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Original Article

Transient Receptor Potential Canonical 4 and 5 Channel Antagonist ML204 Depolarized Pacemaker Potentials of Interstitial Cells of Cajal

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Background/Aims
To investigate an effect of ML204 (an inhibitor of transient receptor potential canonical 4 and 5 [TRPC4/5] channels) on interstitial cells of Cajal (ICCs) and therefore determine whether TRPC4/5 channels act on ICC-generated pacemaker activity.

Methods
We enforced whole cell patch clamp analysis, measurements of the intracellular Ca2+ concentration, and reverse transcription polymerase chain reaction to determine the effect of ML204 (10 μM) or englerin A (a selective activator of TRPC4/5 channeles, 10 μM) and the existence of TRPC4/5 in mouse small intestinal ICC.

Results
Treatment of ICCs with ML204 or englerin A caused the membrane potentials to depolarize. This depolarization effect of membrane potentials by ML204 in ICCs was observed to be concentration-dependent. After treating Ca^{2+}- and Na^{+}-free solutions or flufenamic acid (a non-selective cation channel blocker), the pacemaker potentials in the ICCs were abolished. A specific anoctamin 1 channel blocker did not have any effect on the pacemaker activity in ML204-untreated control cells; however, they blocked ML204-induced pacemaker activity in ICCs. Specific primers designed against TRPC4 and TRPC5 detected the presence of TRPC4/5 in small intestinal ICCs, and the application of ML204 increased raise the frequency of Ca^{2+} oscillations in ICCs, as assessed using Fluo-4 AM.

Conclusion
The results implied that ML204 could not inhibit the pacemaker activity but depolarized the membrane potential of ICCs by regulating intracellular Ca^{2+} oscillations and anoctamin 1 channels.
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Key Words
Anoctamin-1; Interstitial cells of Cajal; Membrane potentials; TRPC cation channels

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Introduction

Interstitial cells of Cajal (ICCs) are special and unique cells in the gastrointestinal (GI) muscle layer.[1] They are connected with each other and with smooth muscle cells by gap junctions electrically. ICCs produce slow waves of rhythmic depolarization, which are transmitted to smooth muscle cells, thus inducing smooth muscle contraction. In addition, ICCs transmit nerve signals to smooth muscle cells.[2] Especially, the number and morphology of ICCs are an important element in intestinal function and in various motility disorders.[3]

Previous reports revealed the pacemaking mechanism of ICCs. Many reports have shown that electrical activity of ICCs is produced by intracellular Ca^{2+} ($[Ca^{2+}]_i$) release from the endoplasmic reticulum (ER) and following Ca^{2+} entry into mitochondria.[4-6] These reports indicate that $[Ca^{2+}]_i$ plays a fundamental role in the pacemaker mechanism. However, periodic activation of plasma channels to produce pacemaker activity is still a matter of debate. Several candidates have been reported, like non-selective cation channels (NSCC), anoctamin 1 (ANO1; also named as transmembrane member 16A [TMEM16A]), and Ca^{2+}-activated Cl^- channels [CACC]).[7-11] Among them, the transient receptor potential canonical 4 (TRPC4) is suggested to be a candidate NSCC involved in $[Ca^{2+}]_i$ oscillation in ICCs. Periodic Ca^{2+} release may lead to depletion of the Ca^{2+} stores, which consequently activates TRP4 spontaneously. Reports that TRP4 is considered to be a store-operated channel support this point of view.[13,14]

To settle this, we tested the pharmacological properties of the pacemaker activity from small intestinal ICCs. Recently, we found compounds that affected the activities of TRPC4/5 channels. Among them, ML204 was described as a novel inhibitor of TRPC4/5 that inhibits calcium influx via the TRPC4 channel.[15] In addition, englerin A is a potent and specific activator of TRPC4/5.[16] Thus, ML204 and englerin A are specific compounds for TRPC4/5, and in this study, we examined their effects on ICCs to determine whether TRPC4/5 channels act on ICC-generated pacemaker activity.

Materials and Methods

Ethics

The mice used in this experiment were fed and treated under the guiding principles approved by the ethics committee of Chosun University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (CIACUC2018-S0028).

Preparation of Tissues and Cell Culture

Either sex of ICR mice aged 4-7 days were sedated with diethyl ether and killed by cervical dislocation. Small intestine (from 1 cm below the pyloric ring to the cecum) and colon (from below cecum to rectum) were cut and pinned to the base of sylgard dish full with ice-cold Ca^{2+} free Hank's solution (see solutions). Tissues were opened along the mesenteric border. After removing luminal contents, the mucosa and submucosa of tissues were peeled away by sharp dissection. Strips of intestinal or colonic muscle were equilibrated in Ca^{2+} free Hank's solution for 30 minutes. The muscle strips were transferred into enzymic solution (see solutions) and incubated in a 37℃ water bath for 14 minutes. Then tissues were washed out 3 times with Ca^{2+} free solution and triturated with blunt pipettes to disperse cell lumps. Cells were plated on poly-L-lysine (200 μL; Sigma, St. Louis, MO, USA) coated sterile glass coverslips in 35 mm culture dishes and incubated at 37℃ in a 95% O_2-5% CO_2 incubator in smooth muscle growth medium (SMGM; Lonza, Walkersville, MD, USA) supplemented with 2% antibiotic-antimycotics (Gibco, Grand Island, NY, USA) and stem cell factor (5 ng/mL; Sigma,St. Louis, MO, USA). After 1 day incubation, the medium was replaced with SMGM without stem cell factor, then incubated further for 24 hours with the same conditions until performing the following experiments.

Electrical Recording

The cells in 35 mm dishes were taken out from the CO_2 incubator after 48 hours of culture and placed into the recording chamber on the inverted microscope. Whole cell patch clamp technique was used to measure the alterations of membrane potentials from cultured intestinal and colonic ICCs. A superfusion system with a temperature controller (Harvard apparatus, Holliston, MA, USA) was used to drive the solution flow, and electronic equipment such as head stage (CV203BU headstage; Axon instruments, Foster, CA, USA), amplifier (Axopatch 200B; Axon instruments), digitizer (Digidata 1322A; Axon instruments), computer, and software (pClamp 9.2; Axon instruments) was used to record and analyze data.

Reverse Transcription Polymerase Chain Reaction

Total cultured cells or picked cells (about 20 cells) with typical ICC morphology (triangular or spindle shapes with several branches) were sucked into a recording pipette under negative

pressure. After picking, the cells were expelled from the recording pipette into phosphate buffered saline by giving positive pressure immediately. The cells were centrifuged at 10 000 rpm at 4℃ for 8 minutes before lysis. According to the technical specifications, total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was obtained using the PrimeScript first strand cDNA synthesis kit (6110A; Takara, Kusatsu, Shiga, Japan). The resultant cDNA was purified using the Maxime PCR PreMix i-StarTaq (iNtRON Biotechnology Inc., Korea) and amplified. The following 3-step process was executed for 40 cycles: 94℃ for 30 seconds for denaturation, 60℃ for 30 seconds for annealing, and 72℃ for 30 seconds for the extension. The polymerase chain reaction products were electrophoresed through a 2% agarose gel following electrophoresis and visualized using ethidium bromide staining.

The primers used were as follows: TRPC4 (Accession number AF019663), forward 5'-GAA TTC ACT CCG GAC ATC AC-3', reverse 5'-GCA GTA AGG AAA GGG TCT TC-3', (with a product size of 248 base pair [bp]); TRPC5 (Accession number AF060107), forward 5'-ATC AGG GGC TAA CAG AAG A-3', reverse 5'-TGC AGC CTA CAT TGA AAG A-3', (with a product size of 210 bp); ANO1 (Accession number NM178642), 5'-AGG CCA AGT ACA GCA TGG GTA TCA-3' and 5'-AGT ACA GGC CAA CCT TCT CAC CAA-3' (with a product size of 213 bp); KIT proto-oncogene receptor tyrosine kinase (c-Kit) (Accession number AY536430), 5'-GCA CAG AAG GAG GCA CTT ATA CCT-3' and 5'-TGA GAC AGG AGT GGT ACA CCT TTG-3' (with a product size of 215 bp); myosin, heavy polypeptide 11, smooth muscle (Myh11) (Accession number NM013607), 5'-AGG CAG ACC TCA TGC AGC TCC AAG A-3' and 5'-CCT CAT TCT GTT CAT CCC GAG CCT G-3' (with a product size of 340 bp); and ubiquitin carboxyl-terminal hydrolase PGP9.5 (Uch-L1) (Accession number AF172334), 5'-GCC AAC AAC CAA GAC AAG CTG GAA-3' and 5'-GCC GTC CAC GTT GTT GAA CAG AAT-3' (with a product size of 213 bp).

Analysis of the Intracellular Ca^{2+} Concentration

After 24 to 30 hours of culture, the ICC cultured on coverslips (25 mm) were well grown and ready for measurement of $[Ca^{2+}]_i$ concentrations. The medium in culture dishes was removed. The cells were washed twice and incubated at 37℃ for 10 minutes with bath solution. Next, the cells were stained by Fluo-4 acetoxymethyl ester (AM) (1 mM, Invitrogen) at 37℃ for 15 minutes and then rinsed for 3 times with normal solution. After mounting on the perfusion chamber, the cells were scanned every 0.4 seconds with Nikon Eclipse TE2000-U (Nikon Inc, Melville, NY, USA) inverted microscope equipped with a PerkinElmer Ultraview confocal scanner (PerkinElmer Inc, Waltham, MA, USA) and a Hamamatsu Orca ER 12-bit CCD camera (×200; Hamamatsu Instrument, Hamamatsu, Shizuoka, Japan). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. The temperature of perfusion chamber remained at 30℃ during the experiment. Relative alterations of $[Ca^{2+}]_i$ fluorescence emission intensity was expressed as the ratio (F1/F0), where F0 was taken from the fluorescence of the first image.

Reagents

Normal solution contains 1.2 mM $MgCl_2$, 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, adjusted to pH 7.4 using Tris. The pipette solution contained 5 mM $MgCl_2$, 140 mM KCl, 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. The chemicals used were ML204, from Tocris (Bristol, UK), and englerin A and flufenamic acid, purchased from Sigma.

Statistical Methods

Data are expressed as means ± SE. Differences among the data were calculated using Student's *t* test. A P-value < 0.05 was indicated a statistically significant difference. The number of cells used for recording was presented by the n values.

Results

ML204 or Englerin A Action on the Pacemaker Activity in Small Intestinal Interstitial Cells of Cajal

Under the current clamp mode ($I = 0$), ICCs generated spontaneous pacemaker potentials. Exposure to ML204 (10 μM), a selective TRPC4/5 inhibitor, resulted in depolarization of the resting membrane potential, together with a decrease in the amplitude of the pacemaker potential (n = 5; Fig. 1A). Treatment with englerin A (10 μM), a specific TRPC4/5 activator, also resulted in depolarization of the resting membrane potential, with a decrease of the amplitude of the pacemaker potential (n = 4; Fig. 1B).

Dose-dependent Action of ML204 on the Pacemaker Activity in Small Intestinal Interstitial Cells of Cajal

Treatment of ICCs with lower concentrations of ML204 (1

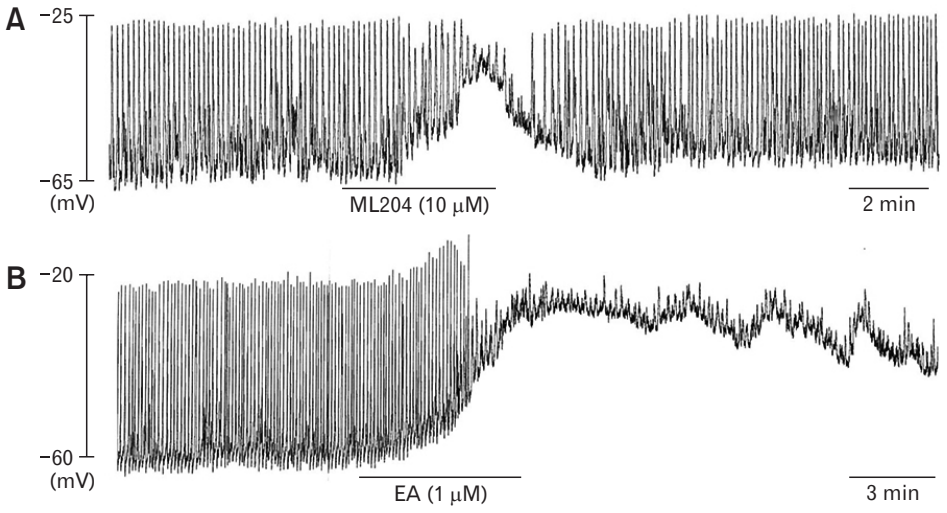

Figure 1. Effects of ML204 (an inhibitor of transient receptor potential canonical [TRPC] 4/5 channels) or englerin A (EA; a selective activator of TRPC4/5 channels) on pacemaker activity in small intestinal interstitial cells of Cajal (ICCs). (A) Pacemaker potentials of ICCs under current-clamp mode in control condition and ML204 (10 μM) action. (B) Pacemaker potentials of ICCs under current-clamp mode in control condition and EA action on pacemaker potentials.

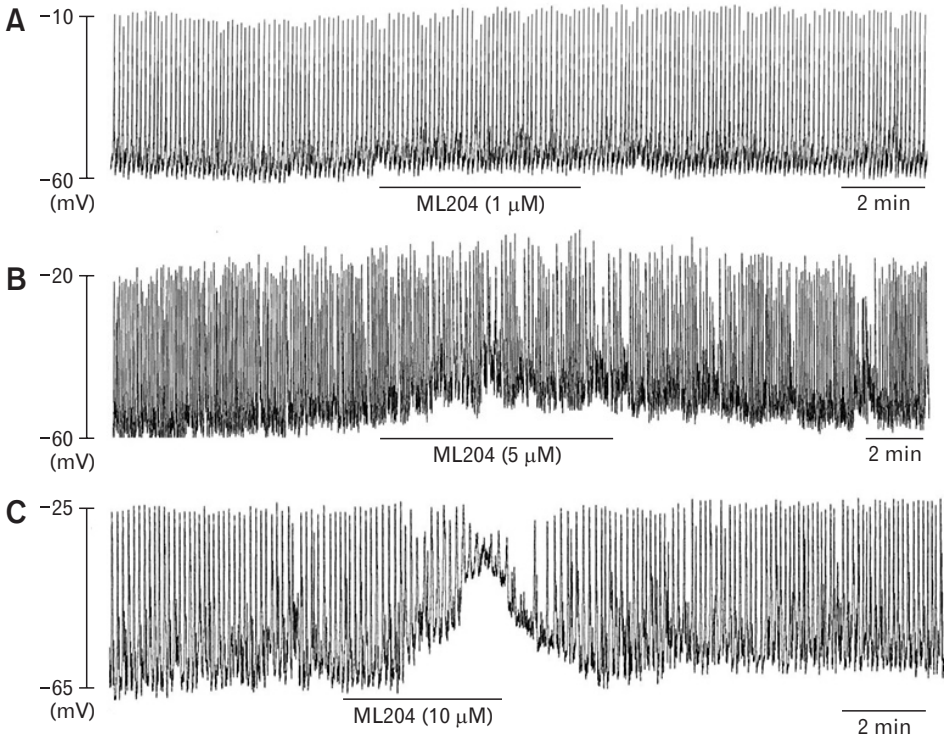

Figure 2. Concentration-dependent effects of ML204 (an inhibitor of transient receptor potential canonical 4 and 5 [TRPC4/5] channels) on pacemaker activity in small intestinal interstitial cells of Cajal (ICCs). (A-C) Pacemaker potentials of ICCs recorded under current-clamp mode and exposed to various concentration of ML204 (1, 5, and 10 μM).

μM and 5 μM) produced weak depolarization of the resting membrane potential, with no decrease in the amplitude of the pacemaker potential (Fig. 2A and 2B). By contrast, exposure with high concentration of ML204 (10 μM) produced tonic depolarization of the resting membrane potential and decreased the amplitude of the pacemaker potential (Fig. 2C).

Effect of an External Na^+-, Ca^{2+}-free Solution or a Non-selective Cation Channel Blocker on the Pacemaker Activity in Small Intestinal Interstitial Cells of Cajal

To study the role of external cations on the pacemaker activity, we tested the effects of an external Na^+-, Ca^{2+}-free solution and flufenamic acid, a NSCC blocker. Exposure to the external Na^+-

free solution abolished the generation of the pacemaker activity completely (Fig. 3A). The exposure of the Ca^{2+}-free solution also abolished the pacemaker activity generated from ICCs (Fig. 3B). In addition, flufenamic acid (50 μM) abolished the generation of pacemaker currents (Fig. 3C). These results suggest that external cations or NSCC are important for the pacemaker activity of ICCs.

Effect of Anoctamin 1 Channel Blockers on the Pacemaker Activity in Small Intestinal Interstitial Cells of Cajal

To understand the involvement of the ANO1 channel in the action of ML204, a TMEM16A (another name for ANO1) blocker or a CACC blocker were used. Treatment with the TMEM16A

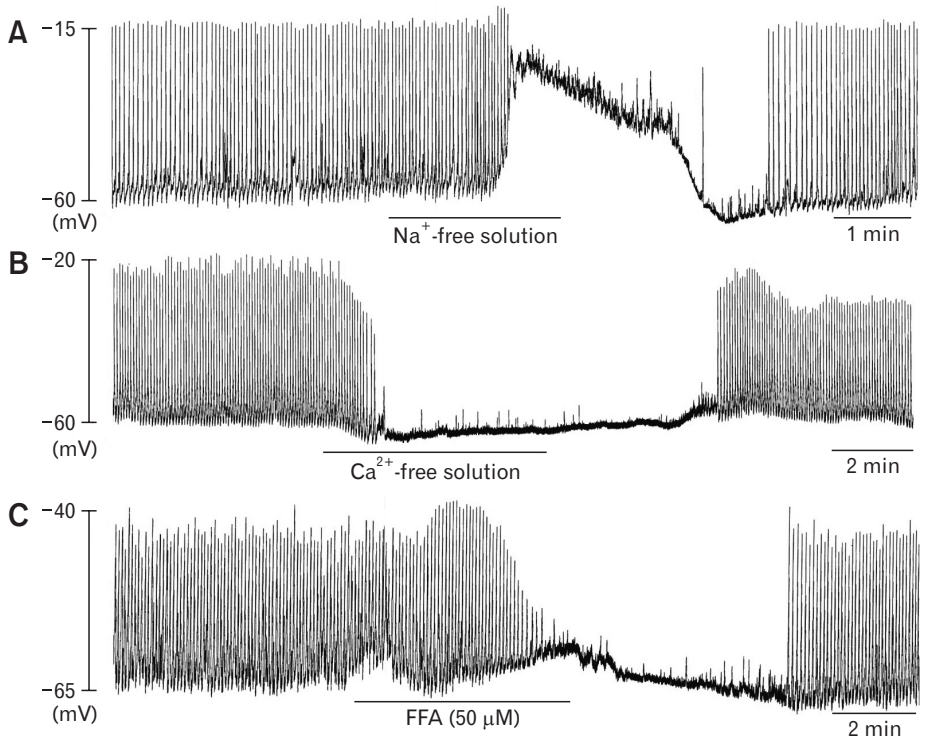

Figure 3. Effects of external Na^+-free, Ca^{2+}-free or flufenamic acid (FFA) on pacemaker activity in small intestinal interstitial cells of Cajal. (A) Application of Na^+-free solution abolished the generation of pacemaker activity. (B) Application of Ca^{2+}-free solution abolished the generation of pacemaker activity. (C) Application of FFA (50 μM) abolished the generation of pacemaker activity.

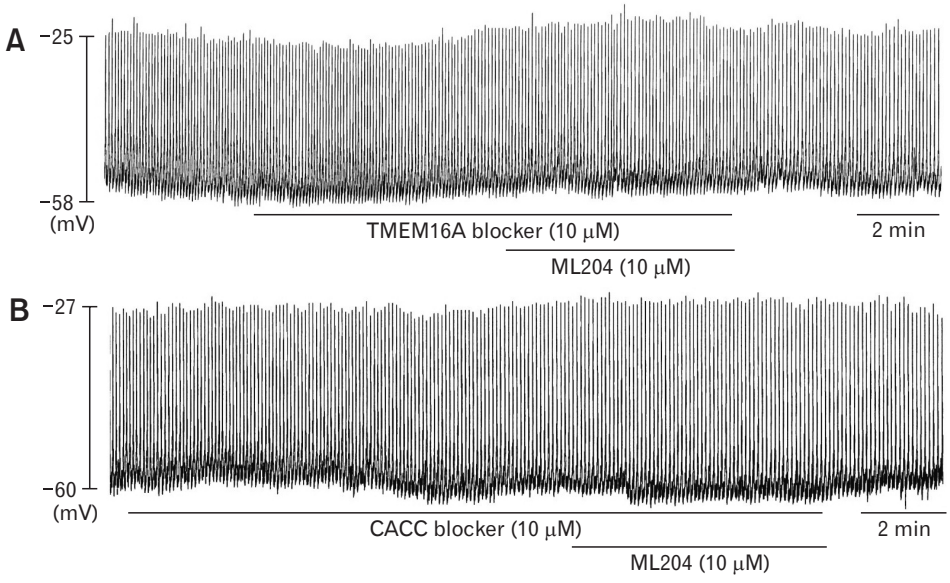

Figure 4. Effects of anoctamin 1 (ANO1) or Ca^{2+}-activated Cl^- channel blocker on ML204 (an inhibitor of transient receptor potential canonical 4 and 5 [TRPC4/5] channels)-induced action in small intestinal interstitial cells of Cajal. (A) Transmembrane member 16A (TMEM16A) blocker (ANO1 blocker; 10 μM) did not show any influence on pacemaker potentials but blocked ML204-induced action. (B) Ca^{2+}-activated Cl^- channel (CACC) blocker (10 μM) did not show any influence on pacemaker potentials but blocked ML204-induced action.

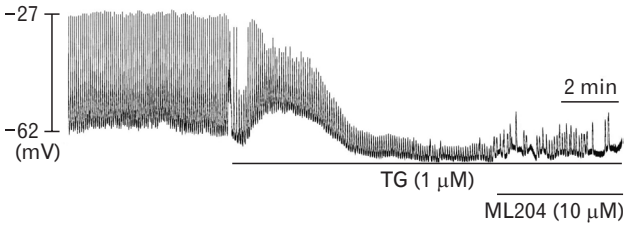

Figure 5. Effects of a Ca^{2+}-ATPase inhibitor on ML204 (an inhibitor of transient receptor potential canonical 4 and 5 [TRPC4/5] channels)-induced action in small intestinal interstitial cells of Cajal. Thapsigargin (TG; 1 μM), a Ca^{2+}-ATPase inhibitor, abolished the generation of pacemaker potentials, and in the presence of TG, ML204-induced action was blocked.

blocker (10 μM) or the CACC blocker (10 μM) had no effect on the pacemaker activity under control conditions. However, the ML204-induced depolarization of the resting membrane potential and the decrease in the amplitude of the pacemaker potential were blocked by pretreatment with the TMEM16A blocker or CACC blocker (Fig. 4A and 4B).

Effect of a Ca^{2+}-ATPase Inhibitor of the Endoplasmic Reticulum on the ML204-induced Responses in Small Intestinal Interstitial Cells of Cajal

To investigate the role of internal Ca^{2+} in the action of ML204, we tested the effects of ML204 in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the ER. Treatment with thapsigargin (1 μM) inhibited the pacemaker activity in small intestinal ICCs and blocked the ML204-induced effect (n = 3; Fig. 5).

Effect of ML204 on Intracellular Ca^{2+} Release in Small Intestinal Interstitial Cells of Cajal

To support the observed effects of thapsigargin, we examined $[Ca^{2+}]_i$ changes in small intestinal ICCs using a Ca^{2+} marker. Under normal conditions, we could see spontaneous $[Ca^{2+}]_i$ oscillations from the small intestinal ICCs. The application of ML204 (10 μM) increased the frequency of the $[Ca^{2+}]_i$ oscillations (Fig. 6A). The effects of ML204 on $[Ca^{2+}]_i$ are summarized in Figure 6B.

Identification of Transient Receptor Potential Canonical 4 and 5 Channels in Small Intestinal Interstitial Cells of Cajal

Next, we performed reverse transcription-polymerase chain reaction with primers specific to TRPC4 and TRPC5 to detect their expression in small intestinal ICCs. TRPC4 and TRPC5 amplicons could be amplified from intestinal tissue samples comprising

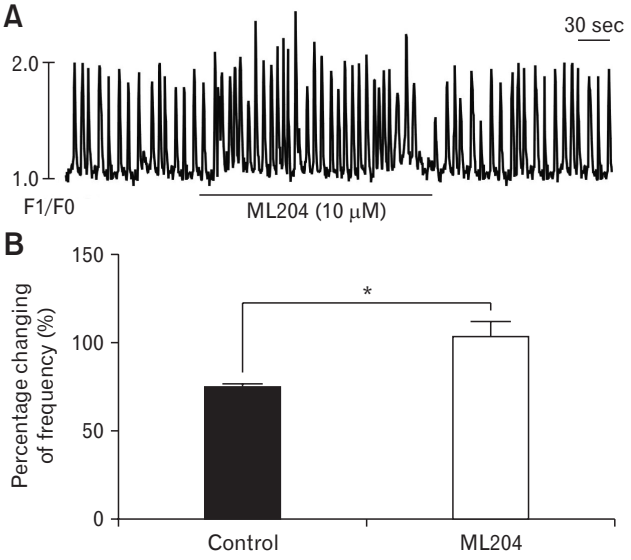

Figure 6. Effects of ML204 (an inhibitor of transient receptor potential canonical 4 and 5 [TRPC4/5] channels) on spontaneous intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations of small intestinal interstitial cells of Cajal (ICCs). (A) The application of ML204 (10 μM) increased spontaneous $[Ca^{2+}]_i$ oscillations. A series of spontaneous $[Ca^{2+}]_i$ oscillations were observed over a time period in ICCs loaded with Fluo-4 acetoxymethyl ester (AM). (B) Summarized frequency changing of ML204 action. Relative alterations of $[Ca^{2+}]_i$ fluorescence emission intensity were expressed as the ratio (F1/F0), where F0 was taken from the fluorescence of first image. Bars represent mean ± SE values (n = 6 per group). *$P < 0.05$.

ICCs, smooth muscle cells, and neurons (Fig. 7A). In addition, TRPC4 and TRPC5 amplicons could be amplified from ICCs, which confirmed their expression in small intestinal ICCs (Fig. 7B).

Discussion

Our results showed that TRPC4/5 channels are expressed in small intestinal ICCs and that treatment with ML204 (a potent TRPC4/5 blocker) resulted in depolarization of the resting membrane potential, together with a decrease in the amplitude of the pacemaker potential. The ANO1 channel might be involved in the effect of ML204 on pacemaker activity in small intestinal ICCs and ML204 might mobilize $[Ca^{2+}]_i$ oscillations. Englerin A (a specific TRPC4/5 activator) also induced depolarization of the resting membrane potentials with a decrease in the amplitude of the pacemaker potential.

Previous studies showed that TRPC4 may regulate the pacemaker activity in ICCs.[12,17] In addition, ICCs are enriched for cave-

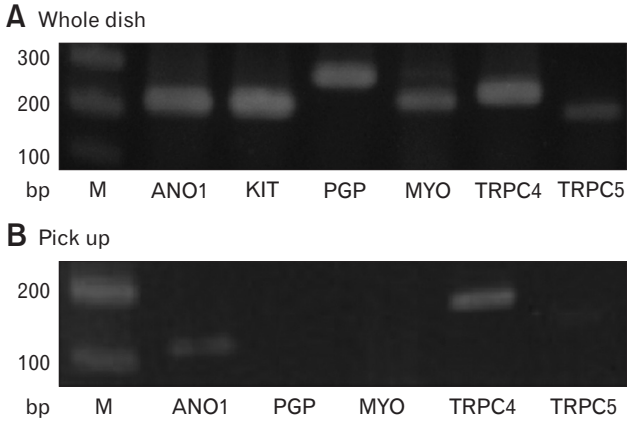

Figure 7. Results of a reverse transcription polymerase chain reaction (RT-PCR) assay to find transient receptor potential canonical 4 and 5 (TRPC4/5) mRNA expression in isolated interstitial cells of Cajal (ICCs) from small intestine. (A) RT-PCR detected the transcripts for TRPC4/5 in whole mounted cultured cells. (B) RT-PCR detected the transcripts for TRPC4/5 in anoctamin 1 (ANO1) positive cultured ICCs. M, marker; KIT, c-Kit; PGP, protein gene product 9.5; MYO, myosin.

olae, which contain many signaling molecules. Our previous studies showed that various neurotransmitters and endogenous compounds could act on the pacemaker activity of ICCs.[18-20] Furthermore, G protein-coupled receptors and receptor tyrosine kinases regulate TRP activity.[11,21] Therefore, it is suggested that TRPC4/5 channels could modulate, or are the key channels for, the pacemaker mechanism in ICCs. Furthermore, stimulation of various receptors on the plasma membrane (PM) induces the release of $[Ca^{2+}]_i$ from the ER, which then activates Ca^{2+} channels on the PM. This event is termed as store-operated Ca^{2+} entry (SOCE).[22-24] Currently, there are 2 candidates for SOCE channels: the signal is maintained by Ca^{2+} influx across the cell membrane by ORAI and TRPC channels. To study this process, we tested ML204, a specific and potent inhibitor of TRPC4/5. However, ML204 did not show inhibitory action on the pacemaker activity, but produced depolarization of the resting membrane potentials. This result produced 2 hypotheses. One is that ML204 is not specific for TRPC4/5 and the other is that TRPC4/5 have no crucial role in the pacemaker activity in ICCs. To address this, we studied how ML204 generated depolarization of the resting membrane potentials in small intestinal ICCs.

NSCC and ANO1 channels are considered as important pacemaker channels in ICCs.[25-27] In the present study, the external application of Ca^{2+}-free or low Na^+ medium abolished the pacemaker activity of the ICCs. This suggests that external cations are important for the pacemaker mechanism. Furthermore, we applied flufenamic acid (an NSCC inhibitor) and found that it suppressed the pacemaker activity. These results suggest that NSCC might be involved in the pacemaker mechanism. The results also indicated that ML204 could not inhibit TRPC4/5 in small intestinal ICCs. Thus, the action of ML204 on the pacemaker activity is not through TRPC4/5 inhibition. Next, we determined the ML204-induced activity in the presence of ANO1 channel blockers (a specific TMEM16A or CACC blocker). As mentioned above, the ANO1 channel has a critical role in ICCs function.[27,28] As shown in Figure 4, the specific ANO1 channel blockers could not block the pacemaker activity under normal conditions, but they inhibited the effects of ML204. This indicates that the ANO1 channel might not be involved in the the mechanisms that regulate basal pacemaker activity. The blocking effect by Ca^{2+}-free, low Na^+ solution, or the NSCC blocker on pacemaker activity supports the importance of NSCC in the spontaneous pacemaker mechanism. We also assessed englerin A, a potent and selective activator of TRPC 4/5 channels[16] to confirm the roles of TRPC 4/5 channels in the pacemaker activity of ICCs. Englerin A also produced tonic depolarization, which was more potent than that induced by ML204. This could be explained by our suggestion that NSCC is important for basic pacemaker mechanism. However, the result that the ANO1 channel blockers blocked the effects of ML204 suggests that the ML204-induced tonic depolarization might involve ANO1 channel regulation.

This suggestion prompted us to hypothesize that ML204 regulates $[Ca^{2+}]_i$ in small intestinal ICCs. Ca^{2+} release from ER is an important initiation for pacemaking and the pacemaker conductance (NSCC or ANO1 channels) in the PM.[5] In particular, we noted that the inositol 1,4,5-triphosphate receptor plays a role in generating spontaneous electrical activity in GI pacemaker cells[4], and the previous suggestion that periodic Ca^{2+} release from $[Ca^{2+}]_i$ stores produces $[Ca^{2+}]_i$ oscillations in ICCs, using cell cluster preparations isolated from mouse ileum,[28] and these actions seen in ICC are considered to be the primary pacemaker activity in the GI tract. In this study, we examined $[Ca^{2+}]_i$ oscillations using a Ca^{2+} dye (Fluo-4 AM) and found that ML204 increased the oscillating Ca^{2+} wave frequency. This was similar to the activity of carbachol observed in our previous study.[20] We suggest that the modulation of pacemaker activity by carbachol is mediated by an $[Ca^{2+}]_i$ release mechanism. Therefore, ML204 could modulate the pacemaker activity by modulating $[Ca^{2+}]_i$ oscillations, but not through TRPC4/5. Determining the exact mechanism of how ML204 modulates $[Ca^{2+}]_i$ oscillations in small intestinal ICCs requires further study.

In conclusion, this study suggested that ML204, a potent

TRPC4/5 inhibitor, modulates pacemaker activity by regulating $[Ca^{2+}]_i$ fluctuation in ICCs. ICCs are the pacemaker cells for GI motility; therefore, ML204 can affect GI motility by stimulating or regulating ICCs.

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Conflicts of interest: None.

Author contributions: Jun Hyung Lee and Wen-Hao Wu performed the experiments and analyzed the data; Xing-You Huang participated in preparation of animals; and Jae Yeoul Jun and Seok Choi wrote the paper and contributed to the experiment determination.

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