further elucidate the mechanisms of ghrelin acting at the AP in homeostatic regulation.

Perspectives and Significance

We have provided direct evidence that electrical activity of AP neurons is modulated by the hunger hormone ghrelin. The observation that AP neurons are depolarized, as well as hyperpolarized suggests that the number of neurons modulated by ghrelin is greater than might be predicted by c-Fos labeling alone. While current evidence indicates that exogenous ghrelin acts at both caudal brain stem and hypothalamic sites to alter feeding behavior, further experiments are required to elucidate how these two pathways interact with ghrelin to regulate energy homeostasis in vivo. This research will provide a basis for improving strategies to prevent and treat obesity and obesity-related diseases.

GRANTS

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Present address for M. Fry: Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada, R3T 2N2.

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