Molecules and Cells

The Mechanism of Action of Ghrelin and Motilin in the Pacemaker Potentials of Interstitial Cells of Cajal from the Murine Small Intestine

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Interstitial cells of Cajal (ICCs) are pacemaker cells that exhibit periodic spontaneous depolarization in the gastrointestinal (GI) tract and generate pacemaker potentials. In this study, we investigated the effects of ghrelin and motilin on the pacemaker potentials of ICCs isolated from the mouse small intestine. Using the whole-cell patch-clamp configuration, we demonstrated that ghrelin depolarized pacemaker potentials of cultured ICCs in a dose-dependent manner. The ghrelin receptor antagonist [D-Lys] GHRP-6 completely inhibited this ghrelin-induced depolarization. Intracellular guanosine 5'-diphosphate-β-S and pre-treatment with Ca^{2+}-free solution or thapsigargin also blocked the ghrelin-induced depolarization. To investigate the involvement of inositol triphosphate (IP_3), Rho kinase, and protein kinase C (PKC) in ghrelin-mediated pacemaker potential depolarization of ICCs, we used the IP_3 receptor inhibitors 2-aminoethoxydiphenyl borate and xestospongin C, the Rho kinase inhibitor Y-27632, and the PKC inhibitors staurosporine, Go6976, and rottlerin. All inhibitors except rottlerin blocked the ghrelin-induced pacemaker potential depolarization of ICCs. In addition, motilin depolarized the pacemaker potentials of ICCs in a similar dose-dependent manner as ghrelin, and this was also completely inhibited by [D-Lys] GHRP-6. These results suggest that ghrelin induced the pacemaker potential depolarization through the ghrelin receptor in a G protein-, IP_3-, Rho kinase-, and PKC-dependent manner via intracellular and extracellular Ca^{2+} regulation. In addition, motilin was able to depolarize the pacemaker potentials of ICCs through the ghrelin receptor. Therefore, ghrelin and its receptor may modulate GI motility by acting on ICCs in the murine small intestine.

Keywords: gastrointestinal motility, ghrelin, interstitial cells of Cajal, motilin, pacemaker potentials

INTRODUCTION

Ghrelin is a 28-amino acid peptide originally identified in the rat stomach as an endogenous ligand for growth hormone secretagogue-receptor 1a (GHS-R1a) (Kitazawa et al., 2016). It has been shown to regulate a number of biological processes, including growth hormone (GH)-release, feeding stimulation, lipid metabolism, glucose metabolism, cardiovascular function, and reproductive function (Hosoda et al., 2006; Kojima and Kangawa, 2005; Kojima et al., 1999). Ghrelin stimulates contractility or spontaneous phase III-like contractions in many animals (Depoortere et al., 2005; Fukuda et al., 2004; Kitazawa et al., 2005; Nakamura et al., 2010) as well as in isolated gastrointestinal (GI) smooth muscles, via its action on neural GHS-R1a (Depoortere et al., 2005; Fukuda et al., 2004; Kitazawa et al., 2005). Motilin, a 22-amino-acid peptide, is known to stimulate GI motility and was the first

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interdigestive gut hormone identified (Brown et al., 1971). Motilin is also involved in the regulation of the migrating motor complex in the fasting state (Smet et al., 2009). The amino acid sequence of the motilin receptor is 52% identical to the human ghrelin receptor (Feighner et al., 1999; Thielemans et al., 2001). Numerous studies have demonstrated the presence of motilin receptors on smooth muscle cells (Feighner et al., 1999; Miller et al., 2000), and others have shown that motilin receptor agonists can promote intestinal function and improve gastric emptying in those with gastroparesis (Lu et al., 2010; Tonelli et al., 2009). Recently, an interaction between motilin and ghrelin in regulating GI motility has been reported in *Suncus* (Asian shrew) and dogs (Mondal et al., 2013; Ogawa et al., 2012). These results indicate that both ghrelin and motilin are gut hormones regulating GI motility in mammals.

The interstitial cells of Cajal (ICCs) are the pacemaker cells for GI movement (Huizinga et al., 1995; Kim et al., 2005). Loss of ICCs is frequently associated with several human GI motility disorders, such as diabetic gastroparesis (Yang et al., 2017), Hirschsprung's disease (Gfroerer and Rolle, 2013), slow transit constipation (Kashyap et al., 2011), and achalasia (Müller et al., 2014). However, the mechanism underlying the regulatory effect of ghrelin and motilin on ICCs has not been clearly demonstrated thus far.

In the present study, we investigate the effects of ghrelin or motilin on the pacemaker potentials of ICCs isolated from murine small intestines using a whole-cell patch-clamp technique.

MATERIALS AND METHODS

Ethics

Animal care and experiments were conducted in accordance with the guidelines issued by the Institutional Animal Care and Use Committee (IACUC) at Pusan National University (Busan, Republic of Korea; approval No. PNU-2017-1754) and those issued by the National Institute of Health Guide for the Care and Use of Laboratory Animals (Republic of Korea).

Preparation of ICCs and ICC clusters

Institute of Cancer Research (ICR) mice (Samtako Bio Korea Co., Ltd., Osan, Korea; 4-8 days old; weighing 2.0-2.3 g) of either sex were used as the source of ICCs. Animals were maintained under controlled conditions (21 ± 3°C, relative humidity 50 ± 6%, 12 h light-dark cycle). The small intestines were removed and opened along the mesenteric border and then luminal contents were removed using Krebs-Ringer bicarbonate solution. Mucosae were then removed by sharp dissection, and small tissue strips of intestine muscle were then equilibrated for 30 min in Ca^{2+}-free Hank's solution (containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM glucose, 2.9 mM sucrose, and 11 mM HEPES; pH 7.4). Cells were then dispersed in an enzyme solution containing 1.7 mg ml^{-1} collagenase (Worthington Biochemical, USA), 2.5 mg ml^{-1} bovine serum albumin (Sigma-Aldrich, USA), 3.0 mg ml^{-1} trypsin inhibitor (Sigma-Aldrich), and 0.60 mg ml^{-1} ATP (Sigma-Aldrich). Cells were then cultured at 37°C in a 95% O_2-5% CO_2 incubator in smooth muscle growth medium (Clonetics, USA) supplemented with 2% antibiotics/antimycotics (Gibco, USA) and 5 ng ml^{-1} of murine stem cell factor (Sigma-Aldrich). At first, we attempted to isolate ICCs cultured for < 12 h immunologically, using an anti-c-kit antibody (eBioscience, USA) at a dilution of 1:50 for 20 min (Hong et al., 2015). However, the morphological state of the ICCs did not allow us to subsequently perform patch-clamp experiments. Therefore, because ICCs are morphologically distinct from other cell types in the culture, they were identified by phase contrast microscopy. The ICCs were spindle-shaped, with several branches emanating from a central soma, and connected to neighboring cells, producing a network. The patch-clamp technique was tested on ICCs that showed these network-like structures in culture. We subsequently performed immunostaining with the anti-c-kit antibody (Fig. 1) (Jun et al., 2005), which revealed that the vast majority of the cells were c-kit positive. Spontaneous rhythmicity was routinely recorded from cultured ICC clusters under current-clamp conditions, and the ICCs within the networks had a more robust electrical rhythmicity. Tissue-like spontaneous slow waves were recorded from these cells (Koh et al., 1998).

Patch-clamp experiments

We used whole-cell patch-clamp methods to record the effects of ghrelin or motilin on the pacemaker potentials of the ICCs. The physiological salt solution used to bathe cultured ICC clusters contained 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES (adjusted to pH 7.4 with NaOH). The pipette solution used to examine pacemaker potentials contained 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM NaGTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM EGTA (adjusted to pH 7.2 with KOH). Patch-clamp techniques were conducted using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments, USA). Command pulses were applied using an IBM-compatible personal computer and pClamp soft-

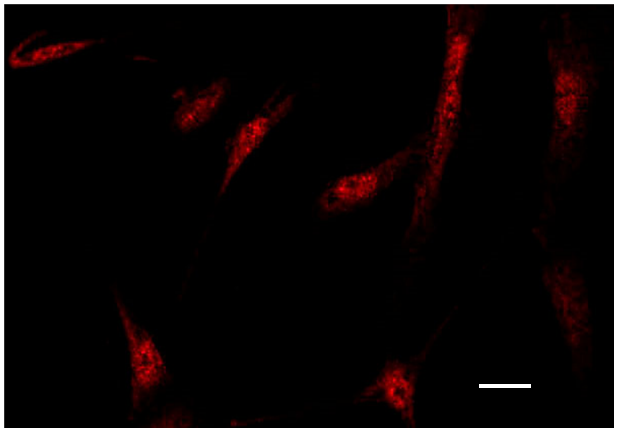

Fig. 1. Cultured ICCs from the murine small intestine. The tunica muscularis of the small intestine was digested with collagenase, and the dispersed cells were cultured for 12 h. The confocal microscope image shows the c-kit-immunopositive ICC network in the culture. Scale bar = 10 μm.

ware (ver. 6.1 and ver. 10.0; Axon Instruments). Data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and/or a Gould 2200 pen recorder (Gould, USA). Results were analyzed using pClamp and Origin software (ver. 6.0; Microcal, USA). All experiments were performed at 30°C to 32°C.

Drugs

Motilin, MA2029, staurosporine (ST), Go6976, and rottlerin (ROT) were purchased from Tocris Bioscience (United Kingdom), while ghrelin, [D-Lys] growth hormone-releasing peptide (GHRP-6), guanosine 5'-diphosphate (GDP)-β-S, thapsigargin, 2-aminoethoxydiphenyl borate (2-APB), xestospongin C, Y-27632, and all other drugs were obtained from Sigma-Aldrich. For stock solutions, all drugs were dissolved in distilled water or dimethylsulfoxide (DMSO) and stored at −20°C. The final concentration of DMSO in the bath solution was always < 0.1%, and we confirmed that it did not affect the results at this concentration. Furthermore, the addition of these chemicals did not alter the pH of the bath solution.

Statistical analysis

Results are expressed as the means ± SEMs, and n refers to the number of cells used in the experiments. For multiple comparison analysis, we used one-way ANOVA with Bonferroni's *post hoc* comparison. For statistical analyses, we used Prism 6.0 (GraphPad Software Inc., USA) and Origin (ver. 8.0; OriginLab Corporation, USA). *P* values of < 0.05 were considered statistically significant.

RESULTS

Effects of ghrelin on the pacemaker potentials of ICCs from murine small intestines

We investigated the characteristics of cultured ICC clusters using the whole-cell patch-clamp technique. ICC clusters formed network-like structures after culture for < 12 h and were viable as demonstrated by the generation of spontaneous rhythmic contractions. Under the current clamp mode ($I = 0$), the ICCs generated pacemaker potentials with a mean resting membrane potential of −57.4 ± 1.2 mV and a mean amplitude of 24.4 ± 1.1 mV (Fig. 2). Ghrelin (1-10 μM) depolarized the pacemaker potentials and decreased their amplitudes in a dose-dependent manner (Figs. 2A-2C). The mean degrees of depolarization in the presence of 1, 5, and 10 μM ghrelin were 2.5 ± 0.5 mV, 11.0 ± 0.8 mV, and 24.5 ± 1.3 mV (n = 11; Fig. 2D), and the mean amplitudes were 23.7 ± 1.2 mV, 8.1 ± 0.8 mV, and 3.5 ± 0.5 mV (n = 11; Fig. 2E), respectively. The effects of ghrelin on the pacemaker potentials are summarized in Figures 2D and 2E. These results suggest that ghrelin depolarizes the pacemaker potentials of ICCs in a dose-dependent manner.

Involvement of the ghrelin receptor in ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines

To investigate whether the effect of ghrelin on ICCs was via the G protein-coupled ghrelin receptor, we exposed ICCs to the ghrelin receptor antagonist [D-Lys] GHRP-6 (5 μM for 5 min). [D-Lys] GHRP-6 completely inhibited the effect of ghrelin on the pacemaker potentials of ICCs (Fig. 3A). In the presence of 1, 3, and 5 μM [D-Lys] GHRP-6, the mean degrees of depolarization were 13.2 ± 0.7 mV ($P < 0.01$), 5.9 ± 1.1 mV ($P < 0.01$), and 1.5 ± 0.4 mV ($P < 0.01$), respectively (n = 7; Fig. 3B). These results suggest that ghrelin affects ICC pacemaker potentials via the ghrelin receptor.

Involvement of G proteins in ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines

To investigate whether G proteins are required for ghrelin-induced pacemaker potential depolarization, we used GDP-β-S,

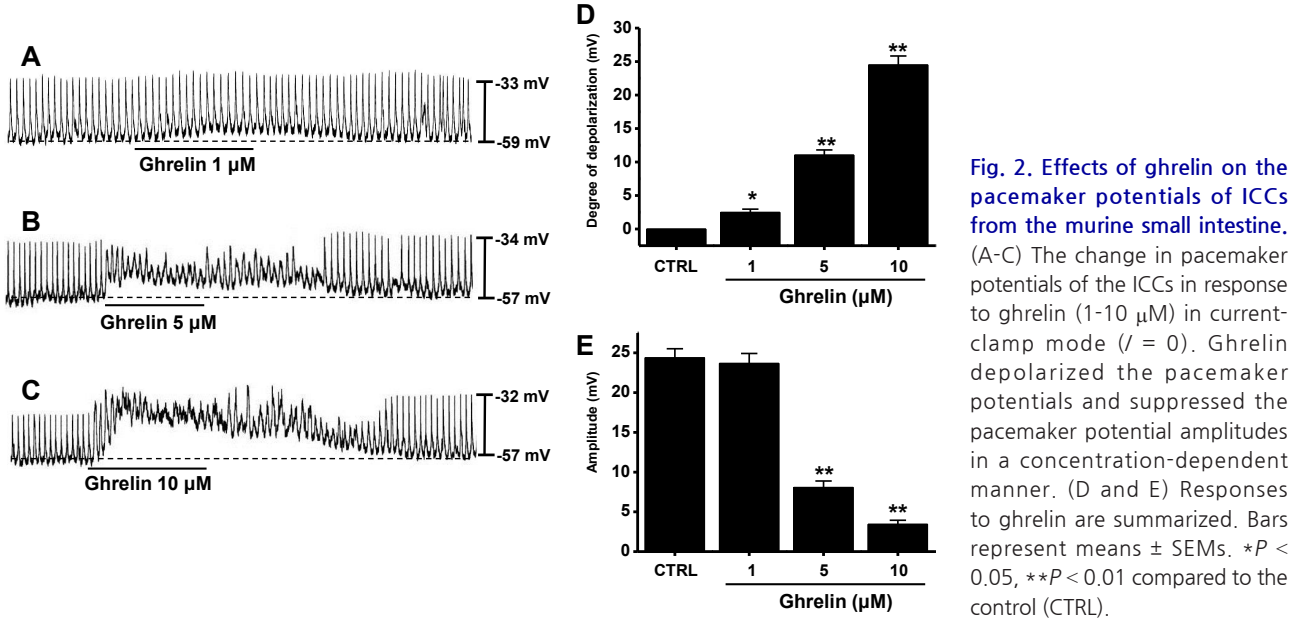

Fig. 2. Effects of ghrelin on the pacemaker potentials of ICCs from the murine small intestine. (A-C) The change in pacemaker potentials of the ICCs in response to ghrelin (1-10 μM) in current-clamp mode ($I = 0$). Ghrelin depolarized the pacemaker potentials and suppressed the pacemaker potential amplitudes in a concentration-dependent manner. (D and E) Responses to ghrelin are summarized. Bars represent means ± SEMs. $*P < 0.05$, $**P < 0.01$ compared to the control (CTRL).

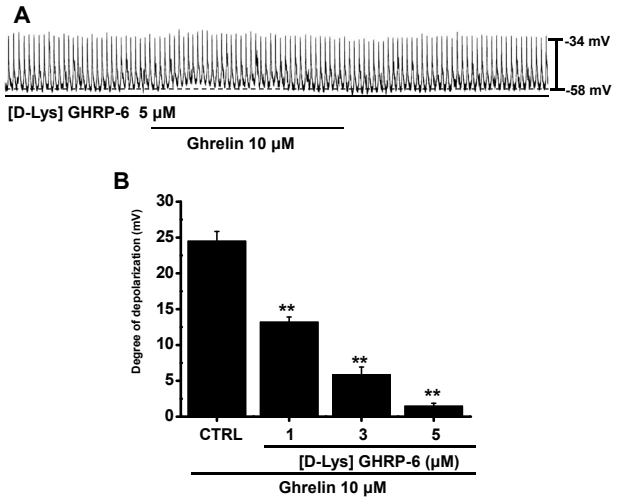

Fig. 3. Effects of the ghrelin receptor antagonist on ghrelininduced pacemaker potential depolarization of ICCs from murine small intestines. (A) In the presence of the ghrelin receptor antagonist [D-Lys] GHRP-6 (5 μM), the ghrelin-induced depolarization was inhibited. (B) Responses to ghrelin in the presence of [D-Lys] GHRP-6 are summarized. Bars represent means ± SEMs. $**P < 0.01$ compared to the non-treated control (CTRL).

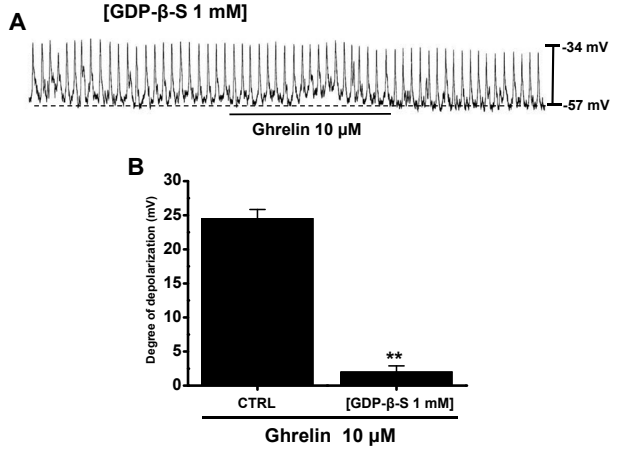

Fig. 4. Effects of GDP-β-S on ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines. (A) Under intracellular application of GDP-β-S (1 mM), ghrelin did not depolarize the ICC pacemaker potentials. (B) Ghrelin-induced responses in the presence of GDP-β-S are summarized. Bars represent the means ± SEMs. $**P < 0.01$ compared to the non-treated control (CTRL).

which permanently inactivates G protein-binding proteins (Komori et al., 1992; Ogata et al., 1996). When GDP-β-S (1 mM) was applied intracellularly, ghrelin (10 μM) induced only a slight pacemaker potential depolarization (2.1 ± 0.8 mV, n = 7; Fig. 4). These results suggest that G proteins are involved in the ghrelin-induced pacemaker potential depolarization of ICCs.

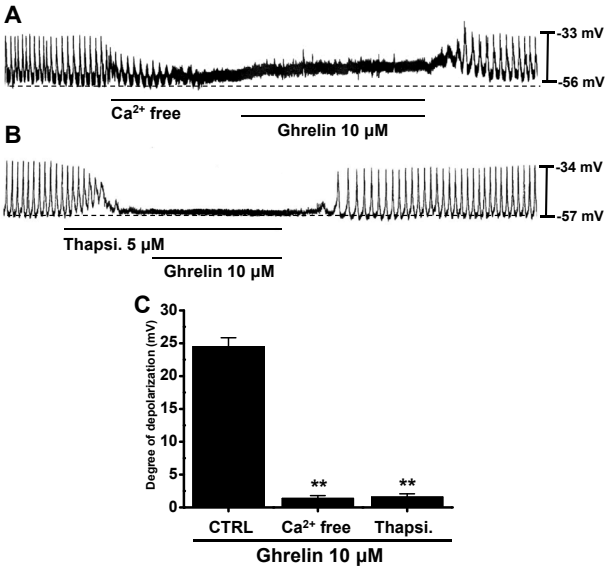

Fig. 5. Effects of extracellular and intracellular Ca^{2+} regulation on ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines. (A) Ghrelin did not induce depolarization under external Ca^{2+}-free solution conditions. (B) Ghrelin did not induce depolarization in the presence of thapsigargin (Thapsi.). (C) Ghrelin-induced responses are summarized. Bars represent the means ± SEMs. $**P < 0.01$ compared to the untreated control (CTRL).

Involvement of extracellular and intracellular calcium ions in ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines

Both intracellular and extracellular Ca^{2+} have important roles In GI motility modulations (Ward, 2000). To investigate the involvement of intracellular and extracellular Ca^{2+} in the ghrelin-induced pacemaker potential depolarization of ICCs, we performed experiments under external Ca^{2+}-free conditions or in the presence of thapsigargin, an inhibitor of Ca^{2+}-ATPase in the endoplasmic reticulum. Pre-treatment with the external Ca^{2+}-free solution (Fig. 5A) or thapsigargin (Fig. 5B) abolished the pacemaker potentials, and inhibited ghrelin-induced pacemaker potential depolarization of ICCs (n = 5; Fig. 5). The effects of intracellular and extracellular Ca^{2+} on ghrelin-induced pacemaker potential depolarization are summarized in Figure 5C. These results suggest that ghrelin-induced pacemaker potential depolarization of murine ICCs is dependent on intracellular and extracellular Ca^{2+} regulation.

Involvement of the inositol triphosphate (IP_3) and Rho kinase pathways in ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines

Ghrelin induces smooth muscle contraction via IP_3- and Rho kinase-dependent pathways (Dimitrova et al., 2007). Therefore, we investigated whether these two pathways are required for ghrelin-induced pacemaker potential depolar-

ization in ICCs using the Rho kinase inhibitor Y-27632 and the IP_3 receptor inhibitors 2-APB and xestospongin C. Ghrelin did not depolarize pacemaker potentials in the presence of 2-APB (100 μM), xestospongin C (1 μM) (n = 6; Figs. 6A and 6B) or Y-27632 (1 μM) (n = 5; Fig. 6C). In the presence of 10, 50, and 100 μM 2-APB, the mean degrees of depolarization were 19.2 ± 0.8 mV ($P < 0.05$), 11.8 ± 1.6 mV ($P < 0.01$), and 1.4 ± 0.4 mV ($P < 0.01$), and the mean amplitudes were

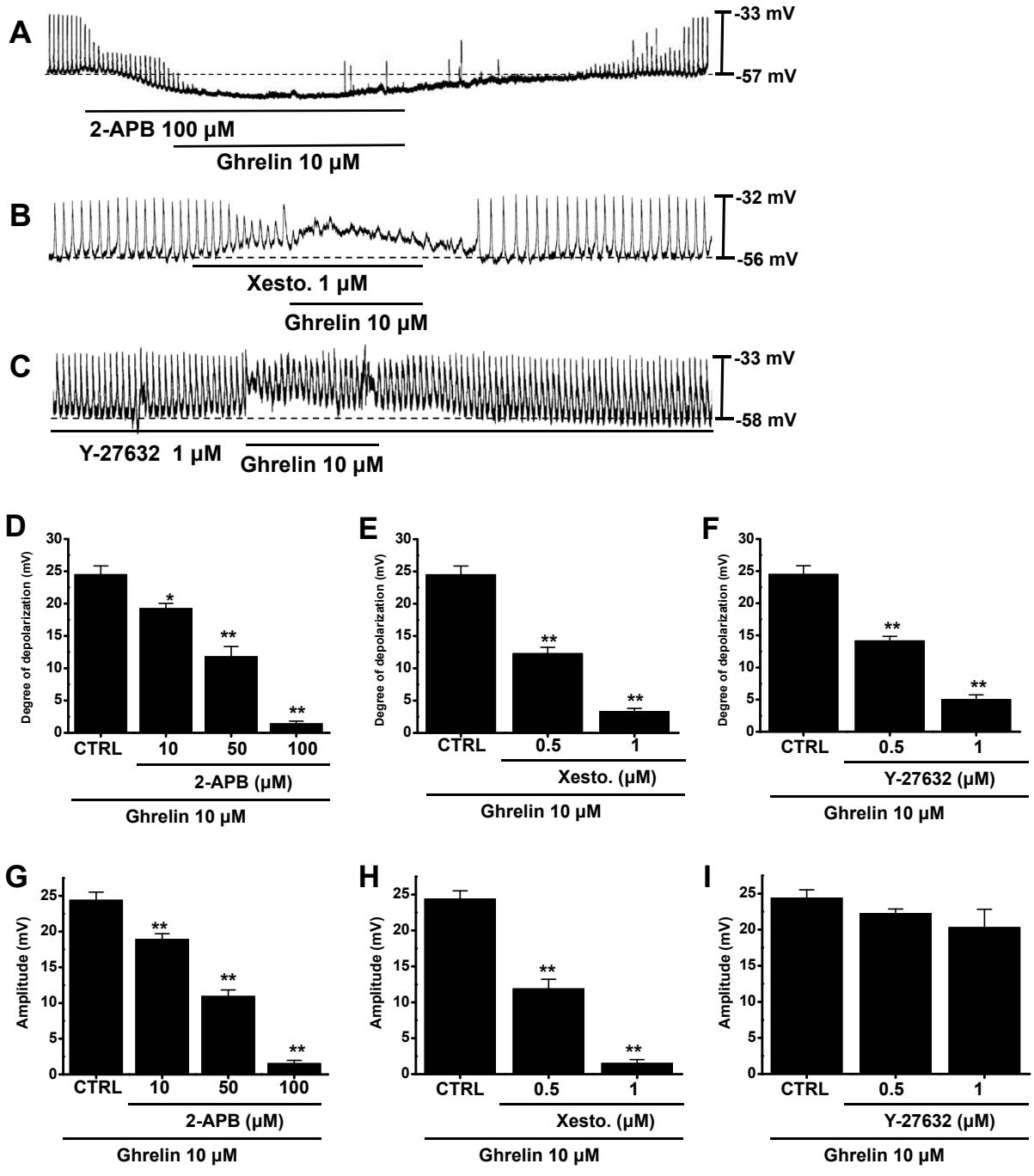

Fig. 6. Effects of the IP_3 receptor inhibitors 2-APB and xestospongin C (Xesto.), and the Rho kinase inhibitor Y-27632 on ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines. (A and B) 2-APB or xestospongin C treatment blocked ghrelin-induced depolarization. (C) Y-27632 treatment blocked ghrelin-induced depolarization. Depolarization responses under 2-APB (D), xestospongin C (E), and Y-27632 (F) treatments are summarized. Amplitude responses under 2-APB (G), xestospongin C (H), and Y-27632 (I) treatments are summarized. Bars represent the means ± SEMs. $*P < 0.05$, $**P < 0.01$ compared to the non-treated control (CTRL).

18.9 ± 0.8 mV ($P < 0.01$), 10.9 ± 0.9 mV ($P < 0.01$), and 1.5 ± 0.4 mV ($P < 0.01$), respectively (n = 9; Figs. 6D and 6G). In the presence of 0.5 and 1 μM xestospongin C, the mean degrees of depolarization were 12.3 ± 1.0 mV ($P < 0.01$) and 3.3 ± 0.5 mV ($P < 0.01$), and the mean amplitudes were 11.9 ± 1.3 mV ($P < 0.01$) and 1.5 ± 0.5 mV ($P < 0.01$), respectively (n = 7; Figs. 6E and 6H). In the presence of 0.5 and 1 μM Y-27632, the mean degrees of depolarization were 14.2 ± 0.7 mV ($P < 0.01$) and 5.0 ± 0.7 mV ($P < 0.01$) and the mean amplitudes were 22.3 ± 0.6 mV and 20.3 ± 2.5 mV, respectively (n = 8; Figs. 6F and 6I). The effects of the IP_3 and Rho kinase inhibitors on ghrelin-induced pacemaker potential depolarization are summarized in Figures 6D and 6E. These results suggest that the IP_3 and Rho kinase pathways are involved in ghrelin-induced pacemaker potential depolarization of ICCs.

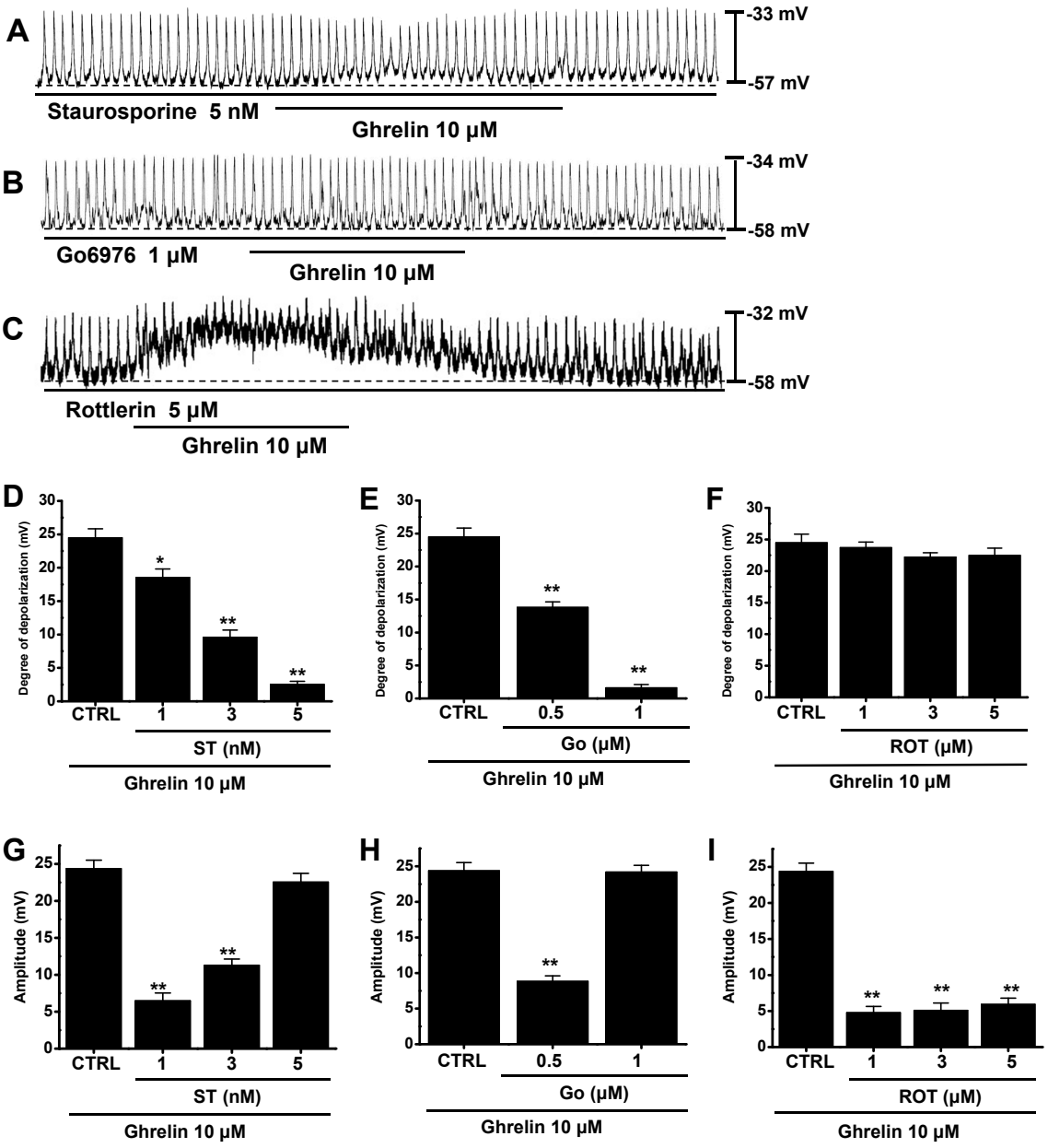

Fig. 7. Effects of the PKC inhibitors staurosporine (a broad-spectrum PKC inhibitor), Go6976 (a calcium-dependent PKCα/β inhibitor), and rottlerin (a calcium-independent PKCδ inhibitor) on ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines. (A and B) Staurosporine or Go6976 treatment blocked the ghrelin-induced depolarization. (C) Rottlerin treatment did not block the ghrelin-induced depolarization. Depolarization responses under staurosporine (D), Go6976 (E), and rottlerin (F) treatments are summarized. Amplitude responses under staurosporine (G), Go6976 (H), and rottlerin (I) treatments are summarized. Bars represent the means ± SEMs. ST, staurosporine; Go, Go6976; ROT, rottlerin. $*P < 0.05$, $**P < 0.01$ compared to the non-treated control (CTRL).

Involvement of the protein kinase C (PKC) pathway in ghrelin-induced pacemaker potential depolarization of ICCs from murine small intestines

Finally, we investigated whether the PKC pathway is required for ghrelin-induced pacemaker potential depolarization in ICCs using the PKC inhibitors ST (a broad-spectrum PKC inhibitor), Go6976 (a calcium-dependent PKCα/β inhibitor), and ROT (a calcium-independent PKCδ inhibitor). In the presence of ST (5 nM) and Go6976 (1 μM), ghrelin did not depolarize the pacemaker potentials (n = 8; Figs. 7A and 7B). However, in the presence of ROT (5 μM), ghrelin was still able to induce the pacemaker potential depolarization (n = 7; Fig. 7C). In the presence of 1, 3, and 5 nM ST, the mean degrees of depolarization were 18.6 ± 1.2 mV ($P < 0.05$), 9.6 ± 1.1 mV ($P < 0.01$), and 2.5 ± 0.5 mV ($P < 0.01$), and the mean amplitudes were 6.5 ± 1.0 mV ($P < 0.01$), 11.3 ± 0.8 mV ($P < 0.01$), and 22.6 ± 1.2 mV, respectively (n = 13; Figs. 7D and 7G). In the presence of 0.5 and 1 μM Go6976, the mean degrees of depolarization were 13.8 ± 0.8 mV ($P < 0.01$) and 1.6 ± 0.5 mV ($P < 0.01$), and the mean amplitudes were 8.9 ± 0.8 mV ($P < 0.01$) and 24.2 ± 0.9 mV, respectively (n = 12; Figs. 7E and 7H). In the presence of 1, 3, and 5 μM ROT, the mean degrees of depolarization were 23.7 ± 0.9 mV, 22.2 ± 0.6 mV, and 22.5 ± 1.2 mV, and the mean amplitudes were 4.8 ± 0.9 mV ($P < 0.01$), 5.1 ± 1.0 mV ($P < 0.01$), and 5.9 ± 0.8 mV ($P < 0.01$), respectively (n = 12; Figs. 7F and 7I). These results suggest that the Ca^{2+}-dependent PKC pathway is involved in ghrelin-induced pacemaker potential depolarization of ICCs.

Effects of motilin on the pacemaker potentials of ICCs from murine small intestines

In rodents, only pseudogenes for the motilin receptor have been identified (Sanger et al., 2011). However, close structural identity exists between ghrelin and motilin, their precursor peptides, and their receptors (Sanger and Furness, 2016). Ghrelin and motilin are also linked by their predominant distribution in the upper GI tract and their release during hunger to influence GI functions (Sanger and Furness, 2016). Therefore, we also investigated the effects of motilin on the pacemaker potentials of ICCs from murine small intestines. Motilin (1-5 μM) was able to depolarize the pacemaker potentials of the ICCs, decreasing their frequencies in a dose-dependent manner (Figs. 8A-8C). In the presence of 1, 3, and 5 μM motilin, the mean degrees of depolarization were 2.7 ± 0.5 mV, 12.7 ± 1.3 mV, and 26.1 ± 1.0 mV (n = 12; Fig. 8D), and the mean frequencies were 16.1 ± 1.2 cycles/min, 11.6 ± 1.3 cycles/min, and 3.5 ± 0.8 cycles/min (n = 12; Fig. 8E), respectively. The effects of motilin on the pacemaker potentials are summarized in Figures 8D and 8E. These results suggest that motilin depolarizes the pacemaker potentials of ICCs in a dose-dependent manner.

Involvement of the ghrelin receptor in motilin-induced pacemaker potential depolarization of ICCs from murine small intestines

We next used the ghrelin receptor antagonist [D-Lys] GHRP-6 to investigate whether motilin mediates its effect on ICCs via the ghrelin receptor. Exposure of ICCs to [D-Lys] GHRP-6 (5 μM for 5 min) completely inhibited motilin-induced depolarization (Fig. 9A). In the presence of 1, 3, and 5 μM [D-Lys] GHRP-6, the mean degrees of depolarization were 15.6 ± 1.5 mV ($P < 0.01$), 8.0 ± 1.4 mV ($P < 0.01$), and 2.3 ± 0.5 mV ($P < 0.01$), respectively (n = 7; Fig. 9B). These results suggest that motilin affects ICC pacemaker potentials through the ghrelin receptor.

DISCUSSION

In the present study, we elucidated the mechanism by which

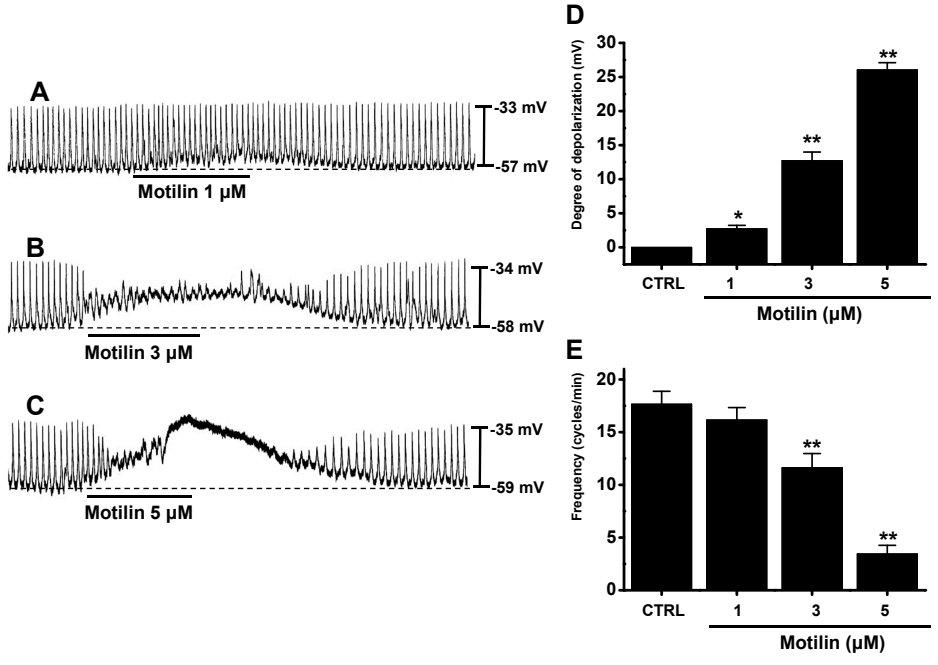

Fig. 8. Effects of motilin on the pacemaker potentials of ICCs from murine small intestines. (A-C) The change in pacemaker potentials of the ICCs induced by motilin (1-5 μM) in current-clamp mode ($I = 0$). Motilin depolarized the pacemaker potentials and suppressed the pacemaker potential amplitudes in a concentration-dependent manner. (D and E) Responses to motilin are summarized. Bars represent means ± SEMs. $*P < 0.05$, $**P < 0.01$ compared to the control (CTRL).

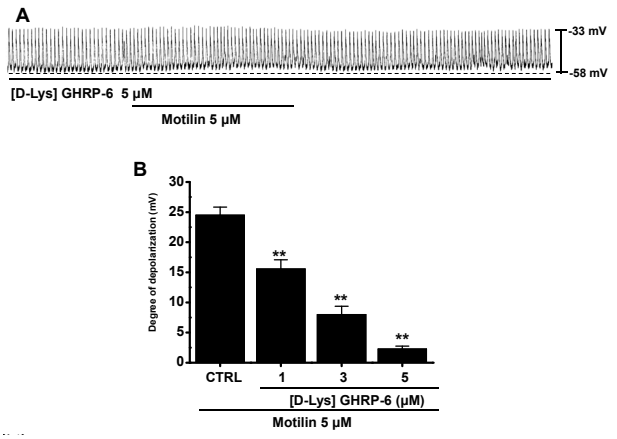

Fig. 9. Effects of the ghrelin receptor antagonist on motilin-induced pacemaker potential depolarization of ICCs from murine small intestines. (A) In the presence of the ghrelin receptor antagonist [D-Lys] GHRP-6, the motilin-induced depolarization was inhibited. (B) Responses to motilin in the presence of [D-Lys] GHRP-6 are summarized. Bars represent means ± SEMs. $^{**}P < 0.01$ compared to the non-treated control (CTRL).

ghrelin depolarizes murine ICC pacemaker potentials. First, we demonstrated that ghrelin depolarized the ICC pacemaker potentials in a dose-dependent manner. We next showed that this was via the G protein-coupled ghrelin receptor, as it was inhibited by the ghrelin receptor antagonist [D-Lys] GHRP-6 (Fig. 3). We also showed that the action of ghrelin on its receptor required intracellular and extracellular Ca^{2+} and the downstream G protein-, IP_3-, Rho kinase-, and PKC-dependent signaling pathways. Intracellular GDP-β-S and pre-treatment with Ca^{2+}-free solution or thapsigargin inhibited the ghrelin-induced depolarization (Figs. 4 and 5). Moreover, ghrelin did not depolarize the ICC pacemaker potentials in the presence of 2-APB, xestospongin C, or Y-27632 (Fig. 6). In addition, ghrelin-induced depolarization was blocked in the presence of ST or Go6976 (Fig. 7). Therefore, ghrelin may modulate GI motility by acting on ICCs in the small intestine. In addition, motilin depolarized the pacemaker potentials of ICCs through the ghrelin receptor in a dose-dependent manner (Figs. 8 and 9).

Ghrelin is a 28-amino acid peptide predominantly produced by endocrine cells in the oxyntic mucosa of the stomach as an endogenous ligand for the GH secretagogue receptor (Kojima et al., 2001; Wang et al., 2002). Motilin is a 22-amino acid peptide synthesized from endocrine cells of the duodeno-jejunal mucosa (Brown et al., 1971). The amino-acid sequences of the motilin and ghrelin precursors share approximately 50% identity. In addition, the receptors of both peptides are members of the same G protein-coupled receptor (GPCR) family and share 53% overall amino-acid sequence identity (Poitras and Peeters, 2008). Based upon their structural similarity, these two peptides are now considered to be members of the new motilin-ghrelin peptide family. Ghrelin has been reported to stimulate GI motility (Kitazawa et al., 2005; Tack et al., 2006), induce phase III-like contractions in the rat stomach (Fujino et al., 2003), and induce premature phase III interdigestive migrating contractions (IMCs) in the human stomach (Tack et al., 2006). Motilin regulates IMC, the motor pattern in the GI tract under fasting conditions (Itoh, 1997). In gastroenterology, ghrelin and motilin are often discussed together because of the genetic and structural similarities between their receptors (Folwaczny et al., 2001; Sanger et al., 2011), their location within the upper GI tract, and their ability to promote gastric motility (Sanger, 2008). However, because the motilin receptor only exists as a pseudogene in rodents (Aerssens et al., 2004; Sanger, 2008), studies on motilin-regulated GI motility in animal models are scarce and have been limited to dogs. Ghrelin was identified at a similar time to motilin by a group who named it the "motilin-related peptide" because of their high sequence similarity (Tomasetto et al., 2000). It then became clear that in addition to the structural similarities between the peptides themselves, their receptors displayed a high sequence homology of 44% overall identity, rising to 87% in the transmembrane regions. Motilin and ghrelin receptors therefore constitute a new subfamily within class A rhodopsin-like GPCRs (Poitras and Peeters, 2008). The absence of functional motilin receptors in mice and rats is consistent with several reports demonstrating an inability of motilin receptor agonists to stimulate GI motility in these species (Depoorteree et al., 2005). However, another study suggested that motilin can stimulate gastric motility, increase intracellular calcium in stomach muscles, and provoke feeding in mice and rats (Feng et al., 2007). One possibility is that rodent ghrelin receptors are responsive to motilin at high concentrations. Another possibility is that certain motilin receptor agonists, which have been derived from the highly complex macrolide structure of erythromycin, might have some affinity for the ghrelin receptor (Nunoi et al., 2012). Generally, low concentrations of motilin stimulate contraction of gut smooth muscle (EC_{50} = 1 nM) and increase pepsin release (Huang et al., 2005). In this study, 1-5 μM motilin depolarized the pacemaker potentials of murine ICCs in a dose-dependent manner (Figs. 8A-8C). Therefore, it seems that the concentrations we used were high enough to stimulate the ghrelin receptor, and/or the particular motilin used in this study (Tocris Bioscience) might have some affinity for the ghrelin receptor. In the future, the characteristics of this motilin will be investigated in more detail. It is also possible that the motilin and ghrelin receptors may have a complementary relationship; where the expression of the motilin receptor is lower, the expression of the ghrelin receptor will be higher. In this study, ghrelin and motilin depolarized the pacemaker potentials of ICCs from murine small intestines (Figs. 2 and 8), and we showed that these effects were through the ghrelin receptor (Figs. 3 and 9). In mice, the ghrelin receptor may make a useful potential target for dysmotility in the GI tract. In addition, ghrelin and motilin have therapeutic potential as pharmacological agents in stimulating GI motility and accelerating gastric emptying (Ohno et al., 2010). Therefore, these two peptides are of great interest to GI physiologists, and their potential value as prokinetic agents may increase in the future.

ICCs act as pacemaker cells in GI motility function (Huizinga et al., 1995; Kim et al., 2005). Therefore, they play a vital

role in the generation and propagation of the electrical properties of smooth muscles (Huizinga et al., 1995; Kim et al., 2005). In this study, ghrelin and motilin depolarized the ICC pacemaker potentials through the ghrelin receptor. These results suggest that ghrelin and motilin may act on other cells (e.g., smooth muscle cells or enteric neurons) through ICCs to increase GI motility, and that the ghrelin receptor has an important role in regulating the pacemaker potentials of ICCs in the mouse small intestine.

In summary, the results of the present study show that: (1) ghrelin depolarized the pacemaker potentials of ICCs in a concentration-dependent manner; (2) a ghrelin receptor antagonist, [D-Lys] GHRP-6, completely inhibited the ghrelin-induced depolarization; (3) intracellular GDP-β-S inhibited the ghrelin-induced depolarization; (4) pre-treatment with Ca^{2+}-free solution or thapsigargin blocked the ghrelin-induced depolarization; (5) 2-APB, xestospongin C (IP_3 receptor inhibitors), Y-27632 (a Rho kinase inhibitor), ST, and Go6976 (PKC inhibitors) blocked the ghrelin-induced depolarization; and (6) motilin depolarized the pacemaker potentials of ICCs in a dose-dependent manner through the ghrelin receptor.

Taken together, these results suggest that ghrelin induced the pacemaker potential depolarization in a G protein-, IP_3-, Rho kinase-, and PKC-dependent manner via intracellular and extracellular Ca^{2+} regulation, and that motilin also depolarized the pacemaker potentials of ICCs in a dose-dependent manner through the ghrelin receptor. Ghrelin and its receptor may modulate GI motility by acting on ICCs in the murine small intestine and may make useful potential targets for the treatment of achalasia, non-achalasia esophageal motility disorders, dyspepsia, gastroparesis, chronic intestinal pseudo-obstruction, irritable bowel syndrome, chronic constipation, and transit disturbances associated with GI motility disorders in mice.

Disclosure
The authors have no potential conflicts of interest to disclose.

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