Low glucose-induced ghrelin secretion is mediated by an ATP-sensitive potassium channel

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Abstract

Ghrelin is synthesized in X/A-like cells of the gastric mucosa, which plays an important role in the regulation of energy homeostasis. Although ghrelin secretion is known to be induced by neurotransmitters or hormones or by nutrient sensing in the ghrelin-secreting cells themselves, the mechanism of ghrelin secretion is not clearly understood. In the present study, we found that changing the extracellular glucose concentration from elevated (25 mM) to optimal (10 mM) caused an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in ghrelin-secreting mouse ghrelinoma 3-1 (MGN3-1) cells ($n=32$, $P<0.01$), whereas changing the glucose concentration from elevated to lowered (5 or 1 mM) had little effect on $[\text{Ca}^{2+}]_i$ increase. Overexpression of a closed form of an ATP-sensitive K^+ (K_{ATP}) channel mutant suppressed the 10 mM glucose-induced $[\text{Ca}^{2+}]_i$ increase ($n=8$, $P<0.01$) and exocytotic events ($n=6$, $P<0.01$). We also found that a low concentration of a K_{ATP} channel opener, diazoxide, with 25 mM glucose induced $[\text{Ca}^{2+}]_i$ increase ($n=23$, $P<0.01$) and ghrelin secretion ($n\geq 3$, $P<0.05$). In contrast, the application of a low concentration of a K_{ATP} channel blocker, tolbutamide, significantly induced $[\text{Ca}^{2+}]_i$ increase ($n=15$, $P<0.01$) and ghrelin secretion ($n\geq 3$, $P<0.05$) under 5 mM glucose. Furthermore, the application of voltage-dependent Ca^{2+} channel inhibitors suppressed the 10 mM glucose-induced $[\text{Ca}^{2+}]_i$ increase ($n\geq 26$, $P<0.01$) and ghrelin secretion ($n\geq 5$, $P<0.05$). These findings suggest that K_{ATP} and voltage-dependent Ca^{2+} channels are involved in glucose-dependent ghrelin secretion in MGN3-1 cells.

Key Words

- ▶ ATP-sensitive potassium channel
- ▶ calcium channel
- ▶ exocytosis
- ▶ ghrelin
- ▶ glucose
- ▶ total internal reflection fluorescence microscopy

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Introduction

Ghrelin, a stomach-derived 28 amino acid peptide hormone, is synthesized and released from X/A-like endocrine cells of gastric mucosa (Kojima *et al.* 1999).

Ghrelin activates growth hormone (GH) secretagogue receptors that are expressed in agouti-related peptide/neuropeptide Y-expressing hypothalamic neurons, which

results in an increase in food intake (Kamegai *et al.* 2001, Nakazato *et al.* 2001). Ghrelin also regulates energy balance by promoting weight gain and adiposity (Tschop *et al.* 2000, Nakazato *et al.* 2001, Theander-Carrillo *et al.* 2006). Although the actions of ghrelin have been investigated intensively by several groups (Masuda *et al.* 2000, Takaya *et al.* 2000, Tschop *et al.* 2000, Kamegai *et al.* 2001, Nakazato *et al.* 2001, Nagaya & Kangawa 2003, Nagaya *et al.* 2006, Theander-Carrillo *et al.* 2006), the mechanism of ghrelin secretion from the ghrelin-secreting X/A-like cells has not yet been elucidated.

Ghrelin-secreting cells are closed-type enteroendocrine cells that do not contact the gastric lumen and are not regulated directly by nutrients that are present in the stomach. However, recent studies have demonstrated that ingested proteins and lipids, which are absorbed in the small intestine, lower the plasma ghrelin concentration (Tschop *et al.* 2000, Williams *et al.* 2003, Foster-Schubert *et al.* 2008). Therefore, ghrelin secretion might be regulated by hormonal, neuronal, and metabolite signals that arise from nutrient sensing and absorption in the intestine. In fact, in support of this view, recent studies have shown that nutrients (i.e., fatty acids and glucose) and hormones (i.e., insulin and oxytocin) regulate ghrelin secretion in fluorescence-activated cell sorting-separated primary mouse ghrelin cells and ghrelinoma cell lines (i.e., SG-1, PG-1, and mouse ghrelinoma 3-1 (MGN3-1)) (Janssen *et al.* 2012, Sakata *et al.* 2012).

Insulin and glucagon are also peptide hormones, and their secretions from pancreatic α and β cells respectively are mediated by nutrients, especially glucose. Although pancreatic β cells secrete insulin when the plasma glucose concentration is high, pancreatic α cells secrete glucagon in response to a decrease in the plasma glucose concentration (MacDonald *et al.* 2007), which is similar to ghrelin secretion from the ghrelin-secreting X/A-like cells. Both pancreatic α and β cells express ATP-sensitive K^+ (K_{ATP}) channels composed of a sulfonylurea receptor 1 and an inward rectifier potassium channel (Kir6.2) (Suzuki *et al.* 1997, 1999), and it has been shown that both insulin and glucagon secretion are regulated by K_{ATP} channels (McTaggart *et al.* 2010). Interestingly, voltage-dependent Ca^{2+} channels are expressed in pancreatic α and β cells in different combinations. A previous paper showed that a blocker of the L-type Ca^{2+} channel inhibited insulin secretion from pancreatic β cells, and a blocker of the N-type and T-type Ca^{2+} channels inhibited glucagon secretion from pancreatic α cells (Quesada *et al.* 2008). Thus, we hypothesize that the K_{ATP} channels and the voltage-dependent Ca^{2+} channels might be involved in

the regulation of glucose-dependent ghrelin secretion from ghrelin-secreting cells, which is similar to the regulation of glucagon secretion in pancreatic α cells.

In the present study, we examined the role of K_{ATP} channels and the voltage-dependent Ca^{2+} channels in ghrelin secretion from MGN3-1 cells, which is the first cell line from a gastric ghrelin-producing cell tumor derived from ghrelin-promoter simian virus 40 T-antigen transgenic mice (Iwakura *et al.* 2010, 2011), and we evaluated how K_{ATP} channels and voltage-dependent Ca^{2+} channels contribute to glucose-dependent ghrelin secretion.

Materials and methods

Chemicals

D-glucose, diazoxide (DZ), diltiazem, and EGTA were purchased from Wako Pure Chemicals (Osaka, Japan). Fluo3 acetoxymethylester (Fluo3-AM), NNC55-0396, tolbutamide (TB), and ω -conotoxin (ω -CgTX) GVIA were purchased from Sigma–Aldrich.

Plasmid construction

Mouse Kir6.2 was amplified from a mouse brain cDNA by PCR. The cDNA fragment of Kir6.2 was inserted into EcoRI/BamHI sites of the pmCherry-N1 vector (Oya *et al.* 2013a). The dominant-negative mutation (G132S) in the selectivity filter of Kir6.2 (Koster *et al.* 2002) was created by sequential PCR (Kir6.2DN-mCherry). Expression vectors that encoded Venus-tagged brain-derived neurotrophic factor (BDNF-Venus) were constructed as described previously (Aoki *et al.* 2010). All of the DNA sequences were validated by sequencing.

Cell culture and transfection

The ghrelin-producing cell line MGN3-1 cells from a gastric tumor derived from ghrelin-promoter simian virus 40 T-antigen transgenic mice (kindly provided by Dr Hiroshi Iwakura, Kyoto University, Kyoto, Japan) were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in 5% CO_2 (Iwakura *et al.* 2010, 2011). To image ghrelin secretion, MGN3-1 cells were plated onto poly-L-lysine-coated coverslips and transfected with 1.5 μ g BDNF-Venus and 1.5 μ g Kir6.2 DN-mCherry expression vectors using 6 μ l Lipofectamine 2000 (Life Technologies) and 200 μ l Opti-MEM (Life Technologies). Transfected cells were cultured in DMEM for

2 days until imaging. Under the present experimental conditions, the transfection efficiency was about 25% for double transfection and about 50% for single transfection.

Visualization of intracellular Ca^{2+} dynamics

Changes in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were measured using Fluo3-AM. MGN3-1 cells and Kir6.2DN-mCherry-expressing MGN3-1 cells on coverslips were loaded with 2.5 μM Fluo3-AM in modified Ringer's buffer (140 mM NaCl, 3.6 mM KCl, 0.5 mM NaH_2PO_4 , 0.5 mM MgSO_4 , 1.5 mM CaCl_2 , 10 mM HEPES, 2 mM NaHCO_3 , and 5 mM glucose, pH 7.4) for 20 min at 37 °C.

The cells were then washed two times before imaging. Imaging of the MGN3-1 cells was performed in Ringer's buffer containing 25 mM glucose. Glucose stimulation was achieved by perfusion with Ringer's buffer containing 1, 5, 10, 15, or 25 mM glucose. Cells were then mounted in a chamber and placed on the stage of an Olympus IX-71 inverted microscope (Olympus). Fluo3-AM-loaded cells were excited at 480 nm at 5 s intervals with a xenon lamp, and emission signals at 515 nm were detected with an electron multiplying charge-coupled device (EM-CCD) camera (Evolve, Photometrics, Tucson, AZ, USA).

Immunocytochemistry

After 2 days of transfection with BDNF-Venus, the MGN3-1 cells were washed in PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. The cells were then sequentially reacted with the anti-N-terminal rabbit acyl-ghrelin antibody (1:100 000 dilution, produced by Prof. M K, Kurume University, Fukuoka, Japan) and the anti-rabbit Alexa568-conjugated secondary antibody (1:3000 dilution, Life Technologies). Confocal images were obtained by a Nipkow-disk confocal scanner (CSU-10, Yokogawa, Tokyo, Japan) equipped with an EM-CCD camera (C9100-02, Hamamatsu Photonics, Hamamatsu, Japan) and controlled by MetaMorph Software version 7.7 (Molecular Devices, Sunnyvale, CA, USA).

Total internal reflection fluorescence imaging

To observe the release of BDNF-Venus at the single-vesicle level, we used a total internal reflection fluorescence (TIRF) microscope similar to those described previously (Mori *et al.* 2011, Oya *et al.* 2011, 2013a,b, Sato *et al.* 2012, Kitaguchi *et al.* 2013). Briefly, a high numerical aperture objective lens (CFI Apochromat TIRF, 100 $\times$, NA=1.49, Nikon, Tokyo, Japan) was mounted on an inverted

microscope (Ti-E-Perfect focus system, Nikon), and incident light for total internal reflection illumination was introduced from the high numerical aperture objective lens through a single-mode optical fiber and two illumination lenses (TI-TIRF, Nikon). To observe the BDNF-Venus fluorescence images, we used an optically pumped semiconductor 488 nm laser (Sapphire 488LP, 30 mW, Coherent, Santa Clara, CA, USA) for total internal fluorescence illumination and a band-pass filter (HQ535/30m, Chroma, Bellows Falls, VT, USA) as an emission filter. The laser beam was passed through an electromagnetically driven shutter (TI-TIRF, Nikon), and the shutter was opened synchronously with EM-CCD camera (iXon, Andor, Belfast, UK) exposure, which was controlled by either NIS-Elements (Nikon) or MetaMorph Software. Images were acquired every 500 ms. Imaging was performed in Ringer's buffer. Glucose stimulation was achieved by perfusion with Ringer's buffer containing 1, 5, 10, 15, or 25 mM glucose. To distinguish between fusion events and retreated vesicles, we focused on fluorescence changes just before the disappearance of fluorescent signals. A fusion event was demonstrated by a rapid transient increase in fluorescence intensity (to peak intensity almost two times greater than the original fluorescence intensity within 1 s). By contrast, vesicle movements were shown by the fluorescence intensity gradually decreasing to the background level, as described previously (Tsuboi *et al.* 2006). The number of fusion events during a 20 min period was counted manually.

Measurement of ghrelin secretion

MGN3-1 cells were plated at a density of 3.0×10^5 cells/well in six-well plates and cultured in DMEM containing 25 mM glucose for 2 days. Before treatment, cells were switched to Ringer's buffer. The cells were incubated for 1 h in Ringer's buffer that contained various concentrations of glucose: 5 or 25 mM glucose plus 1 μM TB or 1 μM DZ; or 10 mM glucose plus 1 μM diltiazem, 1 μM ω -CgTX, 1 μM NNC55-0396, or 2 mM EGTA. The amount of secreted acyl- or desacyl-ghrelin into the Ringer's buffer was measured by an ELISA kit specific for acyl- or desacyl-ghrelin (SCETI, Tokyo, Japan). Data are reported as means $\pm$ S.E.M.

Statistical analysis

Data are reported as means $\pm$ S.E.M. Means were compared by the Student's *t*-test (for two groups) or by ANOVA

followed by a Dunnett's test using GraphPad Prism Software (GraphPad Software, La Jolla, CA, USA).

Results

K_{ATP} channels are involved in low glucose-induced Ca²⁺ increase

To examine the effect of glucose on the [Ca²⁺]_i changes in ghrelin-secreting cells *in vitro*, we used a novel ghrelin-producing cell line, MGN3-1 cells, from a gastric ghrelin-producing cell tumor derived from ghrelin-promoter simian virus 40 T-antigen transgenic mice (Iwakura *et al.* 2010, 2011). When the glucose concentration was lowered from 25 mM to either 10 or 15 mM, ghrelin-secreting MGN3-1 cells displayed an increase in [Ca²⁺]_i (Fig. 1A and B; G10: 125.7 ± 3.9%, *n* = 32 cells, *P* < 0.01 and G15: 114.9 ± 3.8%, *n* = 23 cells, *P* < 0.05). Meanwhile, lowering the glucose concentration from 25 mM to either 1 or 5 mM had little effect on the [Ca²⁺]_i, and maintaining the glucose concentration at 25 mM also showed little change (Fig. 1A and B; G1: 107.1 ± 1.3%, *n* = 41 cells, *P* = 0.75; G5: 113.5 ± 2.8%, *n* = 24 cells, *P* = 0.06; and G25: 103.5 ± 1.1%, *n* = 25 cells).

Pancreatic α cells secrete glucagon in response to a decrease in glucose concentration, and they express K_{ATP} channels to enable glucagon secretion (Gromada *et al.* 2007). To examine whether K_{ATP} channels were also involved in ghrelin secretion from X/A-like endocrine cells, we overexpressed a dominant-negative form of the K_{ATP} channel subunit Kir6.2 (Kir6.2DN, substitution of

Figure 1

Effect of glucose on [Ca²⁺]_i changes in MGN3-1. (A) Typical time course showing [Ca²⁺]_i changes during the application of various concentrations of glucose to MGN3-1 cells. Stimuli were applied at the times indicated by the dotted line. Basal fluorescence intensity was normalized to 100. (B) Lowering the glucose concentration caused an increase in [Ca²⁺]_i. Normalized amplitude was calculated from the peak amplitude induced by various concentrations of glucose. Data are shown as means ± s.e.m. (*n* = at least 23 cells in each condition). **P* < 0.05 and ***P* < 0.01.

Figure 2

The K_{ATP} channel is involved in low glucose-induced [Ca²⁺]_i increase. (A) Typical time course showing [Ca²⁺]_i during the application of either 10 or 25 mM glucose to MGN3-1 cells that expressed a dominant-negative form of the K_{ATP} channel subunit Kir6.2 (Kir6.2DN). Stimuli were applied at the times indicated by the dotted line. Basal fluorescence intensity was normalized to 100. (B) Lowering the glucose concentration had little effect on the [Ca²⁺]_i increase in the MGN3-1 cells that expressed Kir6.2DN. Normalized amplitude was calculated from the peak amplitude induced by the glucose concentration change. Data are shown as means ± s.e.m. (*n* = at least eight cells in each condition). NS, not significant. ***P* < 0.01.

glycine with serine at position 132; Seino *et al.* 2000) in MGN3-1 cells and monitored the effect on low glucose-induced [Ca²⁺]_i changes (Fig. 2A). We found that the increase in [Ca²⁺]_i that was caused by lowering the extracellular glucose concentration was suppressed in Kir6.2DN-expressing MGN3-1 cells (Fig. 2B; control in G10: 125.7 ± 3.9%, *n* = 32 cells and Kir6.2DN in G10: 99.7 ± 2.1%, *n* = 8 cells, *P* < 0.01). These results suggest that lowering extracellular glucose concentration increases [Ca²⁺]_i via the K_{ATP} channel-mediated pathway in MGN3-1 cells.

Low glucose induces ghrelin secretion in MGN3-1 cells

We observed the dynamics of single ghrelin exocytotic events in live MGN3-1 cells by TIRF microscopy. To visualize ghrelin secretion, we transfected fluorescent protein (FP)-tagged BDNF because FP-tagged BDNF (i.e., BDNF-Venus)-positive vesicles were shown to well co-localize with ghrelin-containing vesicles in MGN3-1 cells by immunocytochemistry (Fig. 3A; 75.6 ± 4.8%, *n* = 20 cells). After lowering the extracellular glucose concentration, highly punctate BDNF-Venus fluorescent spots suddenly brightened and subsequently dimmed (Fig. 3B), which shows that ghrelin was secreted to the extracellular space. Similar to the [Ca²⁺]_i increase that is caused by lowering glucose, the number of BDNF-Venus exocytotic

Figure 3

Effect of glucose on ghrelin exocytosis from MGN3-1 cells. (A) Typical confocal image of paraformaldehyde-fixed MGN3-1 cells showing the distribution of BDNF-Venus (green) and ghrelin (red). Scale bar = 10 μm . (B, left) Typical TIRF image of BDNF-Venus-expressing MGN3-1 cells. The position of the vesicle before exocytosis is outlined by a square. Scale bar = 10 μm . (B, right) Five sequential TIRF images showing exocytotic release of BDNF-Venus after the application of 10 mM glucose. Scale bar = 1 μm . (C) The number of exocytotic events during the application of glucose at various concentrations. Data are shown as means $\pm$ S.E.M. (n = at least six cells in each condition). (D) The number of exocytotic events during the application of either 10 or 25 mM glucose to MGN3-1 cells that expressed Kir6.2DN. Data are shown as means $\pm$ S.E.M. (n = at least six cells in each condition). NS, not significant. * P < 0.05 and ** P < 0.01.

events was increased by 5, 10, and 15 mM glucose stimulation (Fig. 3C; G25: 1.9 ± 0.5 events/200 μm^2 , n = 8 cells; G1: 8.6 ± 2.9 events/200 μm^2 , n = 8 cells, P = 0.33; G5: 13.5 ± 3.1 events/200 μm^2 , n = 7 cells, P < 0.05; G10: 15.1 ± 4.7 events/200 μm^2 , n = 7 cells, P < 0.05; and G15: 13.9 ± 3.3 events/200 μm^2 , n = 6 cells, P < 0.05). We next monitored exocytotic events in Kir6.2DN-expressing MGN3-1 cells and found that 10 mM glucose-induced BDNF-Venus exocytotic events were significantly inhibited

(Fig. 3D; control in G10: 15.1 ± 4.7 events/200 μm^2 , n = 7 cells and Kir6.2DN in G10: 2.9 ± 0.8 events/200 μm^2 , n = 6 cells, P < 0.01). Additionally, the amount of secreted ghrelin was measured by ELISA. Because there are two types of ghrelin (i.e., acyl- and desacyl-ghrelin) circulating in the blood, each of which show distinct functions (Kojima *et al.* 1999), we measured the amount of both acyl- and desacyl-ghrelin secreted. The amount of secreted acyl-ghrelin increased when low glucose stimulation was applied (Fig. 4, left panel; G25: $100.0 \pm 4.1\%$, n = 5 trials; G1: $146.2 \pm 16.1\%$, n = 6 trials, P < 0.05; G5: $190.5 \pm 15.6\%$, n = 6 trials, P < 0.01; G10: $210.6 \pm 6.0\%$, n = 6 trials, P < 0.01; and G15: $229.1 \pm 11.1\%$, n = 6 trials, P < 0.01), whereas the amount of secreted desacyl-ghrelin increased when the glucose concentration was lowered from 25 to 10 mM or 15 mM (Fig. 4, right panel; G25: $100.0 \pm 5.7\%$, n = 6 trials; G1: $103.4 \pm 10.5\%$, n = 6 trials, P = 0.99; G5: $157.0 \pm 19.8\%$, n = 6 trials, P = 0.26; G10: $228.9 \pm 35.9\%$, n = 6 trials, P < 0.01; and G15: $214.7 \pm 29.1\%$, n = 6 trials, P < 0.01). These results suggest that acyl- and desacyl-ghrelin secretion were induced in similar but slightly distinct manners. Together with our evidence that the $[\text{Ca}^{2+}]_i$ increase by low glucose involves K_{ATP} channels (Fig. 2), these results indicate that lowering the extracellular glucose concentration increases ghrelin secretion via a $[\text{Ca}^{2+}]_i$ increase by a K_{ATP} channel-mediated pathway in MGN3-1 cells.

Figure 4

The amount of secreted acyl-ghrelin (left) and desacyl-ghrelin (right) after the application of various concentrations of glucose as measured by ELISA. Data are shown as means $\pm$ S.E.M. (n = at least five trials in each condition). * P < 0.05 and ** P < 0.01.

Figure 5

Effect of K_{ATP} channel opener and blocker on $[Ca^{2+}]_i$ changes in MGN3-1 cells. Typical time course showing $[Ca^{2+}]_i$ changes during the application of either 25 mM (A), 5 mM (B), or 10 mM (C) glucose alone, with 1 or 10 μ M diazoxide (DZ), or with 1 or 10 μ M tolbutamide (TB) to MGN3-1 cells. Stimuli were applied at the times indicated by the dotted line. Basal fluorescence intensity was normalized to 100. (D) The application of 1 μ M DZ with 25 mM glucose induced a $[Ca^{2+}]_i$ increase, whereas 1 μ M TB or 10 μ M DZ had little effect on $[Ca^{2+}]_i$. In contrast, 1 μ M TB with 5 mM glucose significantly increased $[Ca^{2+}]_i$, whereas 1 μ M DZ or 10 μ M TB with 5 mM glucose had little effect on $[Ca^{2+}]_i$. The application of either 1 μ M DZ or 1 μ M TB with 10 mM glucose decreased $[Ca^{2+}]_i$. Normalized amplitude was calculated from the peak amplitude induced by various stimuli. Data are shown as means $\pm$ S.E.M. (n = at least 13 cells in each condition). ** P < 0.01.

The modulation of K_{ATP} channel function affects glucose-induced ghrelin secretion

We applied a K_{ATP} channel opener or blocker (i.e., DZ or TB respectively) to modulate K_{ATP} channel activity. We found that the application of 1 μ M DZ with 25 mM glucose induced $[Ca^{2+}]_i$ increase, whereas the application of 1 μ M TB with 25 mM glucose had little effect on $[Ca^{2+}]_i$ change (Fig. 5A and D; G25: $103.5 \pm 1.1\%$, $n = 25$ cells; 1 μ M DZ with G25: $137.3 \pm 3.8\%$, $n = 23$ cells, $P < 0.01$; and 1 μ M TB with G25: $107.0 \pm 5.3\%$, $n = 17$ cells, $P = 0.99$). The application of 1 μ M TB with 5 mM glucose to MGN3-1 cells significantly increased $[Ca^{2+}]_i$, whereas the application of 1 μ M DZ with 5 mM glucose induced little $[Ca^{2+}]_i$ change (Fig. 5B and D; G5: $116.4 \pm 1.6\%$, $n = 29$ cells; 1 μ M DZ with G5: $107.8 \pm 2.9\%$, $n = 17$ cells, $P = 0.62$; and 1 μ M TB with G5: $185.0 \pm 10.9\%$, $n = 15$ cells, $P < 0.01$). Although the application of 1 μ M DZ with 25 mM glucose or 1 μ M TB with 5 mM glucose induced $[Ca^{2+}]_i$ increase,

the application of a high concentration (i.e., 10 μ M) of DZ or TB had little effect on $[Ca^{2+}]_i$ change (Fig. 5A and D; 10 μ M DZ with G25: $118.9 \pm 4.7\%$, $n = 13$ cells, $P = 0.13$ and 10 μ M TB with G5: $122.8 \pm 5.5\%$, $n = 15$ cells, $P = 0.90$). Interestingly, the application of either 1 μ M DZ or 1 μ M TB with 10 mM glucose decreased $[Ca^{2+}]_i$ (Fig. 5C and D; G10: $125.7 \pm 3.9\%$, $n = 32$ cells; 1 μ M DZ with G10: $108.8 \pm 3.0\%$, $n = 35$ cells, $P < 0.01$; and 1 μ M TB with G10: $99.5 \pm 1.8\%$, $n = 30$ cells, $P < 0.01$). Furthermore, to examine the effect of the modulation of K_{ATP} channel activity on ghrelin secretion, we measured the amount of both secreted acyl-ghrelin and secreted desacyl-ghrelin by ELISA. We found that the application of 1 μ M DZ with 25 mM glucose or 1 μ M TB with 5 mM glucose significantly increased the amount of both acyl- and desacyl-ghrelin (Fig. 6; acyl-ghrelin: G25: $99.0 \pm 2.9\%$, $n = 17$ trials; 1 μ M DZ with G25: $141.5 \pm 25.8\%$, $n = 6$ trials, $P < 0.05$; G5: $147.4 \pm 9.1\%$, $n = 9$ trials, $P < 0.01$; 1 μ M TB with G5: $238.4 \pm 60.0\%$, $n = 5$ trials, $P < 0.05$ and desacyl-ghrelin: G25: $100.0 \pm 5.1\%$, $n = 21$ trials; 1 μ M DZ with G25: $129.4 \pm 10.0\%$, $n = 3$ trials, $P < 0.05$; G5: $163.7 \pm 16.3\%$, $n = 14$ trials, $P < 0.01$; 1 μ M TB with G5: $242.4 \pm 45.9\%$, $n = 3$ trials, $P < 0.05$), which is consistent with the $[Ca^{2+}]_i$ changes (Fig. 5). These results suggest that the extent of

Figure 6

Effect of K_{ATP} channel opener and blocker on ghrelin secretion from MGN3-1 cells. The amount of secreted acyl- (left) and desacyl-ghrelin (right) after the application of either 5 or 25 mM glucose with 1 μ M DZ and TB as measured by ELISA. Data are shown as means $\pm$ S.E.M. (n = at least three trials in each condition). * P < 0.05 and ** P < 0.01.

the K_{ATP} channel opening is involved in $[Ca^{2+}]_i$ increase and ghrelin secretion.

Voltage-dependent Ca^{2+} channels are involved in ghrelin secretion from MGN3-1 cells

We examined the $[Ca^{2+}]_i$ effects of the inhibitors of either L-, N-, or T-type Ca^{2+} channels (i.e., diltiazem, ω -CgTX, or NNC55-0396 respectively) on low glucose stimulation. The application of each inhibitor with 10 mM glucose significantly suppressed the low glucose-induced $[Ca^{2+}]_i$ increase (Fig. 7A and B; G25: $103.5 \pm 1.1\%$, $n=25$ cells; G10: $125.7 \pm 3.9\%$, $n=32$ cells, $P<0.01$; 1 μ M diltiazem with G10: $109.9 \pm 2.0\%$, $n=26$ cells, $P<0.01$; 1 μ M ω -CgTX with G10: $100.3 \pm 1.8\%$, $n=27$ cells, $P<0.01$; and 1 μ M NNC55-0396 with G10: $109.3 \pm 1.3\%$, $n=26$ cells, $P<0.01$). The application of EGTA to deplete the extracellular Ca^{2+} ions also significantly suppressed glucose-induced $[Ca^{2+}]_i$ responses (Fig. 7A and B; 2 mM EGTA with G10: $98.7 \pm 0.5\%$, $n=25$ cells, $P<0.01$). Consistent with the $[Ca^{2+}]_i$ increase, we found that the application of 1 μ M diltiazem, 1 μ M ω -CgTX, or 1 μ M NNC55-0396 with 10 mM glucose suppressed the low glucose-dependent secretion of both acyl- and desacyl-ghrelin (Fig. 7C; acyl-ghrelin: G25: $100.0 \pm 3.0\%$, $n=7$ trials; G10: $210.6 \pm 6.0\%$, $n=6$ trials, $P<0.01$; 1 μ M diltiazem with G10: $145.1 \pm 22.2\%$, $n=6$ trials, $P<0.05$; 1 μ M ω -CgTX with G10: $105.5 \pm 14.7\%$, $n=6$ trials, $P<0.01$; 1 μ M NNC55-0396 with G10: $148.1 \pm 25.7\%$, $n=6$ trials, $P<0.05$ and desacyl-ghrelin: G25: $101.1 \pm 4.8\%$, $n=11$ trials; G10: $228.9 \pm 35.9\%$, $n=6$ trials, $P<0.01$; 1 μ M diltiazem with G10: $100.3 \pm 24.0\%$, $n=6$ trials, $P<0.05$; 1 μ M ω -CgTX with G10: $117.2 \pm 13.7\%$, $n=5$ trials, $P<0.01$; 1 μ M NNC55-0396 with G10: $130.5 \pm 21.9\%$, $n=6$ trials, $P<0.05$). The application of 2 mM EGTA with 10 mM glucose also suppressed the amount of acyl- and desacyl-ghrelin secretion (Fig. 7C; acyl-ghrelin: 2 mM EGTA with G10: $88.7 \pm 13.1\%$, $n=5$ trials, $P<0.01$ and desacyl-ghrelin: 2 mM EGTA with G10: $68.8 \pm 10.3\%$, $n=6$ trials, $P<0.01$). These results suggest that L-, N-, and T-type voltage-dependent Ca^{2+} channels induce $[Ca^{2+}]_i$ influx, which ultimately leads to ghrelin secretion from MGN3-1 cells.

Discussion

Increases in glucose concentrations cause the stimulation of various gastrointestinal hormones, including glucagon-like peptide 1 and insulin (Drucker 2006, 2007, Ashcroft & Rorsman 2013). Glucose is believed to act principally to

Figure 7

Effect of voltage-dependent Ca^{2+} channel blocker on ghrelin secretion from MGN3-1 cells. (A) Typical time course showing $[Ca^{2+}]_i$ changes during the application of 10 mM glucose with either 1 μ M diltiazem, 1 μ M ω -conotoxin (ω -CgTX), 1 μ M NNC55-0396, or 2 mM EGTA to MGN3-1 cells. Stimuli were applied at the times indicated by the dotted line. Basal fluorescence intensity was normalized to 100. (B) Diltiazem, ω -CgTX, NNC55-0396, and EGTA decreased the amplitude of the $[Ca^{2+}]_i$ increases as compared to 10 mM glucose alone. Normalized amplitude was calculated from the peak amplitude induced by various stimuli. Data are shown as means $\pm$ S.E.M. (n =at least 25 cells in each condition). (C) The amount of secreted acyl- (left) and desacyl-ghrelin (right) after the application of either 1 μ M diltiazem, 1 μ M ω -CgTX, 1 μ M NNC55-0396, or 2 mM EGTA with 10 mM glucose as measured by ELISA. Data are shown as means $\pm$ S.E.M. (n =at least five trials in each condition). * $P<0.05$ and ** $P<0.01$.

stimulate ATP synthesis by initiating the closure of K_{ATP} channels (Aguilar-Bryan & Bryan 1999), the depolarization of plasma membrane, and Ca^{2+} influx (Safayhi *et al.* 1997). In the present study, we found that overexpressing a closed form of the Kir6.2 subunit or the K_{ATP} channel blocker TB inhibited a 10 mM glucose-induced $[Ca^{2+}]_i$ increase in novel ghrelin-secreting cell line MGN3-1 cells (Figs 2B and 5D), whereas the application of the K_{ATP} channel opener DZ also inhibited 10 mM glucose-induced $[Ca^{2+}]_i$ increase. This complex phenomenon may be

explained by the hypothesis that moderate K_{ATP} channel opening is required for ghrelin secretion from MGN3-1 cells. We speculate that the K_{ATP} channels in MGN3-1 cells are opened a moderate amount in 10 mM glucose. In other words, K_{ATP} channels are closed in 25 mM glucose but opened in 5 mM glucose. Therefore, the application of a K_{ATP} channel opener or blocker in 10 mM glucose disturbs the moderate K_{ATP} channel opening, which leads to an inhibition of the $[Ca^{2+}]_i$ increase and ghrelin secretion. Furthermore, the closed K_{ATP} channels in 25 mM glucose are slightly opened by a low concentration of DZ, and the opened K_{ATP} channels in 5 mM glucose are slightly closed by a low concentration of TB, which induces a moderate K_{ATP} channel opening for ghrelin secretion (Fig. 6). A previous study has shown that the K_{ATP} channel modulators had little effect on ghrelin secretion in the ghrelin-secreting primary cells *in vitro* (Sakata *et al.* 2012). The application of K_{ATP} channel modulators to MGN3-1 cells at a concentration two orders of magnitude lower than that study enabled us in the present study to pinpoint intermediate K_{ATP} channel activity. In fact, when we applied high concentrations of K_{ATP} channel modulators to the MGN3-1 cells, the K_{ATP} channel modulators had little effect on $[Ca^{2+}]_i$ increase and ghrelin secretion (Figs 5 and 6).

Similar to the present results for ghrelin secretion, glucagon secretion has been shown to occur only within a narrow glucose level window in primary cultured pancreatic α cells *in vitro* (MacDonald *et al.* 2007). Cooperative interactions between voltage-dependent N- and T-type Ca^{2+} channels and K_{ATP} channels are implicated in that narrow window. In mouse pancreatic α cells, K_{ATP} channels are involved in the activation of low-voltage-dependent T-type Ca^{2+} channels at low glucose concentrations. Opening of the T-type Ca^{2+} channels induces membrane depolarization and then activates high-voltage-dependent N-type Ca^{2+} and Na^+ channels. N-type Ca^{2+} channel-dependent $[Ca^{2+}]_i$ influx is known to induce glucagon secretion (Gopel *et al.* 2000, Gromada *et al.* 2007, MacDonald *et al.* 2007). In the present study, we found that the inhibitors of L-, N-, or T-type voltage-dependent Ca^{2+} channels suppressed both $[Ca^{2+}]_i$ influx and ghrelin secretion (Fig. 7). Therefore, similar to pancreatic α cells, cooperative interactions between voltage-dependent L-, N-, and T-type Ca^{2+} channels and K_{ATP} channels could create the narrow glucose level window. Further study is needed to clarify how Ca^{2+} channels regulate ghrelin secretion in that narrow window.

Ghrelin (amino acids 24–51) is generated by post-translational processing of the 117 amino acid

preproghrelin precursor protein by prohormone convertase 1/3 (Romero *et al.* 2010). The presence of an eight-carbon fatty acid chain by the enzyme ghrelin-O-acyl-transferase on the serine 3 residue of acyl-ghrelin is required for the activation of the GH secretagogue receptor (Gutierrez *et al.* 2008). In contrast, desacyl-ghrelin has no eight-carbon fatty acid chain on the serine 3 residue (Kojima *et al.* 1999). This presents difficulties in directly labeling ghrelin with FPs. We developed several hormones fused to FPs (i.e., BDNF-Venus, rat tPA-GFP, and rat GH-GFP) that co-localized with ghrelin-containing vesicles (data not shown). We found that BDNF-Venus showed the highest co-localization efficiency with acyl-ghrelin-positive vesicles in MGN3-1 cells, as revealed by the specific anti-acyl-ghrelin antibody and confocal microscopy (Fig. 3A). Therefore, we used BDNF-Venus as an alternative marker for visualizing endogenous ghrelin secretion. In contrast to acyl-ghrelin, desacyl-ghrelin has been described as a functional antagonist of acyl-ghrelin action (Cassoni *et al.* 2001, Thompson *et al.* 2004). Because the receptor for desacyl-ghrelin has not yet been identified (Hosoda *et al.* 2000), the function of desacyl-ghrelin still needs to be elucidated.

Plasma ghrelin concentration increases before meals and decreases after meals in humans and rats (Ariyasu *et al.* 2001, Cummings *et al.* 2001, Tolle *et al.* 2002). Recent studies have shown that water absorption and occlusion of the pylorus of stomach had little effect on plasma ghrelin concentration changes in rodents (Tschop *et al.* 2000, Williams *et al.* 2003), which suggests that gastric distension is not involved in the decrease in plasma ghrelin concentrations after meals. In addition, intra-gastric infusion of glucose in pylorus-occlusion rats did not reduce plasma ghrelin concentrations. Thus, absorbed nutrients (i.e., glucose, amino acids, and fatty acids) are involved in the regulation of plasma ghrelin concentrations. In fact, oral or i.v. administration of glucose has been shown to reduce plasma ghrelin concentrations (Ariyasu *et al.* 2002, McCowen *et al.* 2002, Shiiya *et al.* 2002, Nakai *et al.* 2003, Williams *et al.* 2003, Broglio *et al.* 2004). Consistent with these *in vivo* studies, in the present study, we found that low glucose induced $[Ca^{2+}]_i$ increase and ghrelin secretion in MGN3-1 cells, whereas high glucose did not. Therefore, our *in vitro* results help elucidate the mechanism of ghrelin secretion *in vivo*.

In conclusion, we used mouse ghrelin-secreting MGN3-1 cells to elucidate the molecular mechanisms of glucose-induced ghrelin secretion *in vitro*. In particular, we revealed that K_{ATP} channels were expressed in MGN3-1 cells and mediated low glucose-induced ghrelin secretion.

Because ghrelin is an important hormone for regulating appetite, the modulation of K_{ATP} channel activity in ghrelin-secreting X/A-like cells could prevent glucose intolerance and thereby provide a potential therapeutic application to counteract the progression of obesity or type 2 diabetes mellitus.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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