



Regulation of Intracellular Calcium by Endoplasmic Reticulum Proteins in Small Intestinal Interstitial Cells of Cajal

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Background/Aims

We investigated the role of representative endoplasmic reticulum proteins, stromal interaction molecule 1 (STIM1), and store-operated calcium entry-associated regulatory factor (SARAF) in pacemaker activity in cultured interstitial cells of Cajal (ICCs) isolated from mouse small intestine.

Methods

The whole-cell patch clamp technique applied for intracellular calcium ions ($[Ca^{2+}]_i$) analysis with STIM1 or SARAF overexpressed cultured ICCs from mouse small intestine.

Results

In the current-clamping mode, cultured ICCs displayed spontaneous pacemaker potentials. External carbachol exposure produced tonic membrane depolarization in the current-clamp mode, which recovered within a few seconds into normal pacemaker potentials. In STIM1-overexpressing cultured ICCs pacemaker potential frequency was increased, and in SARAF-overexpressing ICCs pacemaker potential frequency was strongly inhibited. The application of gadolinium (a non-selective cation channel inhibitor) or a Ca^{2+} -free solution to understand Orai channel involvement abolished the generation of pacemaker potentials. When recording intracellular Ca^{2+} concentration with Fluo 3-AM, STIM1-overexpressing ICCs showed an increased number of spontaneous intracellular Ca^{2+} oscillations. However, SARAF-overexpressing ICCs showed fewer spontaneous intracellular Ca^{2+} oscillations.

Conclusion

Endoplasmic reticulum proteins modulated the frequency of pacemaker activity in ICCs, and levels of STIM1 and SARAF may determine slow wave patterns in the gastrointestinal tract.

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Key Words

Interstitial cells of Cajal; Intestinal motility; Stromal interaction molecule 1; Store-operated calcium entry-associated regulatory factor

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Introduction

Various cellular functions such as contraction, proliferation, neurotransmission, and exocytosis are regulated by intracellular calcium ions ($[Ca^{2+}]_i$).¹⁻⁴ Normally, $[Ca^{2+}]_i$ exists in intracellular stores such as the endoplasmic reticulum (ER) and mitochondria. There are 2 ways to increase $[Ca^{2+}]_i$: the influx of Ca^{2+} ions through the plasma membrane (PM) or by release from intracellular stores.⁵⁻⁷

The activation of receptors on the PM releases $[Ca^{2+}]_i$ from the ER and then activates Ca^{2+} channels at the PM. This process is called store-operated Ca^{2+} entry (SOCE). It is well known that SOCE is the primary mechanism for Ca^{2+} signaling and homeostasis.⁸ Many reports have suggested the involvement of diverse proteins in SOCE activity. Until now, stromal interaction molecule (STIM) and Orai proteins are the most important elements for SOCE activity.⁹⁻¹³ STIM is an ER protein and depletion of Ca^{2+} in ER can lead to Orai-STIM binding. This opens Orai channels in the PM and results in Ca^{2+} entry.^{14,15} Recently, an interesting ER protein, store-operated calcium entry-associated regulatory factor (SARAF), was reported. SARAF plays an important role in preventing excessive Ca^{2+} influx.¹⁶ However, the exact role of SARAF in functional cells has not been established.

The gastrointestinal (GI) tract shows spontaneous contraction that is evoked by periodic membrane depolarizations called slow waves. There has been increasing evidence that interstitial cells of Cajal (ICCs) are pacemaking cells that generate slow waves.¹⁷⁻²⁰ ICCs produce spontaneous electrical activity (pacemaker activity) that is transmitted into smooth muscle cells through gap junctions.²¹ Although the exact mechanisms underlying these events are still not understood, many reports indicate that periodic ER Ca^{2+} oscillations are involved in producing ICC pacemaker activity.^{22,23} This implicates $[Ca^{2+}]_i$ as an essential component that enables the production of pacemaker activity in ICCs. However, to date, it is not known how $[Ca^{2+}]_i$ is regulated in ICCs. Therefore, we suggest that 2 ER proteins, STIM and SARAF, could be regulators of $[Ca^{2+}]_i$, and that ICC pacemaker activity was modulated by those ER proteins in this study.

Materials and Methods

Ethics

All experiments were carried out according to the Guiding

Principles for the Care and Use of Animals approved by the ethics committee in Chosun University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the number of animals used and their suffering.

Solutions and Drugs

Cultured ICCs from small intestine were bathed in a solution containing 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 7.2 with Tris. The pipette solution contained 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP , 0.1 mM Na_2GTP , 2.5 mM creatine phosphate disodium, 5 mM HEPES, and 0.1 mM EGTA, adjusted to pH 7.2 with Tris.

Carbachol (CCh) and gadolinium chloride were purchased from Sigma Chemical Co (St. Louis, MO, USA). All drugs were dissolved in distilled water or dimethyl sulfoxide (DMSO) to prepare stock solutions (10 or 100 mM), and were either added to the bath solution or applied to whole-cell preparations by superfusion. The final concentration of DMSO was less than 0.05%.

Preparation of Cells and Tissues

ICR mice (2-3 day-old) of either sex were anesthetized with ether and euthanized by cervical dislocation. After cervical dislocation, an abdominal incision was made and the entire small intestine was removed. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were fixed with pins to a Sylgard dish and the mucosa was removed by sharp dissection. Strips from small intestinal muscle layer were equilibrated in Ca^{2+} -free Krebs-Ringer bicarbonate solution for 30 minutes and cells were agitated with an enzyme solution containing 1.3 mg/mL collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2 mg/mL bovine serum albumin (Sigma Chemical Co), 2 mg/mL trypsin inhibitor (Sigma Chemical Co), and 0.27 mg/mL ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μ g/mL; Falcon/BD) in 35 mm culture dishes. The cells were then cultured at 37°C in a 95% O_2 /5% CO_2 incubator in smooth muscle growth medium (Clonetics Corp, San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (5 ng/mL; Sigma Chemical Co).

Patch Clamp Experiments

The whole-cell configuration of the patch-clamp technique was used to record membrane potentials (current clamp) from cultured ICCs. For transfected ICCs, electrophysiological recordings were performed 24 hours after transfection of the full-length STIM1, SARAF, or empty pcDNA vectors. Potentials were amplified with an Axopatch 200B (Axon Instruments, Foster, CA, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 9.2; Axon Instruments). The data were filtered at 5 kHz and displayed on a computer monitor. Results were analyzed using pClamp and Sigma plot (version 9.0) software. All experiments were performed at 30°C.

Clones Preparation and Transfection

The STIM1 and SARAF expression constructs were obtained from Thermo Fisher Scientific, Seoul, South Korea (SARAF Mm00509538_m1 and STIM1 Mm01158413_m1). STIM1 was cloned into pcDNA7.1 GFP at *EcoRI* (5') and *NotI* sites (3') sites, and SARAF was cloned into pCMV6-AC-myc plasmid at *HindIII* (5') and *MluI* (3') sites, creating a Myc tag at the C-terminus. After 24 hours of ICC culture, transfection of the resulting STIM1, SARAF, and empty pcDNA vectors was performed with Lipofectamine 2000 (Thermo Fisher scientific). All cDNA concentrations were measured with a Colibri Spectrometer (Titertek-Berthold, Pforzheim, Germany).

Reverse Transcription-Polymerase Chain Reaction

Cells with typical ICC morphology, ie, triangular or spindle shapes with multiple branches were collected (about 2-5 cells) by applying negative pressure to the cell in contact with a recording

pipette. After lifting the cell out of the bath, it was immediately expelled from the pipette into phosphate-buffered saline by applying positive pressure. The cells were spun down at 5000 × g for 5 minutes before lysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer specifications. Reverse transcription was performed using Superscript One-Step reverse transcription-polymerase chain reaction (RT-PCR) with Platinum Taq (Invitrogen) at 45°C for 30 minutes followed by 94°C for 5 minutes for denaturation of the cDNA hybrid. The thermal cycler was programmed such that cDNA synthesis was immediately followed by PCR amplification. The primers used are presented in Table. The following 3-step process was executed for 38 cycles: 94°C for 30 seconds for denaturation, 60°C for 30 seconds for annealing, and 72°C for 30 seconds for the extension. The PCR products were visualized on a 2% agarose gel following electrophoresis and ethidium bromide staining.

Measurement of Intracellular Calcium Ions Concentration

Changes in $[Ca^{2+}]_i$ concentration were monitored using Fluo 3-AM, which was initially dissolved in DMSO and stored at -20°C. ICCs cultured on coverslips (25 mm) were rinsed twice with a bath solution (in mM: KCl 5, NaCl 135, CaCl₂ 2, glucose 10, MgCl₂ 1.2, and HEPES 10, adjusted to pH 7.4 with Tris), incubated in the bath solution containing 5 μM Fluo 3-AM with 5% CO₂-95% O₂ at 37°C for 20 minutes, rinsed 2 more times with the bath solution, mounted on a perfusion chamber, and scanned every second with a confocal microscope (×200, Fluoview 300; Olympus, Tokyo, Japan). Fluorescence was excited at 488 nm, and emitted light was observed at 515 nm. During scanning of Ca²⁺ imaging, the temperature of the perfusion chamber containing the

Table. Nucleotide Sequences of the Primers Used for Reverse Transcription-Polymerase Chain Reaction

Gene	Sequences	Accession No.	Size (bp)
ANO1	(F) AGG CCA AGT ACA GCA TGG GTA TCA (R) AGT ACA GGC CAA CCT TCT CAC CAA	NM_178642	213
Myosin	(F) GAGAAAGGAAACACCAAGGTCAAGC (R) AACAAATGAAGCCTCGTTTCCTCTC	NM_010860	264
PGP 9.5	(F) GCCAACAACCAAGACAAGCTGGAA (R) GCCGTCCACGTTGTTGAACAGAAT	AF172334	213
STIM1	(F) AAGAGTCTACCGAAGCAG (R) GTGCTATGTTTCACTGTTGG	NM_009287	199
SARAF	(F) CAACGACCCTGACAGAATAC (R) CACAGCTCACCACAGTTT	NM_026432	251

ANO1, anoctamin-1; PGP 9.5, protein gene product 9.5; STIM1, stromal interaction molecule 1; SARAF, store-operated calcium entry-associated regulatory factor.

cultured ICCs was maintained at 30°C. The variations of intracellular Ca^{2+} fluorescence emission intensity were expressed as $F1/F0$, where $F0$ represents the intensity of the first image and counted the peak points as frequency.

Statistical Methods

Data are expressed as the mean \pm SE. Differences in the data were evaluated by ANOVA followed by a post *hoc* test. Differences with P -values less than 0.05 were considered statistically significant. The n values reported in the text refer to the number of cells used in the patch-clamp experiments.

Results

Effect of Carbachol on Pacemaker Potentials in Cultured Interstitial Cells of Cajal

ICCs were identified with an immunofluorescence kit, and had a distinctive morphology that was easily recognized in cultures (data not shown).²³ We performed the electrophysiological recording from cultured ICCs under the current mode ($I = 0$). Under the

current clamp mode at 30°C, ICCs showed spontaneous regular pacemaker potentials (Fig. 1A). CCh treatment (10 nM), which activates acetylcholine receptors, depolarized the resting membrane potential and decreased the amplitude of the pacemaker potential (Fig. 1B). Under control conditions with the current-clamp mode, the resting membrane potential and pacemaker potential frequency were -59.6 ± 2.6 mV and 14.7 ± 0.8 cycles/minute, respectively. In the presence of CCh, the resting membrane potential and pacemaker potential frequency were -39.4 ± 3.6 mV and 2.6 ± 4.3 cycles/minute, respectively. The summarized values and a bar graph of the effects of CCh on pacemaker potential are shown in Figures 1C and 1D ($n = 5$).

Increased Frequency of Pacemaker Activity by Stromal Interaction Molecule 1 Overexpression in Cultured Interstitial Cells of Cajal

To determine the role of STIM1, STIM1 cDNA was overexpressed in cultured ICCs. Under the current clamp mode at 30°C, pcDNA-overexpressing ICCs showed spontaneous regular pacemaker potentials (Fig. 2A), and pacemaker potential frequency was increased in STIM1-overexpressing ICCs. Further, the regular

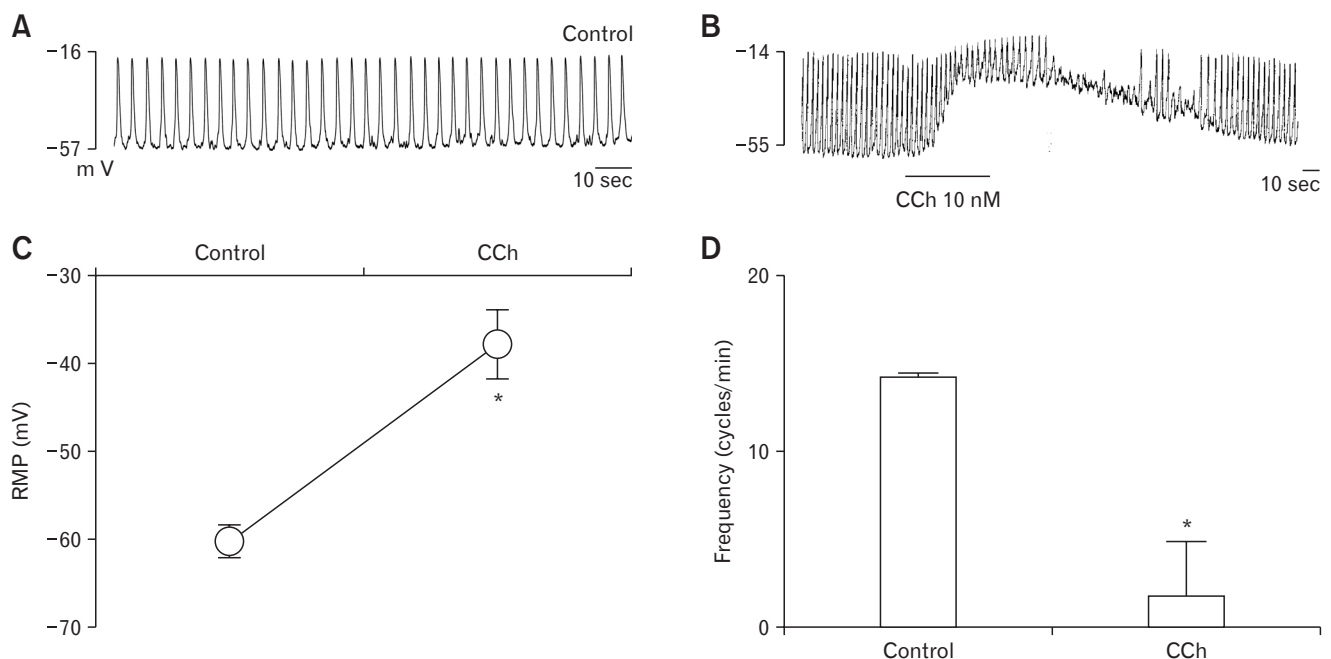


Figure 1. Effects of carbachol (CCh) treatment on pacemaker potentials recorded in cultured interstitial cells of Cajal (ICCs) from mouse small intestine. (A) Pacemaker potentials of ICCs in current clamping mode ($I = 0$). The vertical solid lines represent the scale of pacemaker potential amplitudes, and the horizontal solid lines represent the scale of pacemaker potential recording duration. (B) Pacemaker potentials of ICCs exposed to CCh (10 nM) in the current clamping mode ($I = 0$). Cellular responses to CCh are summarized in (C) and (D). The bars represent mean values \pm SE. Asterisks represent significant differences from untreated control cells (Control) ($*P < 0.01$). RMP, resting membrane potentials.

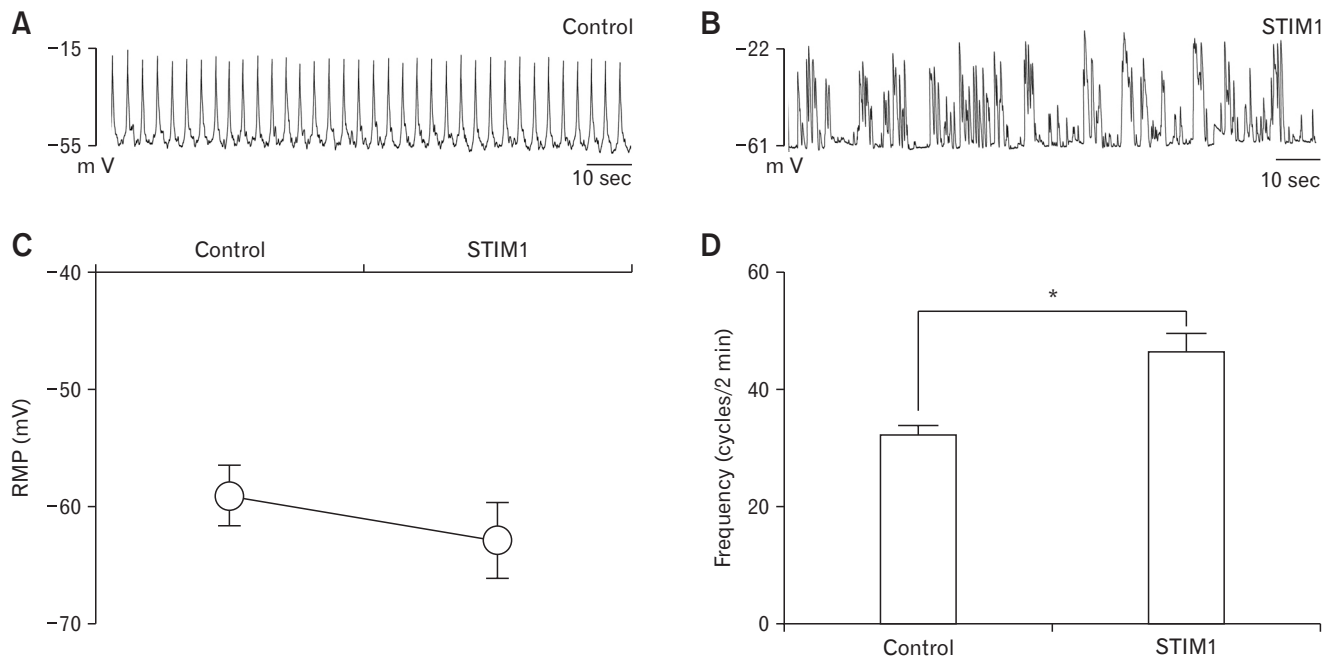


Figure 2. Pacemaker potentials recorded in control or stromal interaction molecule 1 (STIM1)-overexpressing cultured interstitial cells of Cajal (ICCs) from mouse small intestines. (A) Pacemaker potentials of ICCs in the current clamping mode ($I = 0$). Vertical solid lines represent the scale of pacemaker potential amplitudes, and horizontal solid lines represent the scale of pacemaker potential recording duration. (B) Pacemaker potentials of STIM1-overexpressing ICCs in current-clamping mode ($I = 0$). Pacemaker potential responses in STIM1-overexpressing ICCs are summarized in (C) and (D). The bars represent mean values \pm SE. Asterisks represent significant differences from empty vector-expressing control cells (Control) ($*P < 0.01$). RMP, resting membrane potentials.

pattern of pacemaker activity observed in normal ICCs became irregular and a spike-fused shape was observed (Fig. 2B). Under control conditions in pcDNA-transfected ICCs, the resting membrane potentials and frequency of pacemaker potentials were -57.4 ± 2.9 mV and 33.6 ± 6.2 cycles/2 minutes, respectively. In the case of STIM1-overexpressing ICCs, the resting membrane potential and frequency of pacemaker potentials were -62.4 ± 5.4 mV and 48.6 ± 5.2 cycles/2 minutes, respectively. The summarized values and a bar graph with results from STIM1-transfected ICCs are shown in Figures 2C and 2D ($n = 8$).

Decreasing Frequency of Pacemaker Activity by Store-operated Calcium Entry-associated Regulatory Factor Overexpression in Cultured Interstitial Cells of Cajal

To determine the role of SARAF, SARAF cDNA was overexpressed in cultured ICCs. In the current clamp mode at 30°C , pcDNA-overexpressing ICCs showed spontaneous regular pacemaker potentials (Fig. 3A). In case of SARAF overexpressed ICCs, the frequency of pacemaker activity was dramatically inhibited

(Fig. 3B). Under control conditions, resting membrane potentials and frequency of pacemaker potentials in ICCs were -58.1 ± 4.1 mV and 31.8 ± 3.4 cycles/2 minutes, respectively. In the case of SARAF-overexpressing ICCs, the resting membrane potential and frequency of pacemaker potentials were -64.8 ± 5.5 mV and 7.6 ± 2 cycles/2 minutes, respectively. The summarized values and a bar graph with results from SARAF-transfected ICCs are shown in Figures 3C and 3D ($n = 9$).

Molecular Expression of Stromal Interaction Molecule 1 or Store-operated Calcium Entry-associated Regulatory Factor with Interstitial Cells of Cajal

In the present study, RT-PCR analysis revealed the mRNA transcripts for STIM1 and SARAF in whole mount cultured small intestinal cells in Fig. 4A. After pick up cells, we checked only anoctamin-1 (ANO1) positive samples. Those samples showed STIM1 and SARAF were detected in ANO1 positive ICCs isolated from the culture of the small intestine (Fig. 4A).

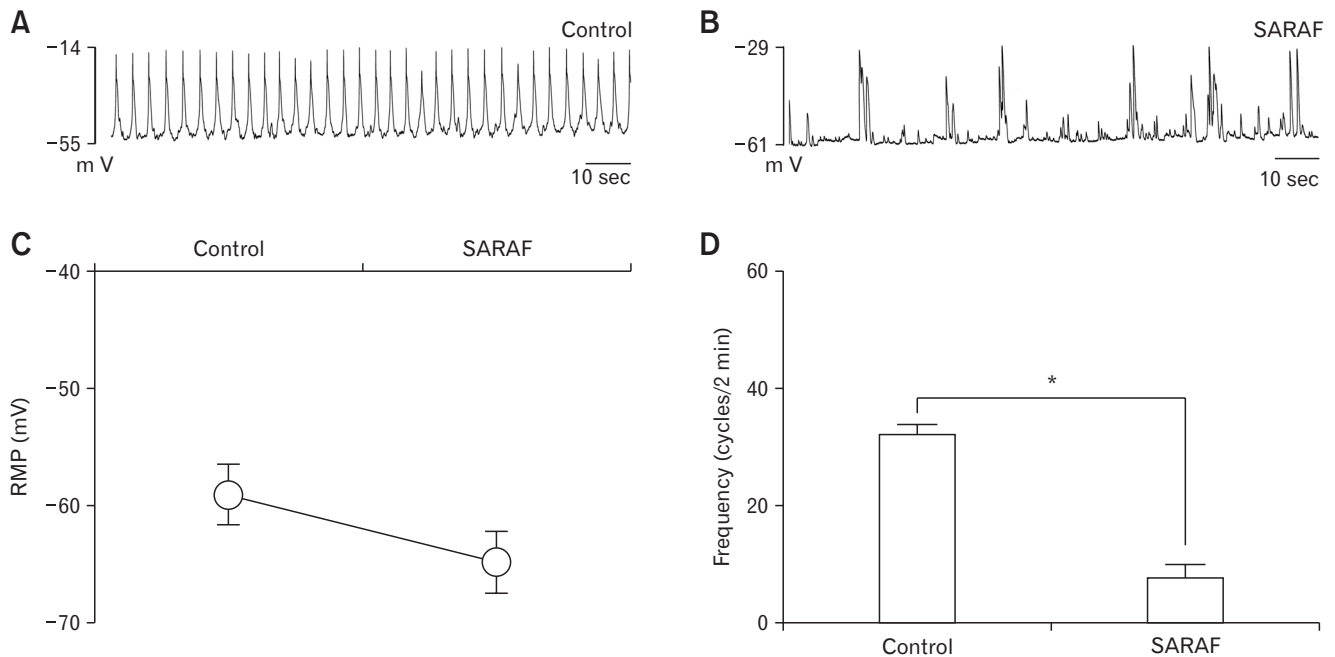


Figure 3. Pacemaker potentials recorded in control or store-operated calcium entry-associated regulatory factor (SARAF)-overexpressing cultured interstitial cells of Cajal (ICCs) from mouse small intestine. (A) Pacemaker potentials of ICCs in the current clamping mode ($I = 0$). Vertical solid lines represent the scale of pacemaker potential amplitudes, and horizontal solid lines represent the scale of pacemaker potential recording duration. (B) Pacemaker potentials of SARAF-overexpressing ICCs in the current clamping mode ($I = 0$). Pacemaker potential responses in SARAF-overexpressing ICCs are summarized in (C) and (D). The bars represent mean values \pm SE. Asterisks represent significant differences from empty vector-expressing control cells (Control) ($*P < 0.01$). RMP, resting membrane potentials.

Effect of Gadolinium or a Calcium Ions-free Solution on Pacemaker Activity in Cultured Interstitial Cells of Cajal

To understand the role of extracellular Ca^{2+} entry mechanism on pacemaker activity, we tested the effect of treating cultured ICCs with gadolinium (Gd^{3+} ; 10 μM) or a Ca^{2+} -free solution at 30°C. In the presence of Gd^{3+} or a Ca^{2+} -free solution, the ICC pacemaker potential frequency was completely blocked (Fig. 4B and 4C). The summarized values and a bar graph with results of treating ICCs with Gd^{3+} or a Ca^{2+} -free solution are shown in Figures 4D and 4E ($n = 6$).

Modulation of Intracellular Calcium Ions Intensity by Overexpressing Stromal Interaction Molecule 1 or Store-operated Calcium Entry-associated Regulatory Factor in cultured Interstitial Cells of Cajal

To directly determine the role of STIM1 or SARAF on $[\text{Ca}^{2+}]_i$, we examined $[\text{Ca}^{2+}]_i$ oscillation in STIM1- or SARAF-transfected ICCs. Spontaneous $[\text{Ca}^{2+}]_i$ oscillations were observed in pcDNA-

transfected ICCs loaded with Fluo 3-AM; the time series data show these spontaneous regular $[\text{Ca}^{2+}]_i$ oscillations (Fig. 5A) at 30°C. In the case of STIM1-overexpressing ICCs, the frequency of $[\text{Ca}^{2+}]_i$ oscillations was increased (Fig. 5B). However, SARAF-overexpressing ICCs showed a decreased frequency of $[\text{Ca}^{2+}]_i$ oscillations compared to the control (Fig. 5C); the time series data are summarized in Figure 5D ($n = 8$).

Discussion

Recently, many studies have attempted to determine which channel generates electrical pacemaker activity of ICCs in the GI tract. Some of the proposed channels include non-selective cation, Ca^{2+} -activated Cl^- or T-type Ca^{2+} channels, among others.²⁵⁻²⁸ However, there is no argument that $[\text{Ca}^{2+}]_i$ oscillations are the initial mechanism for generating pacemaker activity.²⁸ Periodic $[\text{Ca}^{2+}]_i$ release from Ca^{2+} stores produces $[\text{Ca}^{2+}]_i$ oscillations in ICCs and these actions are considered to be the primary pacemaker activity. To date, it is not fully known how $[\text{Ca}^{2+}]_i$ release is modulated. In this study, by examining electrical pacemaker activity in STIM1-

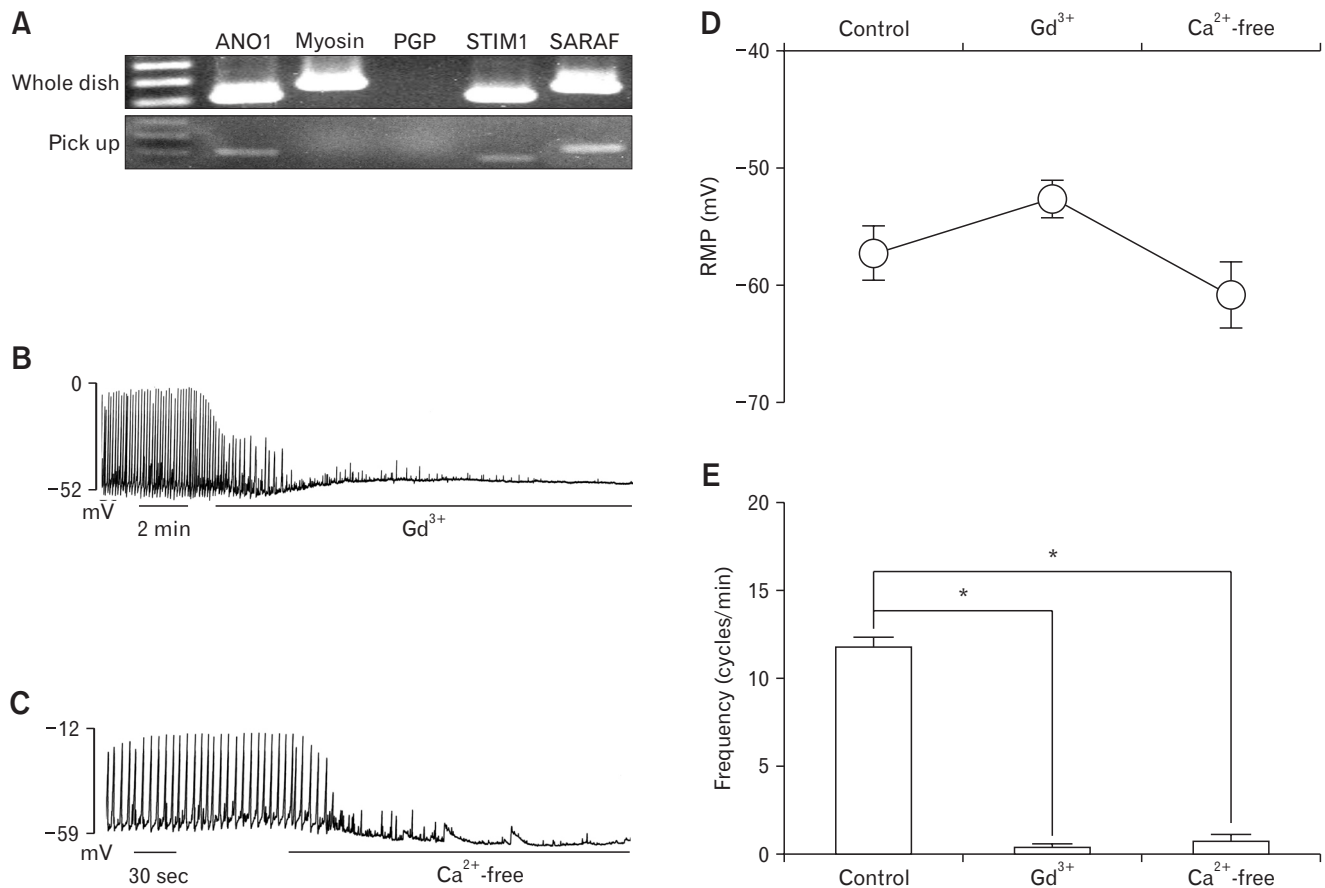


Figure 4. Molecular expression of stromal interaction molecule 1 (STIM1) and store-operated calcium entry-associated regulatory factor (SARAF), and effects of gadolinium or an external calcium ions (Ca²⁺)-free solution on pacemaker potentials in cultured interstitial cells of Cajal (ICCs) from mouse small intestine. (A) Reverse transcription-polymerase chain reaction (RT-PCR) detected the transcripts for STIM1 and SARAF in whole mounted cultured cells (upper panel) and in anoctamin-1 (ANO1) positive cultured ICCs (lower panel). (B) Gadolinium exposure (Gd³⁺; 10 μM) abolished the generation of pacemaker potentials in cultured ICCs. Vertical solid lines represent the scale of pacemaker potential amplitudes and horizontal solid lines represent the scale of pacemaker potential recording duration. (C) External Ca²⁺-free solution exposure inhibited pacemaker potential generation. Pacemaker potential responses to Gd³⁺ or external Ca²⁺-free solution exposure are summarized in (D) and (E). The bars represent mean values ± SE. Asterisks represent significant differences from untreated control cells (Control) (**P* < 0.01). PGP, protein gene product; RMP, resting membrane potentials.

or SARAF-overexpressing ICCs, we strongly suggest that these two proteins can act as essential regulators of pacemaker activity by modulating ER Ca²⁺ concentration in ICCs.

SOCE can be initiated by receptor activation at the PM. Receptor activation leads to Ca²⁺ release from the ER and this activates Ca²⁺ influx at the PM.⁸ Previous studies showed that ICCs possess various receptors for endogenous substances and activation of various receptors by agonists (CCh, substance P, etc) that could influence Ca²⁺ release from ER stores,^{29,30} resulting in the conversion of a pacemaker current into tonic inward currents in ICCs. These phenomena could be blocked by exposure to a Ca²⁺-free solution. This indicates the possibility that ICCs have a SOCE

system. As is shown in Figure 1, we confirmed the action of CCh on pacemaker activity in ICCs and found tonic pacemaker potential upon CCh treatment.

There are 2 essential components for SOCE activation: STIM1 in the ER and Orai1 in the PM.^{13,31,32} STIM1 is the Ca²⁺ sensor in the ER that measures Ca²⁺ depletion in the ER. Upon Ca²⁺ store depletion, STIM1 binds with the Orai1 channel in the PM, resulting in Ca²⁺ influx through Orai1.³³ We speculate the existence of a [Ca²⁺]_i modulating component like STIM1 in ICCs because ICCs have spontaneous electrical activity by [Ca²⁺]_i oscillation and various receptors are activated via [Ca²⁺]_i regulation. As shown in Figure 2, we found that overexpressing STIM1 ICCs by

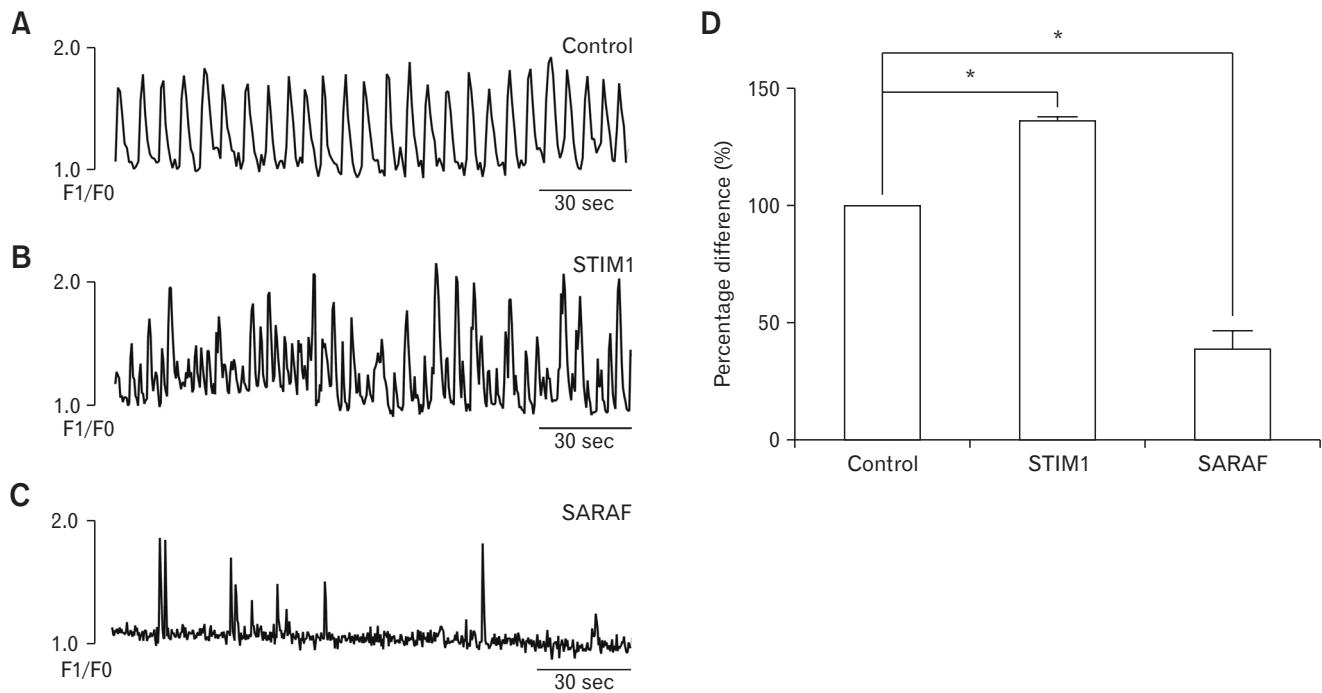


Figure 5. Effects of stromal interaction molecule 1 (STIM1) or store-operated calcium entry-associated regulatory factor (SARAF) overexpression in cultured interstitial cells of Cajal (ICCs) from mouse small intestine on intracellular calcium ions (Ca^{2+}) oscillation. (A-C) Fluorescence intensity changes in cultured mouse ICCs overexpressing empty vector (A), STIM1 (B), or SARAF (C). Horizontal solid lines represent the scale of fluorescence intensity change duration. Pacemaker potential responses in STIM1- or SARAF-overexpressing ICCs are summarized in (D). The bars represent percentage difference. Asterisks represent significant differences from the empty-vector expressing control (Control) ($*P < 0.01$). The variations of intracellular Ca^{2+} fluorescence emission intensity were expressed as $F1/F0$, where $F0$ represents the intensity of the first image and counted the peak points as frequency.

transfection resulted in a dramatically increased frequency of pacemaker activity in these cells. Furthermore, fused pacemaker peaks were observed, possibly due to overlapping pacemaker activity as a result of the increased frequency. This suggests that the frequency of pacemaker activity in ICCs is decided by Ca^{2+} concentration in the ER, and that STIM1 can be a modulator for this in ICCs. Furthermore, exposure to Gd^{3+} that is considered as a relative inhibitor of SOCE or Ca^{2+} -free solution blocked pacemaker activity and CCh-induced action.³⁴ Although Gd^{3+} is not a specific blocker for SOCE channels like Orai, these results provide the possibility that SOCE can be involved in the pacemaker mechanism in ICCs. To verify this, a future study should aim to confirm the interaction between Orai1 and STIM1 in ICCs.

Recently, the ER protein SARAF was reported. It is well known that SARAF is a negative regulator of SOCE.¹⁶ In addition, SARAF is located in the ER membrane where it interacts with STIM1. In resting states, SARAF binds STIM1 and prevents STIM1-Orai1 activation. Upon ER Ca^{2+} depletion, SARAF is dissociated from STIM1 and STIM1-Orai1 is activated.³³ We

found that SARAF-overexpressing ICCs exhibited decreased pacemaker activity frequency. This can be explained because SARAF blocks refilling and decreases the concentration of ER Ca^{2+} . Namely, the STIM1-Orai1 interaction required for refilling ER Ca^{2+} is interrupted by high SARAF expression in ICCs, and ICCs therefore could not initiate $[\text{Ca}^{2+}]_i$ oscillation to generate pacemaker activity.

The last question we asked is do STIM1 or SARAF really regulate $[\text{Ca}^{2+}]_i$ mobilization? The inositol 1,4,5-triphosphate receptor plays a role in generating spontaneous electrical activity in ICCs, and periodic Ca^{2+} release from intracellular Ca^{2+} stores produces $[\text{Ca}^{2+}]_i$ oscillations in ICCs from mouse ileum. These actions seen in ICCs are considered to be the primary pacemaker activity in the GI tract.²² In our study, we found that STIM1 or SARAF overexpression modulated ICC $[\text{Ca}^{2+}]_i$ directly (Fig. 5). This is important evidence that STIM1 or SARAF can modulate $[\text{Ca}^{2+}]_i$, and this results in an alteration of pacemaker activity frequency in ICCs.

In the GI tract, the patterns of slow waves or pacemaker activity vary by location. This variety can be explained by mechanisms

of smooth muscle, ICCs, or enteric nerves. However, this study also provides evidence that the degree of ER protein expression can determine the frequency of pacemaker activity. Furthermore, this influence may modulate slow waves generated from smooth muscles. In conclusion, this study suggests the possibility that the representative ER proteins, STIM1 and SARAF, exist in ICCs and can modulate pacemaker activity. Additionally, Ca^{2+} concentration in ER stores may determine the frequency of pacemaker activity.

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Conflicts of interest: None.

Author contributions: Chan Guk Park and Mei Jin Wu designed research; Chan Guk Park, Mei Jin Wu, Chansik Hong, Ju Yeon Jo, Han Yi Jiao, Hyun Park, and Jae Yeoul Jun performed research; and Jae Yeoul Jun and Seok Choi wrote the paper and contributed to the experiment determination.

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