Original Article

Ghrelin Uses $G\alpha_{i2}$ and Activates Voltage-Dependent K^+ Channels to Attenuate Glucose-Induced Ca^{2+} Signaling and Insulin Release in Islet β-Cells

Novel Signal Transduction of Ghrelin

Katsuya Dezaki,[1] Masafumi Kakei,[2] and Toshihiko Yada[1]

Ghrelin reportedly serves as a physiological regulator of insulin release. This study aimed to explore signaling mechanisms for insulinostatic ghrelin action in islet β-cells, with special attention to heterotrimeric GTP-binding proteins and K^+ channels. Plasma insulin and growth hormone (GH) concentrations in rats were measured by enzyme-linked immunosorbent assay (ELISA). Islets were isolated from rats, ghrelin-knockout (Ghr-KO) mice, and wild-type mice by collagenase digestion, and insulin release was determined by ELISA. In rat single β-cells, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by fura-2 microfluorometry, and membrane potentials and whole cell currents by patch-clamp technique. In rats, systemic ghrelin administration decreased plasma insulin concentrations, and this effect was blocked by treatment with pertussis toxin (PTX), whereas stimulation of GH release remained unaffected. In rat islets, ghrelin receptor antagonist increased and exogenous ghrelin suppressed glucose-induced insulin release in a PTX-sensitive manner. Glucose-induced insulin release from islets was greater in Ghr-KO than wild-type mice, and this enhanced secretion was blunted with PTX. Ghrelin PTX sensitively increased voltage-dependent K^+ (Kv) currents without affecting ATP-sensitive K^+ channels in rat β-cells. In the presence of Kv channel blockers, ghrelin failed to suppress insulin release. Ghrelin attenuated glucose-induced action potentials and $[Ca^{2+}]_i$ increases in β-cells. Suppressions of $[Ca^{2+}]_i$ increase and insulin release by ghrelin were blunted in β-cells treated with PTX and with antisense oligonucleotide specific for G-protein $G\alpha_{i2}$-subunit. Ghrelin attenuates glucose-induced insulin release via PTX-sensitive $G\alpha_{i2}$-mediated activation of Kv channels and suppression of $[Ca^{2+}]_i$ in β-cells, representing the unique signaling of ghrelin distinct from that for GH release.
***Diabetes* 56:2319–2327, 2007**

Ghrelin, a 28-amino acid gastric-derived hormone, was discovered as the endogenous ligand (1) for the orphan growth hormone (GH) secretagogue-receptor (GHS-R) (2). Ghrelin potently stimulates GH release and feeding and exhibits positive cardiovascular effects, suggesting its possible clinical application (3). Ghrelin and GHS-R are also located in pancreatic islets (4–11). Ghrelin inhibits insulin release in mice, rats, and humans (4,12–14). Low plasma ghrelin levels are associated with elevated fasting insulin levels and insulin resistance in humans (15,16). These findings suggest both physiological and pathophysiological roles for ghrelin in insulin release. In previous reports (4,17), we used the isolated islets and perfused pancreas of rats and found that GHS-R blockade and antiserum against ghrelin markedly enhanced glucose-induced insulin release, whereas exogenous ghrelin suppressed it. Furthermore, ghrelin-knockout (Ghr-KO) mice displayed enhanced glucose-induced insulin release from isolated islets, whereas islet density, size, insulin content, and insulin mRNA levels were unaltered, indicating an increased secretory activity (17). These data indicate that islet-derived ghrelin downregulates glucose-induced insulin release. Furthermore, high-fat diet produced glucose intolerance in wild-type mice; however, it was prevented in ghrelin-KO mice because of enhanced insulin secretory response (17). These findings suggest that manipulation of ghrelin action could provide a novel tool to optimize insulin release and thereby treat type 2 diabetes. Therefore, it is important to elucidate the signaling mechanisms by which ghrelin regulates insulin release.

We previously provided data to support a possible involvement of voltage-dependent K^+ (Kv) channels and Ca^{2+} signaling in the insulinostatic action of ghrelin in β-cells (4). However, the mechanisms that couple GHS-R to these electrophysiological activities in β-cells are unknown. Expression study revealed that GHS-R is coupled to G_{11}-phospholipase C (PLC) signaling, leading to production of inositol triphosphate (IP_3) and Ca^{2+} release from IP_3-sensitive store (2). Synthetic GHSs and ghrelin evoke

From the [1]Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, Tochigi, Japan; and the [2]Department of Internal Medicine, Saitama Medical Center, Jichi Medical University, Saitama, Japan.

Address correspondence and reprint requests to Toshihiko Yada, Division of Integrative Physiology, Department of Physiology, Jichi Medical University School of Medicine, Yakushiji 3311-1, Shimotsuke, Tochigi 329-0498, Japan. E-mail: tyada@jichi.ac.jp.

Received for publication 16 March 2007 and accepted in revised form 8 June 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 15 June 2007. DOI: 10.2337/db07-0345.

$[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; db-cAMP, dibutylyl-cyclic AMP; ELISA, enzyme-linked immunosorbent assay; GH, growth hormone; GHS-R, growth hormone secretagogue-receptor; GTT, glucose tolerance test; HKRB, HEPES-added Krebs-Ringer bicarbonate buffer; IAP, islet-activating protein; IP_3, inositol triphosphate; JSPS, Japan Society for the Promotion of Science; K_{ATP} channel, ATP-sensitive K^+ channel; Kv current/channel, voltage-dependent K^+ current/channel; PLC, phospholipase C; PTX, pertussis toxin; ScTx, stromatoxin; TEA, tetraethylammonium.

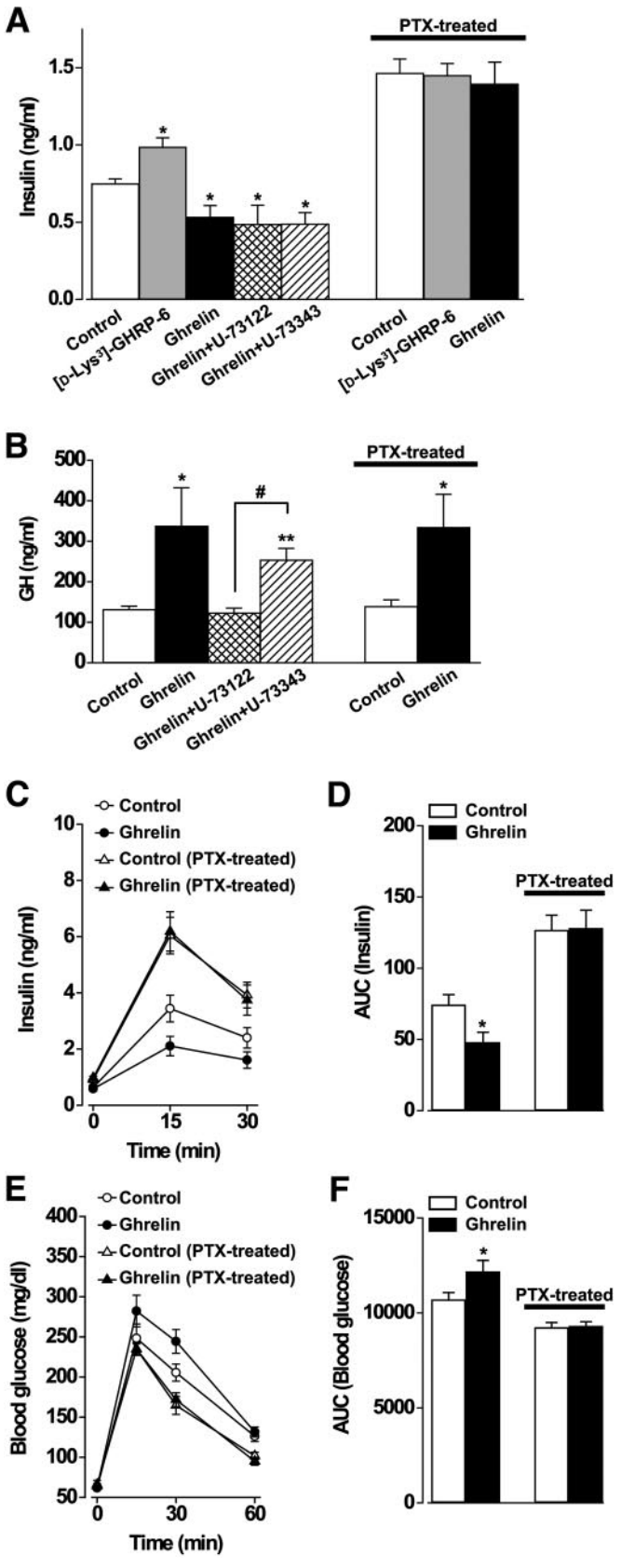

FIG. 1. Ghrelin decreases plasma insulin concentrations in a PTX-sensitive manner. *A*: A GHS-R antagonist, [D-Lys^3]-GHRP-6 (10 μmol/kg), increased plasma insulin concentrations at 30 min after intraperitoneal administration in rats fasted overnight, indicating suppression of insulin levels by endogenous ghrelin. Conversely, administration of ghrelin (10 nmol/kg i.p.) significantly decreased plasma insulin levels. The insulinostatic effect of ghrelin was unaltered by pretreatment with PLC inhibitor U-73122 (2 μmol/kg i.p.) and its inactive compound U-73343 (2 μmol/kg i.p.). The effects of endogenous and exogenous ghrelin on insulin levels were not observed in PTX (5 μg/kg i.v.)-treated rats. Data represent means ± SE of eight rats. *P < 0.05 vs. control. *B*: Administration of ghrelin (10 nmol/kg i.p.) increased plasma GH concentrations at 15 min. The ghrelin-induced GH release was inhibited by pretreatment with U-73122 (2 μmol/kg i.p.) but not an inactive analog U-73343 (2 μmol/kg i.p.). The ghrelin-induced GH release was unaltered in PTX-treated rats (n = 8). *P < 0.05; **P < 0.01 vs. control, #P < 0.05. *C* and *E*: Ghrelin (10 nmol/kg i.p.) decreased plasma insulin levels (*C*) and increased blood glucose levels (*E*) during GTTs (glucose, 2 g/kg i.p.) in normal rats but failed to do so in rats treated with PTX (5 μg/kg i.v.) (n = 8–10). *D* and *F*: Ghrelin attenuated the integrated insulin release for 30 min (*D*) and enhanced the integrated blood glucose concentrations for 60 min (*F*) after glucose challenge, as expressed by the area under the curve (AUC) in control rats, whereas these changes were not observed in PTX-treated rats (n = 8–10). *P < 0.05 vs. control.

GH release via activation of PLC-mediated increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in pituitary cells (18–22). However, ghrelin-induced attenuation of insulin release appears not to be mediated by PLC signaling pathways, because PLC activation operated by muscarinic acetylcholine and GPR40 receptors potentiates, but does not attenuate, glucose-induced increases in $[Ca^{2+}]_i$ and insulin release in β-cells (23,24). Thus, ghrelin may elicit different signaling pathways in a tissue-specific manner, which could underlie the opposing effects of ghrelin on GH release and insulin release. Heterotrimeric G-proteins, which consist of α,- β-, and γ-subunits, function as signal transducers that link the membrane receptor activation to intracellular effectors. It is known that pertussis toxin (PTX) specifically ADP-ribosylates the α-subunit of G_i family of G-proteins ($G\alpha_i$) and thereby abolishes its ability to be linked to G-protein–coupled receptors. We show here a novel signaling mechanism for ghrelin that operates in islet β-cells; PTX-sensitive G_i-protein links GHS-R to activation of Kv channels and attenuation of glucose-induced $[Ca^{2+}]_i$ increase and insulin release.

RESEARCH DESIGN AND METHODS

Male Wistar rats (Japan SLC, Hamamatsu, Japan), Ghr-KO mice (17), and wild-type C57BL/6J mice (Charles River Laboratories Japan, Yokohama, Japan) were housed in accordance with our institutional guidelines and with the Japanese Physiological Society's guidelines for animal care. PTX (5 μg/kg) (Sigma-Aldrich, St. Louis, MO) or saline was intravenously injected. Three days after injections, animals were used for in vivo and in vitro studies.

Measurements of plasma insulin and GH concentrations. Ghrelin (Peptide Institute, Osaka, Japan) and [D-Lys^3]-GHRP-6 (Sigma-Aldrich) were administered intraperitoneally to male Wistar rats (8 weeks old) after overnight fasting. U-73122 (Wako Pure Chemical, Osaka, Japan) and U-73343 (Wako) were administered intraperitoneally 15 min before ghrelin administration. In glucose tolerance tests (GTTs), 2 g/kg glucose with or without ghrelin (10 nmol/kg) was injected intraperitoneally into rats. Blood was obtained from tails, and plasma insulin and GH concentrations were measured using ELISA kits (Morinaga Institute of Biological Science, Yokohama, Japan) and enzyme immunoassay kits (GE Healthcare Bio-Science, Piscataway, NJ), respectively.

Preparation of pancreatic islets and single β-cells from rats and mice. Islets of Langerhans were isolated by collagenase digestion from male Wistar rats aged 8–12 weeks and male Ghr-KO and wild-type mice aged 10 weeks, as reported previously (4,25) with slight modification. Animals were anesthetized by intraperitoneal injection of pentobarbitone at 80 mg/kg, followed by injection of collagenase at 1.05 mg/ml (Sigma-Aldrich) into the common bile duct. Collagenase was dissolved in 5 mmol/l Ca^{2+}-containing HEPES-added Krebs-Ringer bicarbonate buffer (HKRB) solution (129 mmol/l NaCl, 5 mmol/l $NaHCO_3$, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 2 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, and 10 mmol/l HEPES, at pH 7.4 with NaOH). HKRB was added with 0.1% BSA except for patch-clamp experiments. The pancreas was dissected out and incubated at 37°C for 16 min. Islets were collected and either used for insulin release experiments or dispersed into single cells in Ca^{2+}-free HKRB. The single cells were plated sparsely on coverslips or 12-well plates and maintained in short-term culture for up to 2 days at 37°C in an atmosphere of 5% CO_2 and 95% air in Eagle's minimal essential medium

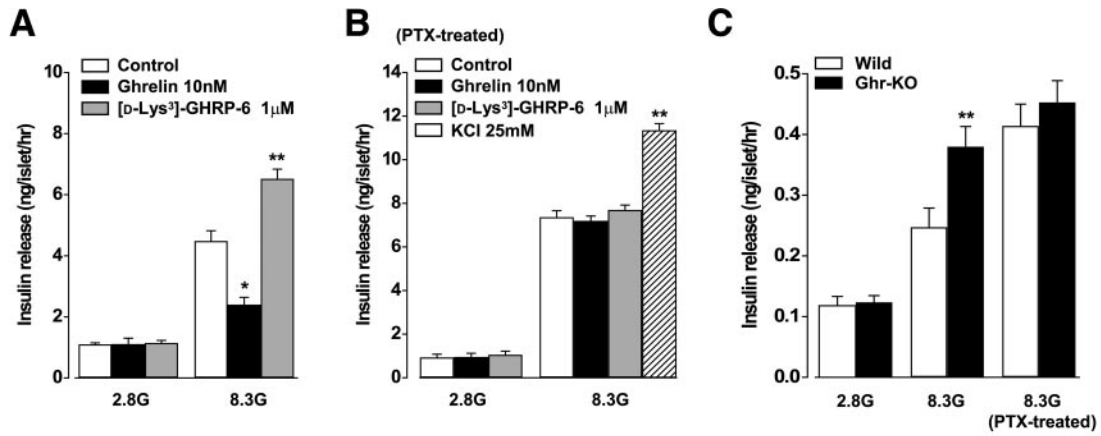

FIG. 2. *A*: [D-Lys^3]-GHRP-6 (1 μmol/l) enhanced, whereas exogenous ghrelin (10 nmol/l) inhibited, glucose (8.3 mmol/l)-induced insulin release in islets isolated from rats (n = 10). *P < 0.05; **P < 0.01 vs. control. *B*: Neither ghrelin (10 nmol/l) nor [D-Lys^3]-GHRP-6 (1 μmol/l) altered glucose (8.3 mmol/l)-induced insulin release in islets isolated from PTX-treated rats (n = 7). The addition of 25 mmol/l KCl enhanced insulin release as a positive control (n = 8). **P < 0.01 vs. control. *C*: Glucose (8.3 mmol/l)-induced insulin release was greater in islets isolated from Ghr-KO mice than those from wild-type mice. This enhancement in islets of Ghr-KO mice was blunted in islets pretreated with PTX (n = 11–12). Extent of the enhancement of glucose-induced insulin release with PTX was somewhat larger than that seen in Ghr-KO mice. **P < 0.01 vs. wild-type mice (Wild).

containing 5.6 mmol/l glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin.

Measurements of insulin release in rat and mouse islets. 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. Ghrelin, [D-Lys^3]-GHRP-6, tetraethylammonium (TEA; Wako), or stromatoxin (ScTx; Sigma-Aldrich) was present throughout the incubation. As a positive control, islets were stimulated by addition of 25 mmol/l KCl (final concentration 29.7 mmol/l) in HKRB in the presence of 8.3 mmol/l glucose. Insulin concentrations were determined by ELISA (Morinaga Institute of Biological Science).

Measurements of $[Ca^{2+}]_i$ in rat single β-cells. Isolated and dissociated single β-cells on coverslips were mounted in an open chamber and superfused in HKRB. $[Ca^{2+}]_i$ 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. The ratio image was produced on an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan).

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 reported (26). Membrane currents, recorded using an amplifier (200B; Axopatch, Foster, CA), were stored online in a computer using pCLAMP9 software. Voltage clamp in perforated mode was considered to be adequate when the series resistance was <20 MΩ. Patch pipettes were pulled from glass tubings (Narishige, Tokyo, Japan); the resistances of the pipettes ranged from 4 to 7 MΩ when filled with pipette solution that contained 40 mmol/l K_2SO_4, 50 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.5 mmol/l EGTA, and 10 mmol/l HEPES at pH 7.2 with KOH. Cell capacitance recorded from single β-cells was 7.7 ± 0.22 pF (n = 64). β-Cells were voltage-clamped at a holding potential of −70 mV and then shifted to test potentials from −60 to 40 mV in 10-mV steps with pulses of

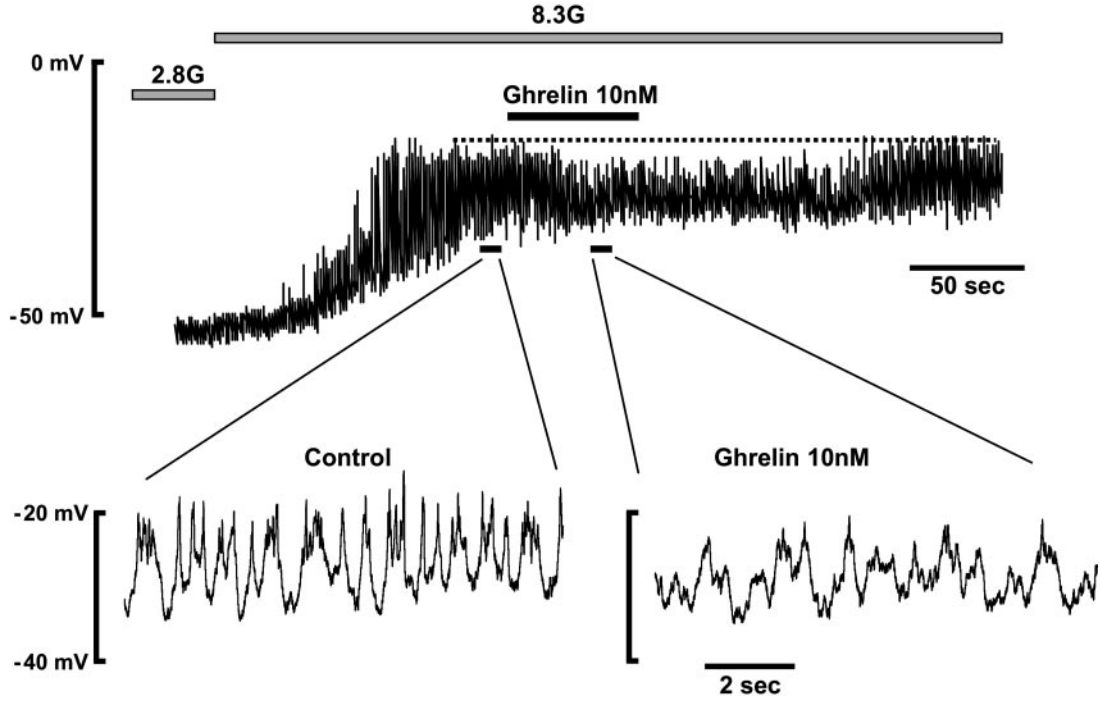

FIG. 3. Ghrelin decreases action potential firing in rat islet β-cells. *Top*: After establishing a perforated whole-cell clamp in HKRB solution containing 2.8 mmol/l glucose, membrane potentials in β-cells were continuously monitored in current-clamp mode. Glucose (8.3 mmol/l) elicited membrane depolarization associated with subsequent action potential firings, and ghrelin attenuated the firing in a reversible manner in a β-cell. *Bottom*: Action potential profiles in specified periods at top traces were shown in an expanded timescale. The results are representative of three cells.

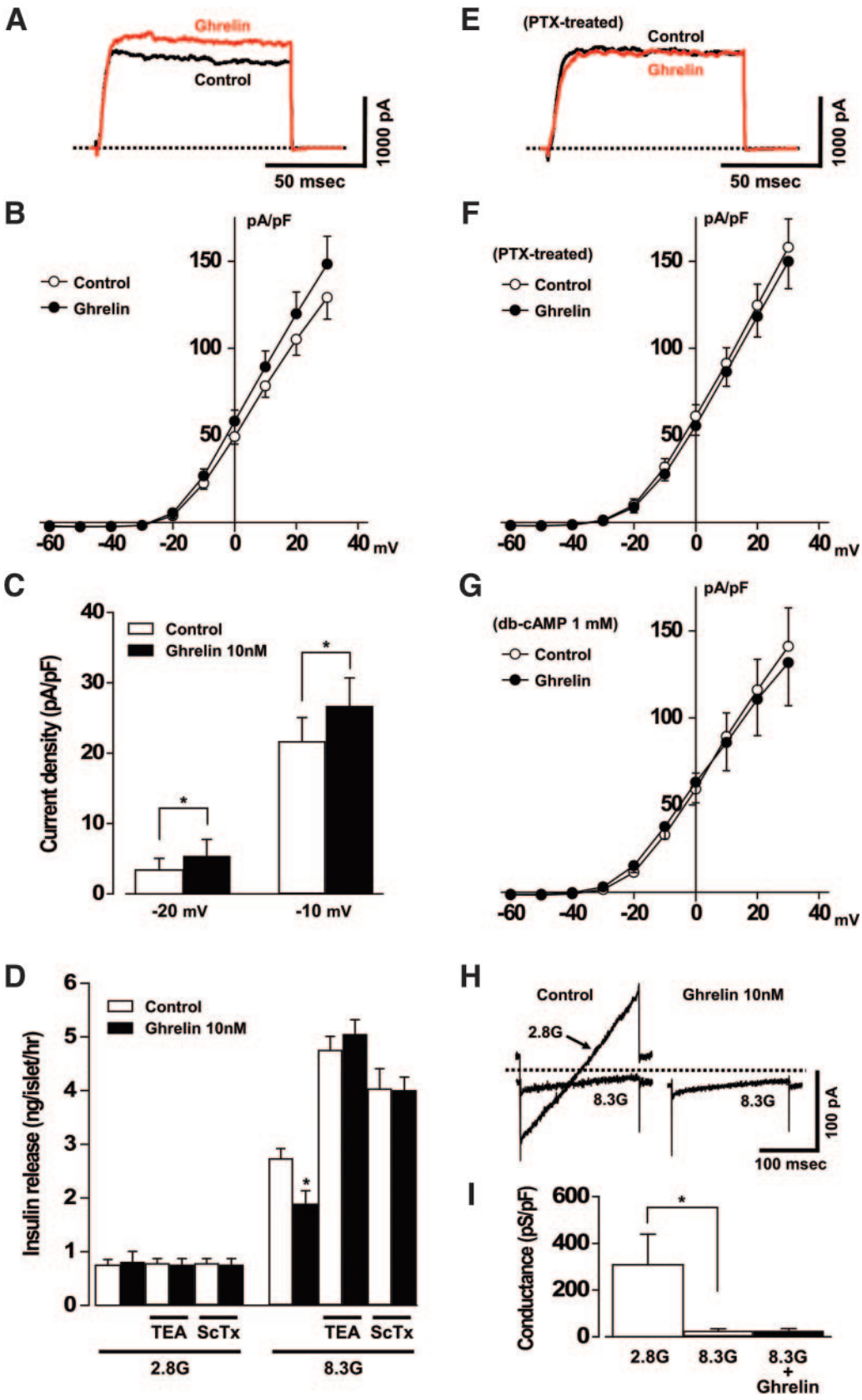

FIG. 4. Ghrelin enhances Kv-channel conductance in rat islet β-cells, and these changes are linked to ghrelin-induced attenuation of glucose-induced insulin release. *A*: Current traces evoked by a step pulse to 20 mV from a holding potential of −70 mV were displayed in perforated whole-cell clamp mode. Data were recorded after exposure to 8.3 mmol/l glucose in the presence of 100 μmol/l tolbutamide (control; black trace) and during exposure to 10 nmol/l ghrelin (red trace). The dotted lines indicate zero current level. Exposure to ghrelin increased the amplitudes of delayed outward currents. *B*: Current levels measured at the end of test pulses in control and ghrelin-treated cells were plotted as a current density (pA/pF), which was the value of currents normalized by cell capacitance, against membrane potentials (n = 7). *C*: Peak amplitudes of current density at −20 and −10 mV of membrane potentials. Ghrelin (10 nmol/l) significantly activated delayed outward Kv

100-ms durations at 5- to 8-s intervals. Because the ATP-sensitive K^+ (K_{ATP}) channel has voltage-independent kinetics and is a major determinant of resting potential of β-cells, the currents elicited by voltages around the resting potential are those through the K_{ATP} channels. Thus, to record the K_{ATP} channel current, the β-cells were voltage-clamped to the holding potential of −70 mV, stepped to −100 mV to apply voltage ramp from −100 to −50 mV at a speed of 25 mV/100 ms, and stepped back to −70 mV every 10 s. The currents evoked by the ramp pulses during superfusion with 2.8 mmol/l glucose were substantially inhibited by exposure to tolbutamide or by increasing glucose concentration to 8.3 mmol/l. These properties are consistent with features of K_{ATP} channels. The membrane potentials were recorded by switching to current-clamp mode from whole-cell clamp mode on the amplifier. Electrophysiological experiments were performed at room temperature (25°C).

Antisense study. Antisense oligonucleotides specific for the $G\alpha_{i1}$-, $G\alpha_{i2}$-, and $G\alpha_{i3}$-subunits of G-proteins were obtained from BIOGNOSTIK (Göttingen, Germany). Dissociated single β-cells were incubated for 48 h with 5 μmol/l antisense or control oligonucleotide in tissue culture medium at 37°C. Cells were used for $[Ca^{2+}]_i$ and insulin release studies. Knockdown of each protein was determined by immunocytochemical analysis using monoclonal antibodies as follows. Cells were fixed in 4% paraformaldehyde and then incubated overnight with mouse monoclonal anti-insulin antibodies (Sigma-Aldrich) at dilutions of 1:1,000 at 4°C. Samples were then incubated in Alexa Fluor 488-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR). Preparations were then incubated overnight with monoclonal antibodies against the $G\alpha_{i1}$-, $G\alpha_{i2}$-, or $G\alpha_{i3}$-subunit of G-proteins (Calbiochem, San Diego, CA) at a dilution of 1:1,000 at 4°C. Antibody binding was detected with Alexa Fluor 568–labeled goat anti-rabbit IgG (Molecular Probes). Control experiments were carried out without the primary antibodies. Immunofluorescences for insulin and G-protein were observed with photomultipliers of a multiphoton laser-scanning microscope (FluoView FV300-TP; Olympus, Tokyo, Japan).

Statistical analysis. Data represent the means ± SE. Statistical analyses were performed using the Student's *t* test or one-way ANOVA. *P* values <0.05 were considered statistically significant.

RESULTS

Ghrelin decreases plasma insulin concentrations in a PTX-sensitive manner. Intraperitoneal administration of ghrelin (10 nmol/kg) decreased plasma insulin concentrations at 30 min in rats fasted overnight (Fig. 1*A*). Conversely, intraperitoneal administration of a specific GHS-R antagonist, [D-Lys^3]-GHRP-6 (10 μmol/kg), significantly increased plasma insulin levels, reflecting counteraction of endogenous ghrelin activity to inhibit insulin release. On the other hand, ghrelin (10 nmol/kg i.p.) increased plasma GH concentrations at 15 min (Fig. 1*B*), and this effect was completely blocked by treatment with a PLC inhibitor U-73122 (2 μmol/kg i.p.), but not with its inactive analog U-73343 (2 μmol/kg i.p.). These effects are consistent with the current concept that the effects of ghrelin are mediated by G_{11}-PLC signaling (2). In contrast, the ghrelin action to inhibit insulin release was unaffected by the PLC inhibitor (Fig. 1*A*). The insulinostatic effects of both endogenous and exogenous ghrelin were blunted in rats treated with PTX (5 μg/kg), whereas ghrelin-induced GH release was unaltered (Fig. 1*A* and *B*). Thus, the G-protein coupling for the action of ghrelin on insulin release is distinct from that on GH release.

To further confirm the PTX sensitivity of the insulinostatic function of ghrelin, we examined GTTs in rats fasted overnight. In control experiments without PTX, ghrelin (10 nmol/kg i.p.) injected together with glucose (2 g/kg) markedly attenuated plasma insulin responses (Fig. 1*C* and *D*) and enhanced blood glucose responses (Fig. 1*E* and *F*) to GTT in comparison with control values without ghrelin. In rats treated with PTX (5 μg/kg), insulin responses during glucose challenge were higher and glucose responses were lower than those in control rats, and the actions of ghrelin to attenuate insulin responses and to enhance glucose responses were not observed (Fig. 1*C–F*).

Insulinostatic effects of endogenous ghrelin in islets. In isolated rat islets, 8.3 mmol/l glucose-induced insulin release was augmented by GHS-R blockade, whereas it was inhibited by exogenous ghrelin (Fig. 2*A*), as previously reported. These effects of endogenous and exogenous ghrelin were blunted in islets isolated from PTX-treated rats, whereas addition of 25 mmol/l KCl enhanced insulin release from these islets (Fig. 2*B*).

Glucose (8.3 mmol/l)-induced insulin release from isolated islets of Ghr-KO mice was significantly greater than that of control mice (Fig. 2*C*), as already reported. The enhancement of glucose-induced insulin release from islets of Ghr-KO mice was undetectable after treatment with PTX (Fig. 2*C*), indicating that endogenous ghrelin attenuates insulin release via PTX-sensitive G-proteins.

Ghrelin decreases electrical activities in β-cells. We next electrophysiologically examined the effects of ghrelin in β-cells. Under the condition of perforated whole-cell current-clamp mode, glucose (8.3 mmol/l) elicited firings of action potentials in β-cells. The firings were characterized by spike-like and repetitively occurring action potentials overlapping on slowly depolarized wave as depicted in the time-expanded scale (Fig. 3, control inset). These electrical firings were attenuated by ghrelin administration in a reversible manner (Fig. 3). Ghrelin decreased both the frequency and amplitude of the firings (Fig. 3, right inset). Mean membrane potentials measured at most repolarized levels between slow-wave potentials at 8.3 mmol/l glucose were not significantly changed by ghrelin (−38.1 ± 0.9 mV for control vs. −36.9 ± 4.5 mV for ghrelin treatment, n = 3). These findings suggest that the ghrelin administration does not hyperpolarize the membrane potential but decreases the activity of action potentials in β-cells.

Ghrelin PTX sensitively activates Kv channels but not K_{ATP} channels in β-cells. In the presence of 8.3 mmol/l glucose and 100 μmol/l tolbutamide, outward K^+ currents evoked by depolarizing pulses to +20 mV were increased by exposure to 10 nmol/l ghrelin (Fig. 4*A*), confirming a previous report (4). The current-voltage relationship demonstrated that ghrelin increased the amplitude of current densities through Kv channels (Fig. 4*B*). At holding potentials of −20 and −10 mV, which are in the physiological range of membrane potentials in β-cells stimulated with high glucose, ghrelin significantly enhanced Kv currents (Fig. 4*C*). We examined whether activation of Kv currents is involved in ghrelin-induced suppression of insulin release. The Kv-channel current was largely inhibited by Kv-channel blocker TEA, confirming a previous report (4). In the presence of TEA (10 mmol/l), glucose (8.3 mmol/l)-induced insulin release was potentiated, whereas basal insulin release at 2.8 mmol/l glucose was unchanged (Fig. 4*D*). Ghrelin failed to atten-

currents. *P < 0.05 by paired t test (n = 7). *D*: Ghrelin failed to decrease 8.3 mmol/l glucose-induced insulin release in the presence of nonselective Kv channel blocker TEA at 10 mmol/l and Kv2.1 channel blocker ScTx at 0.1 μmol/l (n = 8). *P < 0.05 vs. control. *E*: Current traces evoked by a step pulse to 20 mV from a holding potential of −70 mV after exposure to 8.3 mmol/l glucose in the presence of 100 μmol/l tolbutamide (control; black trace) and during exposure to 10 nmol/l ghrelin (red trace) in PTX-treated β-cells. Ghrelin failed to increase the currents in β-cells after overnight treatment with PTX (1 μg/ml). *F*: The action of ghrelin to increase amplitudes of delayed outward currents was not observed in the PTX-treated β-cells (n = 10). *G*: Ghrelin failed to potentiate Kv currents in the presence of db-cAMP at 1 mmol/l (n = 6). *H*: Ghrelin had no effects on the K_{ATP} channel currents monitored by ramp pulses from −100 to −50 mV in rat β-cells. The dotted line indicates zero current level. *I*: K_{ATP} channel conductances in the presence of 8.3 mmol/l glucose were not significantly changed by ghrelin (10 nmol/l) (n = 5). *P < 0.05.

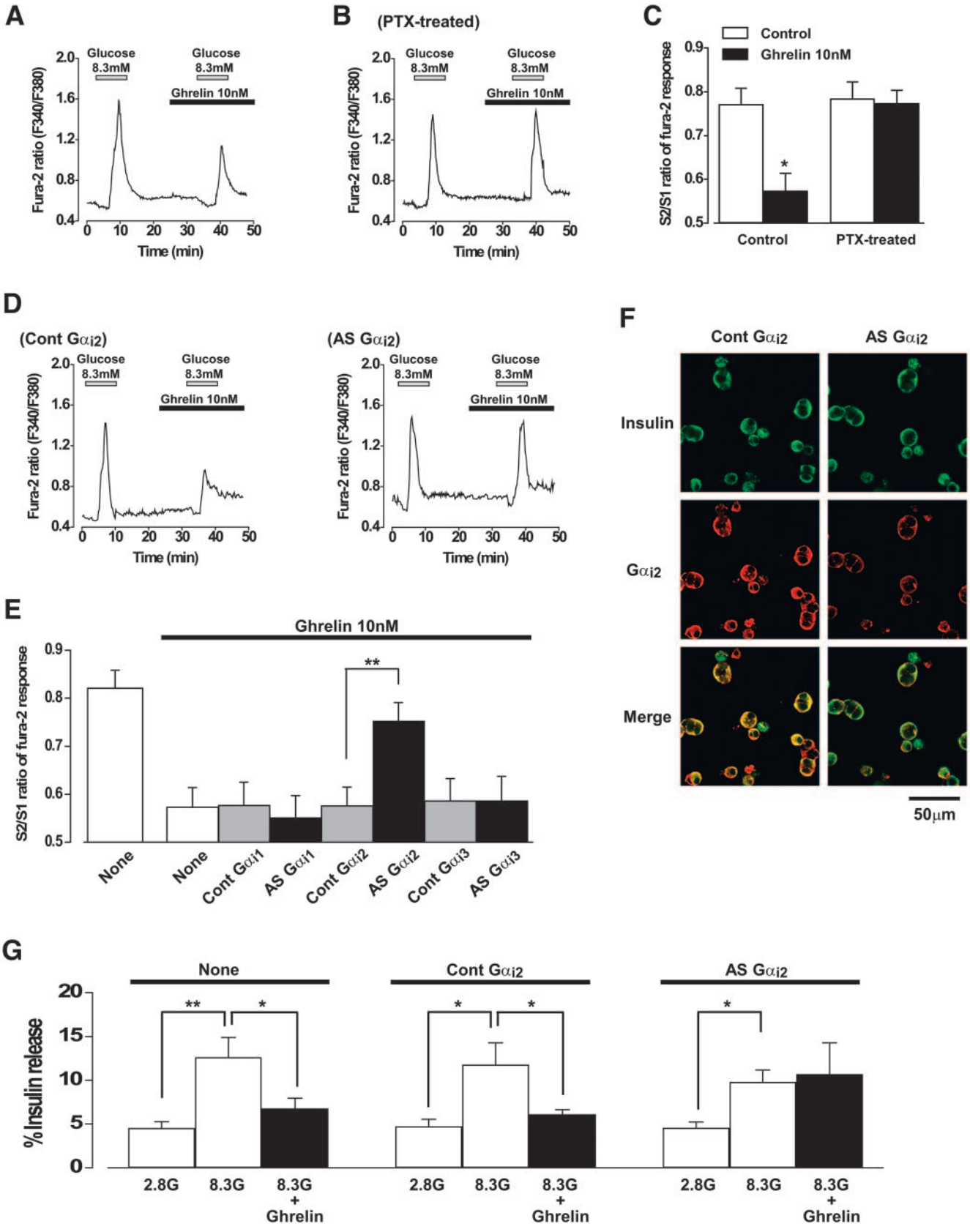

FIG. 5. Ghrelin inhibits $[Ca^{2+}]_i$ and insulin responses to glucose in rat β-cells via the PTX-sensitive and $G\alpha_{i2}$-dependent pathway. *A*: Ghrelin (10 nmol/l) attenuated the $[Ca^{2+}]_i$ responses to 8.3 mmol/l glucose. β-Cells were repetitively stimulated with glucose, whereas ghrelin was added 5 min before the second glucose stimulation. *B*: Effects of ghrelin (10 nmol/l) on $[Ca^{2+}]_i$ responses to 8.3 mmol/l glucose in β-cells after overnight treatment with PTX (1 μg/ml). *C*: The ratio of the peak $[Ca^{2+}]_i$ responses to the second glucose (8.3 mmol/l) stimulation (S2) over the first glucose stimulation (S1). Ghrelin (10 nmol/l) decreased the S2-to-S1 ratio, indicating inhibition of $[Ca^{2+}]_i$ responses to glucose. This inhibition of $[Ca^{2+}]_i$ responses was abolished in PTX-treated β-cells (n = 89–100). *$P < 0.05$ vs. control. *D*: Effects of ghrelin (10 nmol/l) on $[Ca^{2+}]_i$ responses to 8.3 mmol/l glucose in β-cells after treatment with an antisense oligonucleotide specific for the G-protein $G\alpha_{i2}$-subunit and control oligonucleotide

uate glucose (8.3 mmol/l)-induced insulin release in the presence of TEA (Fig. 4*D*), suggesting that the enhancement of Kv channel conductance is linked to insulinostatic ability of ghrelin. Furthermore, ScTx (0.1 μmol/l), an inhibitor of the Kv2.1 channel (27), potentiated glucose (8.3 mmol/l)-induced insulin release, and in the presence of ScTx ghrelin, it failed to attenuate glucose-induced insulin release (Fig. 4*D*). This result suggests that ghrelin inhibits insulin release by increasing the Kv2.1 channel conductance.

We next examined whether the ghrelin-induced Kv channel potentiation is sensitive to PTX. As shown in Fig. 4*E*, in β-cells treated with PTX (1 μg/ml), Kv currents evoked by depolarizing pulses to 20 mV were not increased by exposure to 10 nmol/l ghrelin. The ghrelin action to enhance Kv currents was blunted in the entire range of potentials in β-cells treated with PTX (Fig. 4*F*). These results indicate that PTX-sensitive G-protein is required for ghrelin activation of Kv currents. Moreover, in the presence of membrane-permeable cyclic AMP analog dibutylyl-cyclic AMP (db-cAMP) at 1 mmol/l, ghrelin did not potentiate Kv currents (Fig. 4*G*), suggesting that ghrelin activation of Kv channels is mediated by cAMP signaling pathway.

Effects of ghrelin on the K_{ATP} channel currents, which were evoked by ramp pulses, were determined in rat single β-cells. K_{ATP} channel currents were decreased by changing glucose concentrations from 2.8 to 8.3 mmol/l. The conductance density of the K_{ATP} channel current measured at a slope during ramp pulses was not altered by ghrelin (Fig. 4*H* and *I*), indicating that K_{ATP} channels may not contribute to the inhibition of β-cell membrane excitability by ghrelin.

Ghrelin inhibits $[Ca^{2+}]_i$ and insulin release via $G\alpha_{i2}$-dependent pathway. In islet β-cells, $[Ca^{2+}]_i$ is considered the major regulator of insulin secretion. Repetitive glucose (8.3 mmol/l) stimulation induced repeated $[Ca^{2+}]_i$ increases in rat single β-cells. Ghrelin (10 nmol/l), added 5 min before the second glucose stimulation, suppressed $[Ca^{2+}]_i$ responses (Fig. 5*A* and *C*), and this effect was abolished by treatment with PTX (Fig. 5*B* and *C*). Thus, the effects of ghrelin on insulin release, Kv channels, and $[Ca^{2+}]_i$ were all inhibited by PTX, suggesting that certain subtype(s) of PTX-sensitive G-proteins are crucial in the signal transduction of ghrelin in β-cells. Treatment of primary cultured rat β-cells with antisense oligonucleotide specific for the $G\alpha_{i2}$-subunit of G-proteins markedly decreased $G\alpha_{i2}$-subunit expression (Fig. 5*F*). In the antisense-treated, but not control oligonucleotide-treated, β-cells, ghrelin failed to attenuate $[Ca^{2+}]_i$ responses to glucose (Fig. 5*D* and *E*). The inhibitory effect of ghrelin on $[Ca^{2+}]_i$ responses was unaltered in β-cells treated with antisense oligonucleotides specific for $G\alpha_{i1}$ and $G\alpha_{i3}$ (Fig. 5*E*). Furthermore, in primary cultured β-cells treated with antisense oligonucleotide for $G\alpha_{i2}$, ghrelin failed to suppress glucose (8.3 mmol/l)-induced insulin release, whereas significant suppression was observed in the control oligonucleotide-treated and untreated cells (Fig. 5*G*). These results suggest that $G\alpha_{i2}$-mediated signaling is crucial for the action of ghrelin to suppress glucose-induced $[Ca^{2+}]_i$ increase and insulin release and that the ghrelin-induced attenuation of $[Ca^{2+}]_i$ increase is linked to that of insulin release.

DISCUSSION

In this study, we present the novel ghrelin signaling in β-cells, which is distinct from that for GH release. The function of endogenous ghrelin was assessed by the effects of GHS-R antagonist in vivo and in islets. In rats, ghrelin of both endogenous and exogenous origins resulted in PTX-sensitive decrease in plasma insulin concentrations, contrasting with PTX-insensitive increase in GH levels by ghrelin. In rat isolated islets, endogenous and exogenous ghrelin suppressed glucose-induced insulin release. Moreover, isolated islets from Ghr-KO mice displayed PTX-sensitive enhancement of glucose-induced insulin release. Ghrelin also increased Kv currents and inhibited glucose-induced $[Ca^{2+}]_i$ increases in β-cells. Thus, all the effects of endogenous and exogenous ghrelin on Kv, $[Ca^{2+}]_i$, and insulin release were blunted in the presence of PTX. Moreover, the effects of ghrelin on $[Ca^{2+}]_i$ and insulin release were abolished in the islet β-cells treated with the antisense oligonucleotide specific for $G\alpha_{i2}$-subunit of G-proteins. TEA, a Kv channel blocker, eliminated the ability of ghrelin to suppress insulin release. These findings demonstrate that ghrelin suppresses glucose-induced insulin release via $G\alpha_{i2}$- and Kv channel–mediated attenuation of Ca^{2+} signaling in β-cells.

PTX, an inhibitor of $G_{i/o}$ subtypes of trimeric G-proteins, was formally recognized as the islet-activating protein (IAP) because this agent increased insulin release from isolated islets (28). The effect of IAP has suggested the presence of intra-islet substance that activates $G_{i/o}$-protein–coupled receptors to decrease insulin release. However, the endogenous islet substance whose insulinostatic action is antagonized by PTX/IAP is as yet unidentified. This study demonstrated that the insulinostatic effect of endogenous ghrelin in islets, as evidenced by enhancement of insulin release due to GHS-R antagonist and ghrelin gene knockout, was blunted by PTX treatment. The enhancement of insulin release by GHS-R antagonist and that by ghrelin gene knockout were as large as 70–80% of the enhancement by PTX/IAP treatment (Fig. 2*A* vs. *B* and *C*). These findings indicate that a large part, but not all, of the PTX/IAP effect is exerted by blocking the action of endogenous ghrelin in islets. The apparently ghrelin-independent portion of the PTX/IAP effect could be due to inhibition of the signaling of somatostatin, an islet hormone that is released from δ-cells and inhibits insulin release via PTX-sensitive G-proteins (29), although a paracrine role for somatostatin in islets has long been controversial (30).

This study revealed a novel ghrelin signaling coupled to inhibition of glucose-induced insulin release in β-cells. The effects of ghrelin on Kv, $[Ca^{2+}]_i$, and insulin release were all completely blocked by PTX. Moreover, the effects of ghrelin on $[Ca^{2+}]_i$ and insulin release were blunted in the

***E*: Antisense oligonucleotide-induced knockdown of $G\alpha_{i2}$ expression blocked ghrelin attenuation of $[Ca^{2+}]_i$ responses to glucose. In cells treated with antisense oligonucleotides against $G\alpha_{i1}$ and $G\alpha_{i3}$, ghrelin-induced suppression of $[Ca^{2+}]_i$ responses did not differ from that seen after treatment with the corresponding control oligonucleotides (n = 71–106). **P < 0.01 vs. control oligonucleotide. *F*: The antisense oligonucleotide specific for the G-protein $G\alpha_{i2}$-subunit decreased the immunofluorescence of $G\alpha_{i2}$-subunit seen in β-cells. *G*: In primary cultured islet β-cells treated with antisense oligonucleotide against $G\alpha_{i2}$, ghrelin failed to inhibit 8.3 mmol/l glucose-induced insulin release. In contrast, ghrelin inhibited glucose-induced insulin release in cells treated with control oligonucleotides for $G\alpha_{i2}$ and in untreated cells (n = 8). *P < 0.05; **P < 0.01.**

islet β-cells treated with the antisense oligonucleotide specific for $G\alpha_{i2}$-subunit of G-proteins. In contrast, neither the antisense oligonucleotide for $G\alpha_{i1}$ nor that for $G\alpha_{i3}$ affected the ghrelin effects on $[Ca^{2+}]_i$. The data indicate that the insulinostatic ghrelin signaling is produced via PTX-sensitive $G\alpha_{i2}$ in β-cells. The mediation by the G-protein $G\alpha_{i2}$-subtype is surprising in light of the current concept that GHS-R signaling is mediated primarily by the G_{11} subtype (2). However, our result is consistent with the reports that $G\alpha_{i2}$ is expressed in β-cells (29,31), whereas $G\alpha_{11}$ is expressed primarily in non–β-cells in islets (32–34).

Voltage-gated potassium channels are involved in repolarization of excitable cells. In pancreatic β-cells, activation of delayed rectifier K^+ (Kv) channels repolarizes cells and attenuates glucose-stimulated action potentials, limiting Ca^{2+} entry through voltage-dependent Ca^{2+} channels to suppress insulin secretion (35). Blockade of Kv channels can promote glucose-dependent insulin secretion (36–38). In the present study, outward K^+ currents were increased by exposure to 10 nmol/l ghrelin, and this effect was blunted in β-cells treated with PTX (Fig. 4). We previously reported that Kv current was largely inhibited by a Kv channel blocker TEA and that the presence of TEA diminished the ability of ghrelin to suppress glucose-induced $[Ca^{2+}]_i$ increases in β-cells (4). Moreover, the present study showed that attenuation of glucose-induced insulin release by ghrelin was also abolished in the presence of a Kv2.1 channel blocker ScTx (Fig. 4*D*). Kv2.1 is expressed at high levels in islets of various species (36,37), and immunohistochemical analysis indicates that expression of Kv2.1 is restricted to islet β-cells (39). Taken together, an increase in Kv2.1 channel conductance is linked to ghrelin-induced inhibition of insulin release and Ca^{2+} signaling. K_{ATP} channel is well known as a key molecule that determines resting membrane potentials and converts the glucose metabolism to the membrane excitation in β-cells (40,41). This channel, however, may not be the target for ghrelin signaling for the following reasons; membrane potentials, which are mainly controlled by K_{ATP} channels, were not significantly changed by ghrelin, and K_{ATP} channel currents at 8.3 mmol/l glucose conditions were not altered by ghrelin application. Therefore, the ghrelin-induced decrease in the action potential firing may be caused largely, if not completely, by activation of Kv channels. It should also be noted that ghrelin significantly activated Kv currents at the membrane potentials of −20 and −10 mV (Fig. 4*C*) that correspond to the peak values of bursting action potentials in glucose-stimulated β-cells (Fig. 3). Collectively, our results corroborate a signaling cascade that ghrelin activates Kv channels via PTX-sensitive mechanisms to cause a rapid repolarization and shortening of bursting action potentials, leading to attenuation of glucose-induced $[Ca^{2+}]_i$ increases and insulin secretion. Moreover, ghrelin did not potentiate the Kv currents in the presence of cyclic AMP analog db-cAMP (Fig. 4*G*), suggesting that ghrelin activation of Kv channels is mediated by cAMP signaling pathway. It is also known that members of the $G\alpha_i$-family are involved in transducing the information of inhibitory hormones and neurotransmitters to cyclic AMP productions (42–44), voltage-dependent Ca^{2+} channels (45,46), and insulin exocytosis (29). Whether the ghrelin action could also involve these processes is unknown and definitely requires further studies. Possible differences in the coupling of G-proteins to signaling pathways between ghrelin and other inhibitory hormones, norepinephrine and somatostatin, also remain to be studied.

Low plasma ghrelin levels are associated with elevated fasting insulin levels, insulin resistance, and type 2 diabetes (16). The inverse relationship between plasma levels of ghrelin and insulin may be explained in part by the inhibition of insulin release by ghrelin. Our present and previous (4,17) studies revealed that ghrelin is a physiological autocrine/paracrine hormone in islets and that its antagonism could promote insulin release and prevent high-fat diet–induced glucose intolerance. Moreover, the present study provided evidence to support both endogenous and exogenous ghrelin attenuating glucose-induced insulin release via $G\alpha_{i2}$-mediated activation of Kv channels and suppression of action potential firings and $[Ca^{2+}]_i$ increases in β-cells. These unique signaling mechanisms and molecules mediating the insulinostatic action of ghrelin in β-cells provide potential therapeutic targets for the prevention and treatment of type 2 diabetes and hyperinsulinemia.

ACKNOWLEDGMENTS

K.D. has received Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and that on Priority Areas (19045026). M.K. has received Grants-in-Aid for Scientific Research from the JSPS. T.Y. has received Grants-in-Aid for Scientific Research from the JSPS and that on Priority Areas (15081101), a grant from the 21st century Center of Excellence (COE) program, a grant from the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan, an Insulin Research Award from Novo Nordisk Pharma, and a grant from Japan Diabetes Foundation.

We thank S. Ookuma and M. Motoshima for technical assistance.

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* 402:656–660, 1999
2. Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong S-S, Chaung L-YP, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJS, Dean DC, Melillo DG, Patchett AA, Nargund RP, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LHT: A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* 273:974–977, 1996
3. Kojima M, Kangawa K: Drug insight: the functions of ghrelin and its potential as a multitherapeutic hormone. *Nat Clin Prac Endocrinol Metab* 2:80–88, 2006
4. Dezaki K, Hosoda H, Kakei M, Hashiguchi S, Watanabe M, Kangawa K, Yada T: Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating Ca^{2+} signaling in β-cells: implication in the glycemic control in rodents. *Diabetes* 53:3142–3151, 2004
5. Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S: Ghrelin is present in pancreatic α-cells of humans and rats and stimulates insulin secretion. *Diabetes* 51:124–129, 2002
6. Gnanapavan S, Kola B, Bustin SA, Morris DG, McGee P, Fairclough P, Bhattacharya S, Carpenter R, Grossman AB, Korbonits M: The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 87:2988–2991, 2002
7. Volante M, Allia E, Gugliotta P, Funaro A, Broglio F, Deghenghi R, Muccioli G, Ghigo E, Papotti M: Expression of ghrelin and of the GH secretagogue receptor by pancreatic islet cells and related endocrine tumors. *J Clin Endocrinol Metab* 87:1300–1308, 2002
8. Wierup N, Svensson H, Mulder H, Sundler F: The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. *Regul Pept* 107:63–69, 2002

9. Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L: Ghrelin cells replace insulin-producing β cells in two mouse models of pancreas development. *Proc Natl Acad Sci U S A* 101:2924–2929, 2004
10. Wierup N, Yang S, McEvilly RJ, Mulder H, Sundler F: Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *J Histochem Cytochem* 52:301–310, 2004
11. Wierup N, Sundler F: Ultrastructure of islet ghrelin cells in the human fetus. *Cell Tissue Res* 319:423–428, 2005
12. Broglio F, Arvat E, Benso A, Gottero C, Muccioli G, Papotti M, van der Lely AJ, Deghenghi R, Ghigo E: Ghrelin, a natural GH secretagogue produced by the stomach, induces hyperglycemia and reduces insulin secretion in humans. *J Clin Endocrinol Metab* 86:5083–5086, 2001
13. Egido EM, Rodriguez-Gallardo J, Silvestre RA, Marco J: Inhibitory effect of ghrelin on insulin and pancreatic somatostatin secretion. *Eur J Endocrinol* 146:241–244, 2002
14. Reimer MK, Pacini G, Ahren B: Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology* 144:916–921, 2003
15. Ikezaki A, Hosoda H, Ito K, Iwama S, Miura N, Matsuoka H, Kondo C, Kojima M, Kangawa K, Sugihara S: Fasting plasma ghrelin levels are negatively correlated with insulin resistance and PAI-1, but not with leptin, in obese children and adolescents. *Diabetes* 51:3408–3411, 2002
16. Poykko SM, Kellokoski E, Horkko S, Kauma H, Kesaniemi YA, Ukkola O: Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes* 52:2546–2553, 2003
17. Dezaki K, Sone H, Koizumi M, Nakata M, Kakei M, Nagai H, Hosoda H, Kangawa K, Yada T: Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent high-fat diet-induced glucose intolerance. *Diabetes* 55:3486–3493, 2006
18. Herrington J, Hille B: Growth hormone-releasing hexapeptide elevates intracellular calcium in rat somatotropes by two mechanisms. *Endocrinology* 135:1100–1108, 1994
19. Bresson-Bepoldin L, Dufy-Barbe L: GHRP6-stimulated hormone secretion in somatotrophs: involvement of intracellular and extracellular calcium sources. *J Neuroendocrinol* 8:309–314, 1996
20. Malagon MM, Luque RM, Ruiz-Guerrero E, Rodriguez-Pacheco F, Garcia-Navarro S, Casanueva FF, Gracia-Navarro F, Castano JP: Intracellular signaling mechanisms mediating ghrelin-stimulated growth hormone release in somatotropes. *Endocrinology* 144:5372–5380, 2003
21. Glavaski-Joksimovic A, Jeftinija K, Jeremic A, Anderson LL, Jeftinija S: Mechanism of action of growth hormone secretagogue, L-692,585, on cultured porcine somatotropes. *J Endocrinol* 175:625–636, 2002
22. Glavaski-Joksimovic A, Jeftinija K, Scanes CG, Anderson LL, Jeftinija S: Stimulatory effect of ghrelin on isolated porcine somatotropes. *Neuroendocrinology* 77:366–378, 2003
23. Gilon P, Henquin JC: Mechanisms and physiological significance of the cholinergic control of pancreatic β-cell function. *Endocr Rev* 22:565–604, 2001
24. Fujiwara K, Maekawa F, Yada T: Oleic acid interacts with GPR40 to induce Ca^{2+} signaling in rat islet β-cells: mediation by PLC and L-type Ca^{2+} channel and link to insulin release. *Am J Physiol* 289:E670–E677, 2005
25. Yada T, Sakurada M, Ihida K, Nakata M, Murata F, Arimura A, Kikuchi M: Pituitary adenylate cyclase activating polypeptide is an extraordinarily potent intra-pancreatic regulator of insulin secretion from islet β-cells. *J Biol Chem* 269:1290–1293, 1994
26. Nakazaki M, Kakei M, Koriyama N, Tanaka H: Involvement of ATP-sensitive K^+ channels in free radical-mediated inhibition of insulin secretion in rat pancreatic β-cells. *Diabetes* 44:878–883, 1995
27. Shiau YS, Huang PT, Liou HH, Liaw YC, Shiau YY, Lou KL: Structural basis of binding and inhibition of novel tarantula toxins in mammalian voltage-dependent potassium channels. *Chem Res Toxicol* 16:1217–1225, 2003
28. Katada T, Ui M: Islet-activating protein: enhanced insulin secretion and cyclic AMP accumulation in pancreatic islets due to activation of native calcium ionophores. *J Biol Chem* 254:469–479, 1979
29. Sharp GW: Mechanisms of inhibition of insulin release. *Am J Physiol* 271:C1781–C1799, 1996
30. Samols E, Stagner JI: Islet somatostatin: microvascular, paracrine, and pulsatile regulation. *Metabolism* 39:55–60, 1990
31. Rucha A, Verspohl EJ: Heterologous desensitization of insulin secretion by GIP (glucose-dependent insulinotropic peptide) in INS-1 cells: the significance of $G\alpha_{i2}$ and investigations on the mechanism involved. *Cell Biochem Funct* 23:205–212, 2005
32. Astesano A, Regnauld K, Ferrand N, Gingras D, Bendayan M, Rosselin G, Emami S: Cellular and subcellular expression of G_{olf}/Gs and Gq/G_{11} α-subunits in rat pancreatic endocrine cells. *J Histochem Cytochem* 47:289–302, 1999
33. Skoglund G, Basmaciogullari A, Rouot B, Marie JC, Rosselin G: Cell-specific localization of G protein α-subunits in the islets of Langerhans. *J Endocrinol* 162:31–37, 1999
34. Portela-Gomes GM, Abdel-Halim SM: Overexpression of Gs proteins and adenylyl cyclase in normal and diabetic islets. *Pancreas* 25:176–181, 2002
35. MacDonald PE, Wheeler MB: Voltage-dependent K^+ channels in pancreatic beta cells: role, regulation and potential as therapeutic targets. *Diabetologia* 46:1046–1062, 2003
36. MacDonald PE, Ha XF, Wang J, Smukler SR, Sun AM, Gaisano HY, Salapatek AM, Backx PH, Wheeler MB: Members of the Kv1 and Kv2 voltage-dependent K^+ channel families regulate insulin secretion. *Mol Endocrinol* 15:1423–1435, 2001
37. MacDonald PE, Sewing S, Wang J, Joseph JW, Smukler SR, Sakellaropoulos G, Wang J, Saleh MC, Chan CB, Tsushima RG, Salapatek AM, Wheeler MB: Inhibition of Kv2.1 voltage-dependent K^+ channels in pancreatic β-cells enhances glucose-dependent insulin secretion. *J Biol Chem* 277: 44938–44945, 2002
38. Herrington J, Zhou YP, Bugianesi RM, Dulski PM, Feng Y, Warren VA, Smith MM, Kohler MG, Garsky VM, Sanchez M, Wagner M, Raphaelli K, Banerjee P, Ahaghotu C, Wunderler D, Priest BT, Mehl JT, Garcia ML, McManus OB, Kaczorowski GJ, Slaughter RS: Blockers of the delayed-rectifier potassium current in pancreatic β-cells enhance glucose-dependent insulin secretion. *Diabetes* 55:1034–1042, 2006
39. Yan L, Figueroa DJ, Austin CP, Liu Y, Bugianesi RM, Slaughter RS, Kaczorowski GJ, Kohler MG: Expression of voltage-gated potassium channels in human and rhesus pancreatic islets. *Diabetes* 53:597–607, 2004
40. Aguilar-Bryan L, Bryan J: Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr Rev* 20:101–135, 1999
41. Tarasov A, Dusonchet J, Ashcroft F: Metabolic regulation of the pancreatic β-cell ATP-sensitive K^+ channel: a pas de deux. *Diabetes* 53:S113–S122, 2004
42. McDermott AM, Sharp GW: Gi2 and Gi3 proteins mediate the inhibition of adenylyl cyclase by galanin in the RINm5F cell. *Diabetes* 44:453–459, 1995
43. Seaquist ER, Armstrong MB, Gettys TW, Walseth TF: Somatostatin selectively couples to $G_{o\alpha}$ in HIT-T15 cells. *Diabetes* 44:85–89, 1995
44. Urano Y, Sakurai T, Ueda H, Ogasawara J, Sakurai T, Takei M, Izawa T: Desensitization of the inhibitory effect of norepinephrine on insulin secretion from pancreatic islets of exercise-trained rats. *Metabolism* 53:1424–1432, 2004
45. Keahey HH, Boyd AE III, Kunze DL: Catecholamine modulation of calcium currents in clonal pancreatic β-cells. *Am J Physiol* 257:C1171–C1176, 1989
46. Hsu WH, Xiang HD, Rajan AS, Kunze DL, Boyd AE III: Somatostatin inhibits insulin secretion by a G-protein-mediated decrease in Ca^{2+} entry through voltage-dependent Ca^{2+} channels in the beta cell. *J Biol Chem* 266:837–843, 1991