Ghrelin Attenuates cAMP-PKA Signaling to Evoke Insulinostatic Cascade in Islet β-Cells

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OBJECTIVE—Ghrelin reportedly restricts insulin release in islet β-cells via the Goε2 subtype of G-proteins and thereby regulates glucose homeostasis. This study explored whether ghrelin regulates cAMP signaling and whether this regulation induces insulinostatic cascade in islet β-cells.

RESEARCH DESIGN AND METHODS—Insulin release was measured in rat perfused pancreas and isolated islets and cAMP production in isolated islets. Cytosolic cAMP concentrations (cAMPi) were monitored in mouse MIN6 cells using evanescent-wave fluorescence imaging. In rat single β-cells, cytosolic protein kinase-A activity (PKAi) and Ca2+ concentration (Ca2+i) were measured by DR-II and fura-2 microfluorometry, respectively, and whole cell currents by patch-clamp technique.

RESULTS—Ghrelin suppressed glucose (8.3 mmol/L)-induced insulin release in rat perfused pancreas and isolated islets, and these effects of ghrelin were blunted in the presence of cAMP analogs or adenylyl cyclase inhibitor. Glucose-induced cAMP production in isolated islets was attenuated by ghrelin and enhanced by ghrelin receptor antagonist and anti-ghrelin antiserum, which counteract endogenous islet-derived ghrelin. Ghrelin inhibited the glucose-induced cAMPi, elevation and PKAi activation in MIN6 and rat β-cells, respectively. Furthermore, ghrelin potentiated voltage-dependent K+(Kv) channel currents without altering Ca2+ channel currents and attenuated glucose-induced Ca2+i increases in rat β-cells in a PKA-dependent manner.

CONCLUSIONS—Ghrelin directly interacts with islet β-cells to attenuate glucose-induced cAMP production and PKA activation, which lead to activation of Kv channels and suppression of glucose-induced Ca2+i increase and insulin release. Diabetes 60:2315–2324, 2011

Ghrelin, an acylated 28-amino acid peptide, is the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (1,2). Ghrelin is produced predominantly in the stomach and stimulates growth hormone release and feeding and exhibits positive cardiovascular effects, suggesting its possible clinical application (3). Ghrelin and GHS-R are located in the pancreatic islets (4–6). Furthermore, ghrelin O-acyltransferase (GOAT), which has been identified as

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RESEARCH DESIGN AND METHODS
Male Wistar rats (Japan SLC, Hamamatsu, Japan) were housed in accordance with our institutional guidelines and with the Japanese Physiological Society’s...
FIG. 1. Ghrelin attenuates glucose-induced insulin release in perfused rat pancreas in a cAMP pathway–dependent manner. Ghrelin was added 10 min prior to 8.3 mmol/L glucose challenge. A: Ghrelin (10 nmol/L) suppressed 8.3 mmol/L glucose (8.3G)-induced insulin release in perfused rat pancreas (n = 6). B: Ghrelin (10 nmol/L) did not alter glucose-induced insulin release from the perfused pancreas of PTX-treated rats (n = 6). C: In the presence of a 1 mmol/L dibutyryl-cAMP (db-cAMP), the glucose-induced insulin release was markedly enhanced and ghrelin failed to suppress it (n = 4–6). D: Following preincubation with an irreversible adenylate cyclase inhibitor MDL-12330A (10 μmol/L) for 30 min, the glucose-induced insulin release was attenuated and ghrelin (10 nmol/L) failed to suppress it (n = 3). E: Ghrelin (10 nmol/L) suppressed both the first and second phases of glucose-induced insulin release. In the pancreata from PTX-treated rats and those treated with a db-cAMP, both phases of glucose-induced insulin release were markedly enhanced and ghrelin failed to alter them. In the presence of MDL-12330A, both phases of the insulin
In vitro perfusion of the pancreas. Saline was intravenously injected into rats. Three days after injections, animals developed diabetes. Glucose (2.8 mmol/L) induced insulin release were suppressed, and ghrelin did not further suppress them. None of these treatments affected basal levels of insulin release at 2.8 mmol/L glucose.

**Preparation of pancreatic islets and single β-cells.** Islets were dispersed into single cells in Ca²⁺-free HKRB, and the single cells were plated sparsely on coverslips (10,000 cells/cm²). Emission wavelengths were detected with interference filters (485/25 nm for cyan fluorescent protein [CFP] excited by 442 nm and 560/40 nm for yellow fluorescent protein [YFP] excited by 514 nm line). Holoenzyme dissociation caused by elevation of cytosolic cAMP concentrations ([cAMP]) results in translocation of Ca²⁺-YFP to the cytoplasm, recorded with evanescent-wave microscopy as selective loss of YFP fluorescence with rise of the CFP-to-YFP fluorescence ratio.

**Measurements of cytosolic PKA activity ([Ca²⁺]), in single β-cells.** Dissociated single β-cells on coverslips were mounted in an open chamber and superfused in HKRB. Cytosolic PKA activity ([PKA]) in rat single β-cells was measured using the fluorescence probe DB-II (Dojindo, Kumamoto, Japan) as previously described (29) with slight modifications using two-photon confocal microscopy. The DB-II-loaded islet cells were superfused in HKRB, and DB-II fluorescence was excited at 780 nm by mode-locked Ti: Sapphire laser every 10 s; the emission signal at 475 nm was detected with a photomultiplier tube of the multiphoton laser-scanning microscope (FluoView FV3000-TP; Olympus, Tokyo, Japan). [Ca²⁺]i in single β-cells were measured by dual-wavelength fura-2 microfluorometry with excitation at 340/380 nm and emission at 510 nm using a cooled charge-coupled device camera (15). The ratio image was produced on an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan). After the [PKA], and [Ca²⁺], measurements, cells were fixed with 4% paraformaldehyde and β-cells were identified by insulin immunostaining. Data were taken exclusively from the insulin-positive cells.

**Patch-clamp experiments in rat single β-cells.** Perforated whole-cell currents were recorded using a pipette solution containing nystatin (150 μg/mL) dissolved in 0.1% DMSO as previously described (5). Membrane currents were recorded using an amplifier (Axopatch 200B, Molecular Devices, Foster, CA) in a computer using pCLAMP10.2 software. For conventional whole-cell clamp experiments measuring Ca²⁺-channel currents, pipette solution contained (in millimoles per liter) 90 aspartate, 10 HEPES, 5 MgCl₂, 10 EGTA, 5 ATP-Mg, 20 TEA-Cl, and 90 CsOH at pH 7.2 with CsOH, and cells were superfused with 10 mmol/L Ca²⁺-containing HKRB. β-cells were voltage clamped at a holding potential of −70 mV and then shifted to the test potentials from −60 to 40 mV in 10 mV steps with the pulses of either 100-ms duration for Kv channel currents or 50-ms duration for Ca²⁺ channel currents at room temperature (25°C).

**Statistical analysis.** Data are means ± SEM. Statistical analyses were performed using Student t test or one-way ANOVA followed by Bonferroni multiple comparison tests. P values < 0.05 were considered statistically significant.

**RESULTS**

Ghrelin attenuates glucose-induced insulin release in a CAMP signaling-dependent manner. In rat perfused pancreas, the first and second phases of glucose (8.3 mmol/L)-induced insulin release were both significantly suppressed by exogenous ghrelin (10 nmol/L) that was administered 10 min prior to 8.3 mmol/L glucose challenge and present through the end of experiments, whereas the basal insulin release at 2.8 mmol/L glucose was not altered (Fig. 1A and E), confirming a previous report (15). In perfused pancreas of PTX-treated rats, both phases of glucose-induced insulin release were markedly enhanced, and ghrelin (10 nmol/L) failed to affect them (Fig. 1B and E). A membrane-permeable CAMP analog, dibutyryl-cAMP (db-CAMP), at 1 mmol/L markedly enhanced both phases of glucose-induced insulin release, and in the presence of db-CAMP ghrelin failed to suppress both phases of insulin release (Fig. 1C and E). In pancreas preincubated with an irreversible adenylyl cyclase inhibitor, MDL-12330A (10 μmol/L), both phases of glucose-induced insulin release were attenuated and were not further altered by administration of ghrelin (10 nmol/L) (Fig. 1D and E). None of these treatments affected basal levels of insulin release at 2.8 mmol/L glucose. Ghrelin (10 nmol/L) affected neither acetylcholine (ACh) (10 μmol/L)-induced (Fig. 1F and G) nor high K⁺ (25 mmol/L KCl)-induced insulin release in rat pancreatic islets, and ghrelin did not further suppress them. None of these treatments affected basal levels of insulin release at 2.8 mmol/L glucose (2.8G). **P < 0.05, **P < 0.01 vs. control; †P < 0.05, ††P < 0.01 vs. 8.3 mmol/L glucose alone (n = 8).**

**Measurements of insulin release and cAMP productions in rat islets.** Measurements of insulin release and cAMP productions in rat islets. For measurements of islet insulin release, groups of 12–15 islets were incubated for 1 h at 37°C in HKRB with 2.8 mmol/L glucose for stabilization, followed by test incubation for 1 h in HKRB with 2.8 or 8.3 mmol/L glucose. In cAMP measurements, islets were incubated for 1 h in HKRB with 500 μmol/L 3-isobutyl-1-methylxanthine (BIBX), a phosphodiesterase (PDE) inhibitor (Sigma-Aldrich), to avoid degradation of CAMP in the samples. Ghrelin (Peptide Institute, Osaka, Japan) with or without dibutyryl-cAMP (Sigma-Aldrich), 6-Pha-cAMP (BioLOG, MDL-12330A (10 μmol/L) superfused islets were voltage clamped at a holding potential of −70 mV and then shifted to the test potentials from −60 to 40 mV in 10 mV steps with the pulses of either 100-ms duration for Kv channel currents or 50-ms duration for Ca²⁺ channel currents at room temperature (25°C).
FIG. 3. Ghrelin suppresses glucose-induced cAMP-PKA signaling in islet β-cells. A: Glucose at 8.3 (8.3G) and 22 mmol/L (22G) induced cAMP productions in islets in the presence of phosphodiesterase inhibitor IBMX (500 μmol/L). Exogenous ghrelin (10 nmol/L) inhibited the glucose-induced cAMP productions, whereas a GHS-R antagonist, [D-Lys³]-GHRP-6 (1 μmol/L), enhanced them. *P < 0.05 vs. control (n = 12). B: Neither ghrelin (10 nmol/L) nor [D-Lys³]-GHRP-6 (1 μmol/L) altered the glucose (8.3 mmol/L)-induced cAMP productions in islets isolated from PTX-treated rats. Glucose at 22 mmol/L (22G) enhanced larger amount of cAMP productions as a positive control (n = 10). C: Immunoneutralization of endogenous ghrelin with an anti-ghrelin antiserum (0.1%) significantly enhanced the glucose (8.3 and 22 mmol/L)-induced cAMP productions compared with the control, a normal rabbit serum (0.1%). *P < 0.05, **P < 0.01 vs. normal rabbit serum (n = 12). D and E: Representative
perfused pancreas at 2.8 mmol/L glucose (Fig. 1H and I). These results suggest that ghrelin selectively attenuates glucose-induced insulin release via PTX-sensitive G-protein that is coupled to modulation of cAMP signaling.

Next, in rat isolated islets, 8.3 mmol/L glucose-induced insulin release was inhibited by exogenous ghrelin (Fig. 2). The glucose-induced insulin release was enhanced by dbcAMP (1 mmol/L). Moreover, 6-Phe-cAMP (10 μmol/L), a membrane-permeable specific PKA activator, enhanced the glucose-induced insulin release. Ghrelin (10 nmol/L) failed to attenuate the insulin release in the presence of these cAMP analogs (Fig. 2). Conversely, the glucose-induced insulin release was significantly suppressed by adenylate cyclase inhibitor MDL-12330A (10 μmol/L), and ghrelin did not affect the insulin release in the MDL-12330A-treated islets (Fig. 2).

**Ghrelin inhibits glucose-induced cAMP production in rat isolated islets.** In the presence of PDE inhibitor IBMX (500 μmol/L), static incubation of islets with 8.3 mmol/L glucose induced modest cAMP productions in islets compared with those with 2.8 mmol/L glucose (P < 0.05) (Fig. 3A). Incubation of islets with higher concentration (22 mmol/L) of glucose induced further increases in the cAMP levels. The glucose (8.3 and 22 mmol/L)-induced cAMP responses were significantly inhibited by exogenous ghrelin (10 nmol/L) and augmented by a GHS-R antagonist [D-Lys6]-GHRP-6 (1 μmol/L) (Fig. 3A). These effects of exogenous and endogenous ghrelin were blunted in islets isolated from PTX-treated rats; neither exogenous ghrelin (10 nmol/L) nor [D-Lys6]-GHRP-6 (1 μmol/L) affected the 8.3 mmol/L glucose-induced cAMP productions, whereas 22 mmol/L glucose induced further cAMP productions (Fig. 3B). Moreover, immunoneutralization of endogenous ghrelin with an anti-ghrelin antisera (0.1%) significantly enhanced the glucose-induced cAMP productions compared with the control with a normal rabbit serum (0.1%) (Fig. 3C).

**Ghrelin suppresses glucose-induced cAMPi elevations in MIN6 β-cells.** To determine the direct effect of ghrelin on the glucose-induced cAMP production, [cAMPi] were monitored in mouse β-cell line MIN6 cells transfected with a fluorescent-translocation biosensor using evanescent-wave microscopy. Raising the glucose concentration from 3 to 11 mmol/L induced a rise in [cAMPi] in an oscillatory manner (Fig. 3D). Ghrelin markedly suppressed the glucose-induced oscillatory rise in [cAMPi] (Fig. 3D). Furthermore, in a MIN6 cell with pronounced oscillations of [cAMPi] during an 11 mmol/L glucose challenge, the [cAMPi] oscillations were inhibited by administration of ghrelin and recovered by its washout (Fig. 3E), showing the reversible effect of ghrelin. Average CFP-to-YFP ratio (AUC/time) revealed that 11 mmol/L glucose increased CFP-to-YFP ratio by 19 ± 4% and ghrelin significantly suppressed it (Fig. 3F), indicating that ghrelin directly inhibits glucose-induced cAMP signaling in β-cells.

**Ghrelin suppresses glucose-induced PKA activation in rat β-cells.** The activity of PKA, a downstream effector of cAMP elevations, was measured in rat single β-cells using PKA-sensitive DR-II fluorescence with confocal microscopy. DR-II fluorescence is inversely related to PKA activity (21). Fluorescence intensity decreased by only 2% during scanning for 30 min, indicating that photo bleaching of DR-II signal was negligible in this experimental condition (data not shown). A rise in the perfusate glucose concentration from 2.8 to 8.3 mmol/L decreased DR-II fluorescence, as shown by an increase in F0-to-F ratio reflecting an increase in PKA activity in rat single β-cells (Fig. 3G). The glucose (8.3 mmol/L)-induced increase in the PKA activity was significantly inhibited by treatment with ghrelin in the majority of β-cells examined (Fig. 3H and I).

**Ghrelin activates Kv channels via PKA-dependent signaling pathway but does not alter L-type Ca2+ channels in β-cells.** The Kv channel currents in rat β-cells under nystatin-perforated whole-cell clamp were measured in the presence of 100 μmol/L tolbutamide to inhibit the ATP-sensitive K+ (KATP) channel and thereby exclude involvement of this channel in the currents. In the presence of 8.3 mmol/L glucose, outward K+ currents evoked by depolarizing pulses from a holding potential of −70 to 20 mV were increased by exposure to 10 mmol/L ghrelin (Fig. 4A), confirming previous reports (5,15). The current-voltage relationships demonstrated that ghrelin significantly increased the current densities through Kv channels at potentials positive to −30 mV (Fig. 4B). At a holding potential of 20 mV, ghrelin significantly increased the current densities to 125.8 ± 12.6 pA/pF from 103.2 ± 9.1 pA/pF (P < 0.05, n = 8) (Fig. 4D). In the presence of 6-Phe-cAMP (10 μmol/L), the Kv channel currents evoked by depolarizing pulse to 20 mV were not increased by ghrelin (Fig. 4A) and ghrelin failed to enhance the current densities in the entire range of potentials (Fig. 4C and D). The Kv channel currents in the 6-Phe-cAMP-treated cells tended to decrease compared with control currents in nontreated cells (Fig. 4B and C), though the difference was not statistically significant (Fig. 4D). We next examined the effect of 6-Phe-cAMP by itself on the β-cells. A constant depolarizing pulse from −70 to 20 mV every 20 s evoked Kv currents in the external solution containing 5.6 mmol/L glucose (Fig. 4E). The currents markedly decreased upon exposure to 6-Phe-cAMP (10 μmol/L) and recovered by washout of it (Fig. 4E and F). In control experiments without the PKA activator, Kv currents were kept constant for >15 min (data not shown).

The effect of ghrelin on the voltage-dependent Ca2+ channel was determined in rat single β-cells. In the control external solution containing 8.3 mmol/L glucose, a depolarizing pulse from holding potential of −70 to 0 mV evoked a long-lasting inward current in rat β-cells (Fig. 4G), and this current was markedly inhibited by an L-type Ca2+ channel blocker, nifedipine (10 μmol/L) (data not shown). Exposure of cells to ghrelin (10 nmol/L) did not alter the current amplitude or current-voltage relationships (Fig. 4G and H), indicating that ghrelin does not directly affect L-type Ca2+ channels in β-cells.

**Ghrelin attenuates glucose-induced [Ca2+]i increases in β-cells in a PKA pathway-dependent manner.** A repetitive glucose (8.3 mmol/L) stimulation induced repeated [Ca2+]i increases in rat single β-cells. The ratio of the peak recordings of glucose-induced changes of [cAMPi] in MIN6 β-cells expressed as CFP-to-YFP ratio by evanescent-wave microscopy. Ghrelin (10 nmol/L) inhibited the glucose-induced elevation of [cAMPi], in a reversible manner. F: Time-average CFP-to-YFP ratio (AUC/time) at 11 mmol/L glucose (11G) was suppressed by ghrelin (10 nmol/L). The basal CFP-to-YFP ratio at 3 mmol/L glucose (3G) was normalized to 1. **P < 0.01 vs. 11G.** G: Typical trace of glucose (8.3 mmol/L)-induced cytosolic PKA activation expressed as the ratio of the initial DR-II fluorescence intensity (F0) to that at each time point (F) in rat β-cells. Increase in F0-to-F ratio indicates PKA activation in cells. H: In the presence of ghrelin (10 nmol/L), the glucose (8.3 mmol/L)-elicited PKA response was diminished in 12 of 13 cells examined. After recording, cells were identified as β-cells by immunocytochemical staining with anti-insulin antibody. I: Peak amplitude of the glucose (8.3 mmol/L)-induced PKA response was markedly suppressed by ghrelin (10 nmol/L). The data were taken only from insulin-positive cells. **P < 0.01 vs. control (n = 12–13).
FIG. 4. Ghrelin enhances Kv channel currents in rat islet β-cells via PKA-dependent signaling pathway. A: Current traces evoked by a step pulse to 20 mV from a holding potential of ~70 mV were measured in a single β-cell under perforated whole-cell clamp mode. Data were recorded in the presence of 8.3 mmol/L glucose and 100 μmol/L tolbutamide (control [black trace]) and during exposure to 10 nmol/L ghrelin (red trace). The dotted lines indicate zero current levels. Upper panel: exposure to ghrelin increased the amplitudes of delayed outward currents. Lower panel: in the presence of PKA activator 6-Phe-cAMP at 10 μmol/L, Kv channel currents were not increased by exposure to 10 nmol/L ghrelin. B: Current levels measured at the end of test pulses were plotted as a current density (pA/pF) against membrane potentials in β-cells (n = 8). C: Ghrelin failed to potentiate Kv channel currents in the presence of 6-Phe-cAMP (n = 7). D: Peak amplitudes of current density at 20 mV of a membrane potential. Ghrelin (10 nmol/L) induced significant increase in the Kv channel currents, which was not observed in the presence of 6-Phe-cAMP. *P < 0.05 by paired t tests (n = 7–8). E: Amplitude of the Kv channel currents in a single β-cell evoked by a step pulse to 20 mV from a holding potential of ~70 mV in the presence of 5.6 mmol/L glucose was decreased by 6-Phe-cAMP in a reversible manner. F: 6-Phe-cAMP (10 μmol/L) significantly decreased peak amplitude of the current density in β-cells (n = 5). *P < 0.05. G: Whole-cell Ca²⁺ channel current evoked by a step pulse to 0 mV from a holding
[Ca^{2+}], response to the second glucose stimulation (S2) to that to the first stimulation (S1), S2/S1, was 0.77 ± 0.04 (n = 93) in the control cells. Ghrelin (10 nmol/L), added to perfused solution 5 min prior to the second glucose stimulation, suppressed [Ca^{2+}] responses, decreasing S2-to-S1 ratio to 0.57 ± 0.04 (P < 0.01, n = 91) (Fig. 5A and G). The effect of ghrelin on [Ca^{2+}] responses was abolished by treatment with db-cAMP (1 mmol/L) (Fig. 5B and G) and 6-Phe-cAMP (10 μmol/L) (Fig. 5C and G). These cAMP analogs alone potentiated the glucose-induced [Ca^{2+}] responses in β-cells, resulting in increases in S2-to-S1 ratio (Fig. 5G). Pretreatment of cells with MDL-12330A (10 μmol/L) significantly suppressed the glucose-induced [Ca^{2+}] increases (Fig. 5D and G), suggesting that activation of adenylyl cyclase is involved in the glucose-induced [Ca^{2+}] increases in β-cells. In MDL-12330A-treated cells, ghrelin did not attenuate [Ca^{2+}] responses to glucose (Fig. 5G). The inhibitory effect of ghrelin on [Ca^{2+}] responses was unaltered in the presence of Epac activator 8-pCPT-2′-O-Me-cAMP at 30 μmol/L (Fig. 5E and G) or phospholipase-C inhibitor U-73122 (1 μmol/L) (Fig. 5F and G). Neither 8-pCPT-2′-O-Me-cAMP (30 μmol/L) nor U-73122 (1 μmol/L) by itself had effect on [Ca^{2+}]. These results suggest that ghrelin acts on the cAMP signaling route linked primarily to PKA but not Epac and thereby suppresses the glucose-induced [Ca^{2+}] increases in β-cells.

**DISCUSSION**

In this study, we have demonstrated that ghrelin suppresses glucose-induced insulin secretion from pancreatic islets in a cAMP-dependent manner. Moreover, ghrelin decreases cAMP levels in islets and β-cells, and the decreased cAMP mediates the electrical and [Ca^{2+}] responses to ghrelin.

In rat perfused pancreas and isolated islets, the insulinosstatic ability of ghrelin was blunted when cellular cAMP level was clamped upward with a cAMP analog and downward with an adenylate cyclase inhibitor. The adenylyl cyclase inhibitor itself mimicked the insulinosstatic ghrelin action. Ghrelin, moreover, reduced the glucose-induced cAMP production in isolated islets, whereas blockade of endogenous ghrelin with a ghrelin receptor antagonist and an antiseraum enhanced it. Since cAMP was measured in the presence of PDE inhibitor, it is likely that ghrelin affects cAMP levels primarily via interacting with the step of cAMP synthesis rather than degradation. Furthermore, evanescent-wave microscopy and two-photon confocal microscopy demonstrated that ghrelin directly interacts with MIN6 and rat β-cells to decrease glucose-induced increases in [cAMP]; and [PKA].

In pancreatic β-cells, Kv channels repolarize cells and attenuate glucose-stimulated action potentials, limiting Ca^{2+} entry through voltage-dependent Ca^{2+} channels to suppress insulin secretion (22). We previously reported that ghrelin activates Kv channels via PTX-sensitive mechanisms to cause rapid repolarization and shortening of bursting action potentials, thereby attenuating glucose-induced [Ca^{2+}] increases and insulin secretion (15). In the current study, we found that the action of ghrelin to suppress glucose-induced [Ca^{2+}] increases was blunted in the β-cells whose cAMP levels were clamped upward by a cAMP analog and downward by an adenylate cyclase inhibitor (Fig. 5). It is known that cAMP potentiates insulin secretion by both PKA-dependent and Epac-dependent mechanisms. 6-Phe-cAMP is a selective activator of PKA (23), and 8-pCPT-2′-O-Me-cAMP relatively activates Epac with high affinity (24). Ghrelin failed to suppress glucose-induced [Ca^{2+}] increases in β-cells and insulin release in isolated islets in the presence of 6-Phe-cAMP (10 μmol/L), whereas 8-pCPT-2′-O-Me-cAMP (30 μmol/L) did not alter the effect of ghrelin on [Ca^{2+}] in β-cells. In consideration of the reports that higher concentrations of cAMP are required for activating Epac pathway (25–27), it is likely that the glucose-induced elevation of cAMP to a moderate level in β-cells is capable of activating PKA but not Epac. Ghrelin, therefore, may attenuate [Ca^{2+}] increases in β-cells at least partly by counteracting the glucose-stimulated cAMP and PKA signaling. However, our data with cAMP analogs cannot exclude an additional involvement of Epac in the ghrelin signaling because Epac activator used in the current study reportedly potentiates glucose-induced insulin release in a PKA-dependent manner (28). Furthermore, potentiation of the β-cell Kv channel currents by ghrelin was blunted when PKA activity was clamped at high levels with a 6-Phe-cAMP. The PKA activator itself inhibited the channel currents (Fig. 4E). These data suggest that ghrelin activates Kv channels by counteracting the cAMP-PKA signaling. In agreement with this finding, it has been shown that incretin hormones GLP-1, glucose-dependent insulino tropic polypeptide, and pituitary adenylyl cyclase-activating polypeptide promote cAMP generation in pancreatic β-cells to potentiate insulin secretion (29–31) and that GLP-1 receptor agonist exendin-4 suppresses Kv channel currents in rat β-cells via PKA-dependent and Epac-independent mechanisms (32). Possible additional inhibitory action of ghrelin on the cAMP-dependent exocytotic machinery remains to be established. It was reported that several hormone receptors of the G-protein G_{i/o}-family regulate the K_{ATP} channel activity and insulin exocytosis (33–35). Whether the ghrelin action could also involve these processes remains to be studied. We found that ghrelin selectively attenuated glucose-induced insulin release without altering ACh-induced insulin release at 2.8 mmol/L glucose in the perfused rat pancreas. In an apparent discrepancy, ghrelin is reported to suppress carbachol-induced insulin secretion at 5.5 mmol/L glucose, a concentration over the threshold for insulin release, in the perfused rat pancreas (36). ACh stimulates β-cells exclusively via muscarinic M3 receptor (37,38) and induces [Ca^{2+}] increases and insulin secretion in a glucose-dependent manner (39,40). Collectively, it is likely that ghrelin suppresses M3 receptor-mediated glucose-dependent insulin release by interfering with glucose signaling but not with muscarinic receptor signaling.

It has been reported that GHS-R is coupled to G_{ blatant phospholipase C signaling, leading to production of inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} release from IP_3-sensitive stores (2), and that ghrelin also activates phospholipase-C pathway for stimulating growth hormone release in pituitary cells (41,42). In contrast, the mechanisms by which GHS-R activates PTX-sensitive G_proteins potential of −70 mV was recorded in a single β-cell in the presence of 8.3 mmol/L glucose (control [black trace]) and during exposure to 10 nmol/L ghrelin (red trace). The dotted line indicates zero current level. H: Ghrelin (10 nmol/L) did not alter the current-voltage relationship for the peak Ca^{2+}-channel current density (n = 3).
FIG. 5. Ghrelin attenuates glucose-induced [Ca^{2+}]_{i} increases in β-cells in a cAMP and PKA pathway-dependent manner. A–F: Representative traces of [Ca^{2+}]_{i} in rat β-cells are expressed by dual-wavelength fura-2 fluorescence ratio (F340 to F380). A: Repeated administration of 8.3 mmol/L glucose induced [Ca^{2+}]_{i} increases repetitively and ghrelin (10 nmol/L) added 5 min prior to the second glucose stimulation attenuated the [Ca^{2+}]_{i} increases. The inhibitory effects of ghrelin were abolished by treatment with db-cAMP (1 mmol/L) (B) and with 6-Phe-cAMP (10 μmol/L) (C). D: Pretreatment of cells with MDL-12330A (10 μmol/L) suppressed [Ca^{2+}]_{i} response to glucose challenge. The inhibitory effect of ghrelin on [Ca^{2+}]_{i} responses was observed in the presence of 8-pCPT-2'-O-Me-cAMP (30 μmol/L) (E) and U-73122 (1 μmol/L) (F). G: The S2-to-S1 ratio of the peak [Ca^{2+}]_{i} response to the second 8.3 mmol/L glucose stimulation (S2) to that to the first glucose stimulation (S1). The data were taken only from insulin-positive cells. *P < 0.05, **P < 0.01 vs. control; †P < 0.05, ††P < 0.01 vs. 8.3 mmol/L glucose alone (n = 66–93).
and decreases cAMP in β-cells are unclear. PTX-sensitive ghrelin signaling is reported in the guinea pig femoral artery smooth muscle cells (43). G-protein–coupled receptors often activate more than one type of G-protein (44). Hence, the ghrelin interaction with GHS-R could activate distinct classes of G-protein signaling including G_{11}- and G_{i}-mediated pathways in a tissue-specific manner. Alternatively, the isoforms of GHS-R (45,46) or heterodimer of GHS-R with other receptors (47) could access the G_{i}-protein complex. The ghrelin receptor in β-cells remains to be further characterized.

In conclusion, ghrelin attenuates the glucose-induced cAMP production and PKA activation, which drives activation of K{\text{y}} channels and suppression of the glucose-induced [Ca^{2+}] increase and insulin release. This is a novel ghrelin signaling that operates in islet β-cells, being distinct from that reported in other tissues including pituitary growth hormone cells. It has been shown that ghrelin receptor antagonists and GOAT inhibitor increase the insulin release and decrease blood glucose in glucose tolerance tests, thereby acting as antidiabetic (5,12,15,48–50). The current study demonstrated that the ghrelin’s signaling cascade and consequent inhibition of insulin release are mediated by attenuation of the cAMP pathway. Hence, counteracting the attenuation of cAMP could provide a new strategy to counteract insulinostatic ghrelin action and thereby to treat type 2 diabetes.

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REFERENCES

1. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656–660
2. Howard AD, Feighner SD, Cully DF, et al. A receptor in putatory and hypothalamus that functions in growth hormone release. Science 1996;273:974–977
3. Kojima M, Kangawa K. Drug insight: the functions of ghrelin and its potential as a multitherapeutic hormone. Nat Clin Pract Endocrinol Metab 2006;2:80–88
4. Granatpan S, Kola B, Bustin SA, et al. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. J Clin Endocrinol Metab 2002;87:2988–2991
5. Dezaki K, Hosoda H, Kakei M, et al. Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca^{2+} signaling in β-cells: implication in the glycemic control in rodents. Diabetes 2004;53:3142–3151
6. Kageyama H, Funahashi H, Hirayama M, et al. Morphological analysis of ghrelin and its receptor distribution in the rat pancreas. Regul Pept 2005;126:267–71
7. Gutierrez JA, Solenberg PJ, Perkins DR, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci USA 2008;105:6320–6325
8. Yang J, Brown MS, Liang G, Grishin NV, Goldstein JL. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 2008;132:387–396
9. An W, Li Y, Xu G, et al. Modulation of ghrelin O-acetyltransferase expression in pancreatic islets. Cell Physiol Biochem 2010;26:707–716
10. Reimer MK, Pacini G, Ahrén B. Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. Endocrinology 2003;144:916–921
11. Tong J, Prigeon RL, Davis HW, et al. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. Diabetes 2010;59:2145–2151
12. Dezaki K, Sone H, Koizumi M, et al. Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. Diabetes 2006;55:3486–3493
13. Yada T, Dezaki K, Sone H, et al. Ghrelin regulates insulin release and glycemia: physiological role and therapeutic potential. Curr Diabetes Rev 2008;4:18–23
14. Dezaki K, Sone H, Yada T. Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. Pharmacol Ther 2008;118:239–249
15. Dezaki K, Kakei M, Yada T. Ghrelin uses G_{b_{12}} and activates voltage-dependent K{\text{y}} channels to attenuate glucose-induced Ca^{2+} signaling and insulin release in islet β-cells: novel signal transduction of ghrelin. Diabetes 2007;56:2319–2326
16. Henquín JC. Triggering and amplifying pathways of regulation of insulin secretion by ghrelin. Diabetes 2000;49:1751–1760
17. Prentki M, Matschinsky FM. Ca^{2+}, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion. Physiol Rev 1987;67:1185–1248
18. Dyachok O, Ilevall-Hagren O, Sägertop J, et al. Ghreline-induced cyclic AMP oscillations regulate pulsatile insulin secretion. Cell Metab 2008;8:26–37
19. Dyachok O, Isakov Y, Sägertop J, Tengholm A. Oscillations of cyclic AMP in hormone-stimulated insulin-secreting β-cells. Nature 2006;439:349–352
20. Hashiguchi S, Yada T, Arima T. A new hypoglycemic agent, JTT-608, evokes protein kinase A-mediated Ca^{2+} signaling in rat islet β-cells: strict regulation by glucose, link to insulin release, and cooperation with glucagon-like peptide-1(7–36)amide and pituitary adenylate cyclase-activating polypeptide. J Pharmacol Exp Ther 2001;296:22–30
21. Higashi H, Sato K, Ohtake A, Omori A, Yoshida S, Kudo Y. Imaging of cAMP oscillations in islet β-cells: strict regulation by glucose, link to insulin release, and cooperation with glucagon-like peptide-1(7–36)amide and pituitary adenylate cyclase-activating polypeptide. J Pharmacol Exp Ther 2001;296:22–30
22. MacDonald PE, Wheeler MB. Voltage-dependent K{\text{y}} channels in pancreatic β-cell role, regulation and potential as therapeutic targets. Diabetologia 2003;46:1046–1062
23. Christensen AE, Selheim F, de Rooij J, et al. cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. J Biol Chem 2003;278:35394–35402
24. Enserink JM, Christensen AE, de Rooij J, et al. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol 2002;4:901–906
25. Bubis J, Neitzel JJ, Saraswat LD, Taylor SS. A point mutation abolishes binding of cAMP to site A in the regulatory subunit of cAMP-dependent protein kinase. J Biol Chem 1988;263:9668–9673
26. Ringheim GE, Taylor SS. Effects of cAMP-binding site mutations on intradomain communication in the regulatory subunit of cAMP-dependent protein kinase I. J Biol Chem 1990;265:19472–19478
27. de Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem 2006;281:20829–20836
28. Chepurny OG, Kelley GG, Dzhura I, et al. PKA-dependent potentiation of glucagon-stimulated insulin secretion by Epac activator 8pCPT-2′-O-Me-cAMP-AM
in human islets of Langerhans. Am J Physiol Endocrinol Metab 2010;298:E622–E633
29. Drucker DJ. Glucagon-like peptides. Diabetes 1998;47:159–169
30. Gremlich S, Porret A, Han E, et al. Cloning, functional expression, and chromosomal localization of the human pancreatic islet glucose-dependent insulinotropic polypeptide receptor. Diabetes 1995;44:1202–1208
31. Klinteberg KA, Karlsson S, Ahrén B. Signaling mechanisms underlying the insulinotropic effect of pituitary adenylate cyclase-activating polypeptide in HIT-T15 cells. Endocrinology 1996;137:2791–2798
32. MacDonald PE, Wang X, Xia F, et al. Antagonism of rat β-cell voltage-dependent K+ currents by exendin 4 requires dual activation of the cAMP/protein kinase A and phosphatidylinositol 3-kinase signaling pathways. J Biol Chem 2003;278:52446–52453
33. Dunne MJ, Bullett MJ, Li GD, Wollheim CB, Petersen OH. Galanin activates nucleotide-dependent K+ channels in insulin-secreting cells via a pertussis toxin-sensitive G-protein. EMBO J 1989;8:413–420
34. Sharp GW. Mechanisms of inhibition of insulin release. Am J Physiol 1996;271:C1781–C1799
35. Wang Y, Park S, Bajpayee NS, et al. Augmented glucose-induced insulin release in mice lacking Go2, but not Go1 or Gi proteins. Proc Natl Acad Sci USA 2011;108:1693–1698
36. Egido EM, Rodriguez-Gallardo J, Silvestre RA, Marco J. Inhibitory effect of ghrelin on insulin and pancreatic somatostatin secretion. Eur J Endocrinol 2002;146:23–29
37. Hennquin JC, Nenguin M. The muscarinic receptor subtype in mouse pancreatic B-cells. FEBS Lett 1988;236:89–92
38. Gautam D, Han SJ, Hamdan FF, et al. A critical role for β cell M3 muscarinic acetylcholine receptors in regulating insulin release and blood glucose homeostasis in vivo. Cell Metab 2006;3:440–461
39. Hermans MP, Henquin JC. Relative importance of extracellular and intracellular Ca2+ for acetylcholine stimulation of insulin release in mouse islets. Diabetes 1989;38:198–204
40. Yada T, Hamakawa N, Yaekura K. Two distinct modes of Ca2+ signalling by ACh in rat pancreatic β-cells: concentration, glucose dependence and Ca2+ origin. J Physiol 1995;488:13–24
41. Glavaski-Joksimovic A, Jeftinija K, Scanen CG, Anderson LL, Jeftinija S. Stimulatory effect of ghrelin on isolated porcine somatotropes. Neuroendocrinology 2003;77:367–379
42. Malagón MM, Luque RM, Ruiz-Guerrero E, et al. Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. Endocrinology 2003;144:5372–5380
43. Mladenov MI, Hristov Kl, Dimitrova DZ, et al. Ghrelin signalling in guineapig femoral artery smooth muscle cells. Acta Physiol (Oxf) 2008;194:195–206
44. Hermans E. Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. Pharmacol Ther 2003;99:25–44
45. Geelissen SM, Beck IM, Darras VM, Kuhn ER, Van der Geyten S. Distribution and regulation of chicken growth hormone secretagogue receptor isoforms. Gen Comp Endocrinol 2003;134:167–174
46. Kineman RD, Gahete MD, Luque RM. Identification of a mouse ghrelin gene transcript that contains intron 2 and is regulated in the pituitary and hypothalamus in response to metabolic stress. J Mol Endocrinol 2007;38:511–521
47. Jiang H, Betancourt L, Smith RG. Ghrelin amplifies dopamine signaling by cross talk involving formation of growth hormone secretagogue receptor/dopamine receptor subtype 1 heterodimers. Mol Endocrinol 2006;20:1772–1785
48. Sun Y, Asnicar M, Saha PK, Chan L, Smith RG. Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. Cell Metab 2006;3:379–386
49. Esser WP, Rudolph J, Claus TH, et al. Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss. Endocrinology 2007;148:5175–5185
50. Barnett BP, Hwang Y, Taylor MS, et al. Glucose and weight control in mice with a designed ghrelin O-acyltransferase inhibitor. Science 2010;330:1689–1692