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Original Paper

Effects of Ca^{2+}-Activated Cl^- Channel ANO1inhibitors on Pacemaker Activity in Interstitial Cells of Cajal

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Key Words
ANO1 • Ca^{2+}-activated Cl^- channel • Pacemaker potential • T-type Ca^{2+} channel • Interstitial cells of Cajal • Colon

Abstract
Background/Aims: Anoctamin1 (Ca^{2+}-activated Cl^- channel, ANO1) is a specific marker of the interstitial cells of Cajal (ICC) in the gastrointestinal tract, and are candidate proteins that can function as pacemaker channels. Recently, novel selective ANO1 inhibitors were discovered and used to study Ca^{2+}-activated Cl^- channels. Therefore, to investigate whether ANO1 channels function as pacemaker channels, selective ANO1 inhibitors were tested with respect to the pacemaker potentials in ICC. ***Methods:*** Whole-cell patch-clamp recording, RT-PCR, and intracellular Ca^{2+} ($[Ca^{2+}]_i$) imaging were performed in cultured ICC obtained from mice. ***Results:*** Though CaCCinh-A01 (5 μM), T16Ainh-A01 (5 μM), and MONNA (5 μM) (selective ANO1 inhibitors) blocked the generation of pacemaker potentials in colonic ICC, they did not do so in small intestinal ICC. Though nifulmic acid (10 μM) and DIDS (10 μM) (classical Ca^{2+}-activated Cl^- channel inhibitors) also had no effect in small intestinal ICC, they suppressed the generation of pacemaker potentials in colonic ICC. In addition, knockdown of ANO1 reduced the pacemaker potential frequency in colonic ICC alone. Though ANO1 inhibitors suppressed $[Ca^{2+}]_i$ oscillations in colonic ICC, they did not do so in small intestinal ICC. T-type Ca^{2+} channels were expressed in the both the small intestinal and colonic ICC, but mibefradil (5 μM) and $NiCl_2$ (30 μM) (T-type Ca^{2+} channel inhibitors) inhibited the generation of pacemaker potentials in colonic ICC alone. ***Conclusion:*** These results indicate that though ANO1 and T-type Ca^{2+} channels participate in generating pacemaker potentials in colonic ICC, they do not do so in small intestinal ICC. Therefore, the mechanisms underlying pacemaking in ICC might be different in the small intestine and the colon.

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Introduction

The interstitial cells of Cajal (ICC) in the gastrointestinal (GI) tract are pacemaker cells that generate and propagate slow waves by producing spontaneous electrical activity called pacemaker potentials [1-3]. Several motility disorders are associated with a loss of ICC function and reduction in the number of ICC [4-7]. Thus, understanding the mechanisms underlying pacemaking in ICC is very important in GI motility research. The handling of intracellular Ca^{2+} ($[Ca^{2+}]_i$) has been known to be a primary pacemaking mechanism of ICC. The inositol triphosphate (IP_3)-dependent cyclical Ca^{2+} release from the endoplasmic reticulum and reuptake of Ca^{2+} into mitochondria are coupled to the periodic activation of membrane pacemaker ion channels [8, 9]. In an initial study regarding the spontaneous pacemaker activity of ICC, the nonselective cation channel (NSCC) was suggested to be a candidate for pacemaker channels in the small intestine. Pacemaking activity was suppressed by reducing external Na^+ concentrations and was blocked by NSCC inhibitors and transient receptor potential (TRP) channels expressed in the cell membrane [10-13]. After this, anoctamin1 (Ca^{2+}-activated Cl^- channel, ANO1) was expressed in human and mouse ICC; hence, it was strongly suggested that anoctamin1 channels functioned as essential pacemaker channels [14, 15]. Hwang et al. [16] found that slow waves were not observed in ANO1 knockout mice. Zhu et al. [17] recorded Ca^{2+}-activated Cl^- currents that were blocked by nifulmic acid and DIDS in small intestinal ICC. Therefore, they suggested that pacemaker current generation might be initiated by ANO1 channels. However, ANO1 knockout mice have very low survival rates and pharmacologicalstudies with nifulmic acid and DIDS were not suitable for studying Ca^{2+}-activated Cl^- channels because of their non-selectivity [18]. DIDS inhibited NSCCs in vascular smooth muscle [19], inhibited L-type Ca^{2+} channels, and delayed rectifier K^+ channels in colonic smooth muscle cells of canines [20]. Nifulmic acid also inhibited NSCCs in pancreatic cells [21]. Recently, newly discovered selective inhibitors of ANO1, T16Ainh-A01, CaCCinh-A01, and MONNA have been reportedly used in diverse tissues [22-25]. However, there have been no reports regarding the effects of these new selective inhibitors of ANO1 on the pacemaker activity of ICC. Thus, we tested new selective inhibitors of ANO1 with regard to the pacemaker potentials, to investigate the functioning of ANO1 channels as pacemaker channels in small intestinal and colonic ICC.

Materials and Methods

Preparation of cells

Mice had free access to water and were fed a standard mouse diet until the day of experimentation. Balb/C mice (5–8 days old) of either sex were anesthetized with ether and sacrificed by cervical dislocation. A 1-cm section of the small intestine was removed from below the pyloric ring to the cecum, and the small intestine was cut open along the mesenteric border. The colonic tissue from below the cecum to the rectum was removed, and tissue from the mid portion of the colon was used. The small intestine and colon were cut open along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish, and the mucosa was removed by sharp dissection. Small strips of intestinal or colonic muscles were equilibrated in Ca^{2+}-free Hank's solution for 30 min, and the cells were dispersed with an enzyme solution containing 1.3mg/ml collagenase (Worthington Biochemical Co, Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma, St. Louis, MO, USA), 2 mg/ml trypsin inhibitor (Sigma), and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 mg/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 (SMGM; Clonetics Corp., San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and 5 ng/ml urine stem cell factor (SCF, Sigma).

Patch-clamp recordings

The patch-clamp technique was used for analysis of membrane potential in ICC; ICC exhibited a network-like structure in culture (2–3 days). The current clamp mode was set and the whole- cell patch-clamp technique was used to record membrane potentials. Membrane potentials were amplified using the 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 the pClamp and GraphPad Prism software (version 2.01, GraphPad Software Inc, San Diego, CA, USA). All patch-clamp experiments were performed at 30°C.

Table 1. Sequences of the primers used for RT-PCR and of small interfering RNAs

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
c-Kit	(F) CACTGTCCAACATAAAGGGT (R) GGAAAGGTGCAAGAGTGTAG	Y00864	276
Alpha 1G	(F) TGCTTCCTCCCTGAGAAT (R) CTGCAGAGCAGTTGGTATAG	NM_009783	256
Alpha 1H	(F) CGTGGTTCGAGCACATTAG (R) GTGTCCGTCCAGAGAGTAT	NM_021415.4	272
Alpha 1I	(F) GTTTGTGTCGCCTCTCTAC (R) TCCTGGGCTGGAGAATAG	NM_001044308	342
Negative control for siRNA	5'-UUCUCCGAACGUGUCACGUTT-3'		
siANO1	5'-GCAAUCGUCCUCGGAGUUATT-3'		

Transient transfection with small interfering RNAs (siRNAs)

The siRNA used for the downregulation of the ANO1 gene and the siRNA used as a negative control were obtained from Shaghai GenePharma Co., Ltd. According to the instructions provided by the transfection reagent's manufacturers, small intestinal and colonic ICC were plated until 60–70 % confluency was achieved, after which ICC were transfected. The siRNA sequences have been shown in Table 1. Before transfection, ICC were starved in FBS-free SMBM media for 1–2 h. Compounds necessary for transfection were prepared for each dish, in accordance with the instructions for using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, siRNA (40 nM) was formulated with lipofectamine reagent, according to the manufacturer's instructions. The recording was performed 24–36 h after transfection.

Reverse Transcription-Polymerase Chain Reaction

Muscles digested using a collagenase solution were chopped finely to prepare a single-cell suspension. Large clumps of cells were removed by spinning at 3000 rpm for 1 min, and the supernatant containing single cells was transferred to a new tube for carrying out the separation process. A Robosep cell separating machine (StemCell Technologies Inc., Vancouver, Canada) was used for this purpose. The cells were incubated with the phycoerythrin (PE)-labeled mouse CD117 antibody, positive selection reagent for magnetic nanoparticles, and PE selection cocktail, as specified by the protocol, and were stored (manually changed) in the automated machine. After washing with PB, pure seperated ICC were obtained. Total RNA was isolated from c-Kit-positive cells using the TRIzol reagent, by following the manufacturer's instructions (Invitrogen). The cDNA was synthesized from total RNA using SuperscriptTM One-Step RT-PCR with Platinum Taq (Invitrogen). The primers used are shown in Table 1. The thermal cycler was so programmed that cDNA synthesis was immediate; this was automatically followed by PCR amplification. The reverse transcription reaction for cDNA synthesis was carried out at 45°C for 30 min, and it was followed by the denaturation of the cDNA hybrid at 94°C for 5 min. Thirty-eight cycles of the three-step cycling process were carried out at 94°C for 30 s for denaturation, 59°C for 30 s for annealing, and 72°C for 30 s for extension. Though the same PCR protocol was used for myosin and CD14, the annealing temperature was changed to 55°C. The PCR products were visualized by 2% agarose gel electrophoresis, followed by ethidium bromide staining.

Measurement of intracellular Ca^{2+} concentration

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations were monitored using fluo-4/AM pre- dissolved in DMSO and stored at -20°C. The ICC cultured on coverslips were rinsed twice with the bath solution mentioned above, and incubated in the bath solution containing 5 μM fluo-4 under 5% CO_2 at 37°C for 5 min. Following two more rinses, they were mounted on a perfusion chamber, and scanned every 0.4 seconds under a confocal microscope (200X; Fluoviews 300, Olympus). Excitation and emission wavelengths of 488/515 nm were used for fluorescence imaging. The variations in $[Ca^{2+}]_i$ fluorescence emission intensity were expressed as F1/F0, where F0 is the intensity of the first imaging process. The temperature of the perfusion chamber containing the cultured ICC was maintained at 30°C.

Solutions and drugs

Cells were bathed in a buffer comprising 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM, and 10 mM HEPES, with the pH adjusted to 7.2 using Tris buffer. The pipette solution was composed of 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 buffer. The drugs used were T16Ainh-A01 (2-[(5-ethyl-1, 6-dihydro-4-methyl-6-oxo-2- pyrimidinyl) thio]-N-[4-(4-methoxyphenyl)-2-thiazolyl]acetamide), CaCCinh-A01 (6-(1, 1- dimethylethyl)-2-[(2-furanyl-carbonyl) amino]-4, 5,6, 7-tetrahydrobenzo [b] thiophene-3- carboxylic acid), and MONNA (N-((4-methoxy)-2-naphthyl)-5-nitroanthranilic acid), and they were purchased from Tocris (Abingdon, UK). Nifulmic acid, 4, 40-diisothiocyanatostilbene- 2, 20-disulfonic acid (DIDS), mibefradil, and NiCl2 were purchased from Sigma Chemicals.

Statistical analysis

Data are expressed in terms of the mean ± standard error (S. E.). Differences in the data were evaluated by ANOVA, followed by a post hoc test. If *P*-values were less than 0.05, the differences were considered statistically significant. The n values reported in the text refer to the number of cells used in the patch-clamp experiments.

Results

Pacemaker potentials of ICC

Under the current clamp mode (*I=0*), periodic spontaneous pacemaker potentials were recorded in cultured small intestinal and colonic ICC (Fig. 1A and B). The pacemaker potentials of small intestinal ICC showed more regularity, higher frequency, and lower amplitude than the pacemaker potentials of colonic ICC. Under control conditions, the resting membrane potential was -60.1 ± 3.4 mV, and the amplitude of pacemaker potential was -26.1 ± 3.5 mV, and the pacemaker potential frequency was -65.5 ± 8.7 cycles/5 min in small intestinal ICC (n = 38). In colonic ICC, the resting membrane potential was -58.7 ± 5.8 mV, the amplitude of pacemaker potential was -35.3 ± 7.6 mV, and the pacemaker potential frequency was -13.3 ± 2.1 cycles/5 min (n = 18, Fig. 1C–E).

Effects of selective ANO1 inhibitors in ICC

To determine whether ANO1 channels were involved in generating pacemaker potentials at the resting state, the selective ANO1 inhibitors CaCCinh-A01, T16Ainh-A01, and MONNA were tested with respect to their effect on pacemaker potentials. Under control conditions at I = 0, CaCCinh-A01 (10 μM, n = 7), T16Ainh-A01 (10 μM, n = 8), and MONNA (10 μM, n = 7) had no effect on the generation of pacemaker potentials in small intestinal ICC (Fig. 2A–C). The values of the resting membrane potential and the pacemaker potential frequency resulting because of ANO1 inhibitors were not significantly different from those of control values in the absence of ANO1 inhibitors (Fig. 2D and E). However, CaCCinh-A01 (5 μM, n = 7), T16Ainh- A01 (5 μM, n = 7), and MONNA (5 μM, n = 8) inhibited the generation of pacemaker potentials in colonic ICC (Fig. 3A–C). In particular, CaCCinh-A01 and MONNA induced hyperpolarization of the resting membrane potentials. The values of the resting membrane potential and the pacemaker potential frequency due to ANO1 inhibitor treatment in colonic ICC are summarized in Fig. 3D and E.

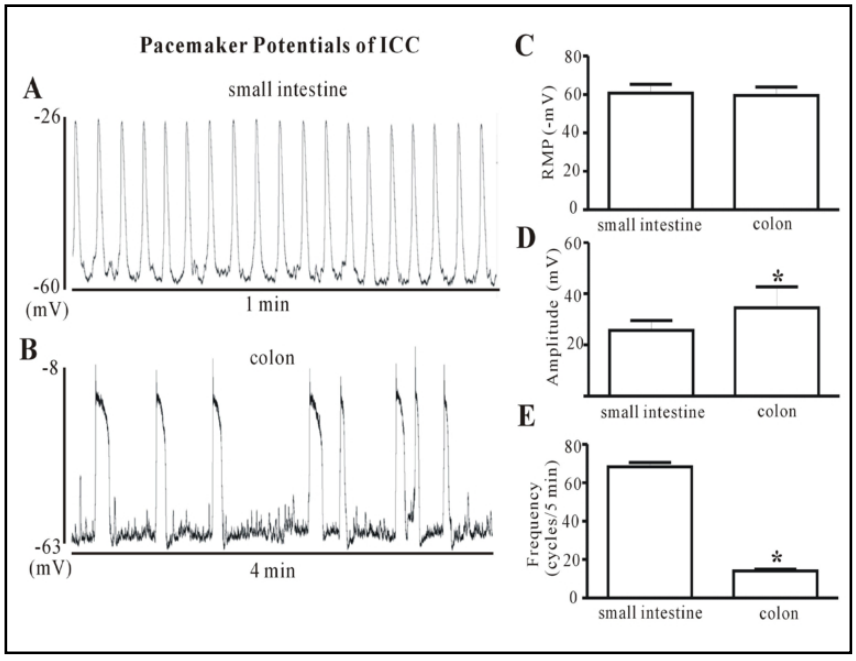

Fig. 1. Pacemaker potentials recorded in cultured interstitial cells of Cajal (ICC) from mice. (A, B) Spontaneous pacemaker potentials recorded in the current clamping mode in small intestinal ICC and colonic ICC. (C–E) The summarized data for pacemaker potentials in colonic and small intestinal ICC. (C), (D), and (E) represent resting membrane potential, amplitude of pacemaker potential, and pacemaker potential frequency, respectively. Bars represent the mean ± SE values. Asterisks indicate values that significantly differ from those of the control ($P < 0.05$). RMP: resting membrane potential.

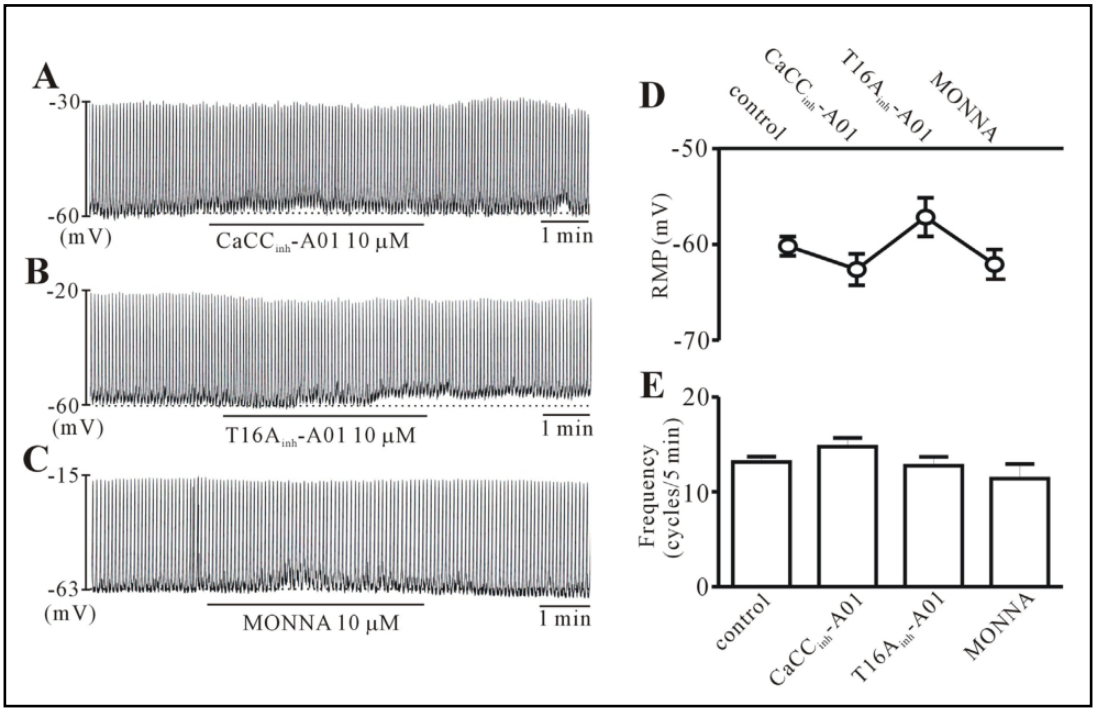

Fig. 2. Effects of selective ANO1 inhibitors on pacemaker potentials in small intestinal ICC. (A–C) At a concentration of 10 μM, CaCCinh-A01, T16Ainh-A01, and MONNA showed no effects on pacemaker potentials in small intestinal ICC. (D, E) The summarized data for the effect of ANO1 inhibitors on pacemaker potentials in small intestinal ICC. CaCCinh-A01, T16A-inh-A01 and MONNA not change the resting membrane potential and frequency of pacemaker potentials. Bars represent the mean ± SE values. RMP: resting membrane potential.

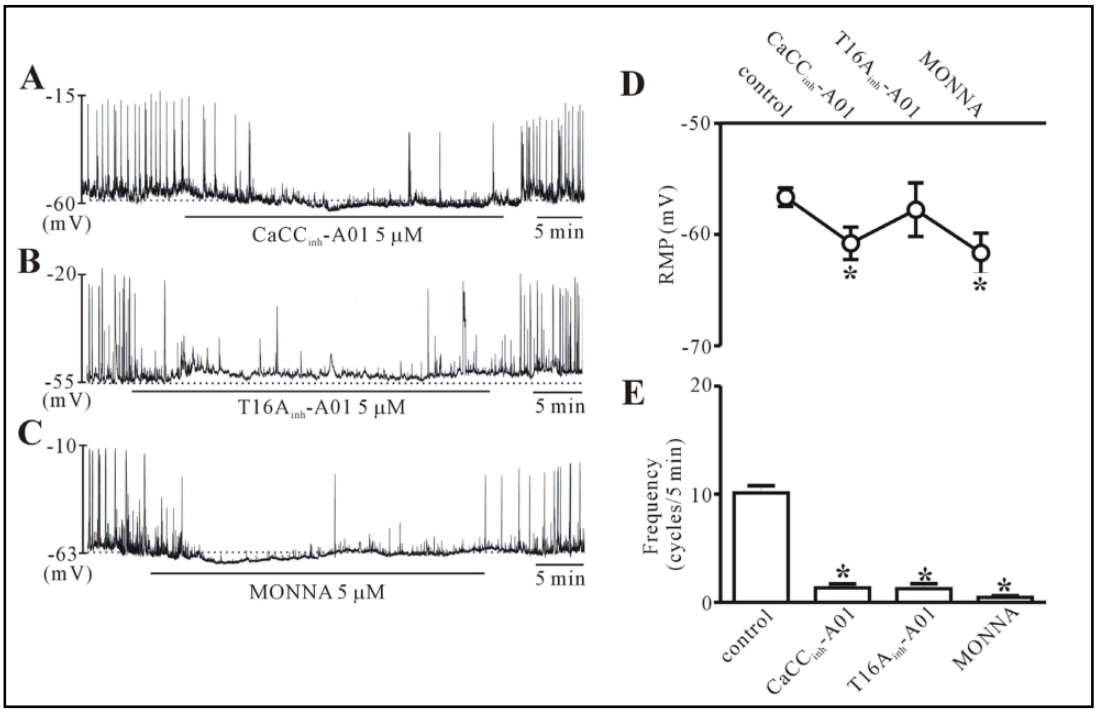

Fig. 3. Effects of selective ANO1 inhibitors on pacemaker potentials in colonic ICC. (A–C) At a concentration of 5 μM, CaCCinh-A01, T16Ainh-A01, and MONNA blocked the generation of pacemaker potentials in colonic ICC. (D, E) The summarized data for the effect of ANO1 inhibitors on pacemaker potentials in colonic ICC. CaCCinh-A01 and MONNA hyperpolarized the resting membrane potentials and reduced the frequency of pacemaker potentials. Bars represent the mean ± SE values. Asterisks indicate values that significantly differ from those of the control values ($P < 0.05$). RMP: resting membrane potential.

Effects of classical Ca^{2+}-activated Cl^- channel inhibitors DIDS and nifulmic acid in ICC

To compare effects of classical nonselective Ca^{2+}-activated Cl^- channel inhibitors with those of ANO1 inhibitors, DIDS and nifulmic were tested on the pacemaker potentials of ICC. Under control conditions, when $I = 0$, DIDS (10 μM, $n = 8$) and nifulmic acid (10 μM, $n = 8$) had no effects on the generation of pacemaker potentials in small intestinal ICC (Fig. 4A and B). The values of the resting membrane potential and frequency of pacemaker potential because of nifulmic acid and DIDS in small intestinal ICC were summarized in Fig. 4E and E. However, in colonic ICC, DIDS (10 μM, $n = 8$) and nifulmic acid (10 μM, $n = 8$) markedly inhibited the generation of pacemaker potentials (Fig. 4C and D). In particular, DIDS resulted in hyperpolarization of the resting membrane potential. The values of the resting membrane potential and frequency of pacemaker potential because of DIDS and nifulmic acid colonic ICC are summarized in Fig. 4G and H.

Effects of siRNA targeting ANO1 in ICC

Because pacemaker activity was inhibited by ANO1 inhibitors in colonic ICC, we tested whether siRNA interference with ANO1 could also affect colonic ICC next. With the negative control, siRNA could not influence the pacemaker activity in small intestinal and colonic ICC ($n = 5$, Fig. 5A and C). In addition, siRNA interference with ANO1 had no effects on the generation of pacemaker potentials in small intestinal ICC ($n = 5$, Fig. 5B). However, siRNA with ANO1 decreased the frequency of pacemaker potentials in colonic ICC ($n = 5$, Fig. 5D). The values of the resting membrane potentials and pacemaker potential frequency because of siRNA interference with ANO1 in small intestinal and colonic ICC are summarized in Fig. 5E-G.

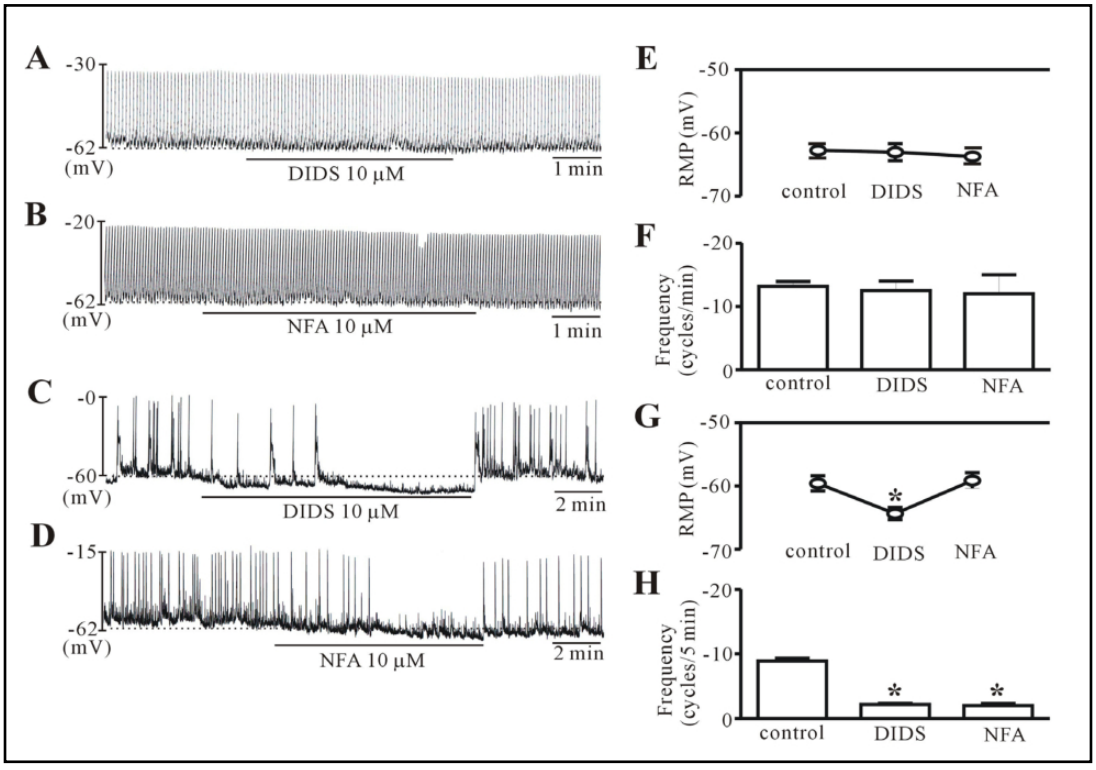

Fig. 4. Effects of classical Ca^{2+}-activated Cl^- channel inhibitors on pacemaker potentials in small intestinal and colonic ICC. (A, B) DIDS (10 μM) and nifulmic acid (10 μM) showed no effects on pacemaker potentials in small intestinal ICC. (C, D) However, DIDS (10 μM) and nifulmic acid (10 μM) inhibited the generation of pacemaker potentials in colonic ICC. (E–H) The summarized data for the effects of classical Ca^{2+}-activated Cl^- channel inhibitors on pacemaker potentials in ICC. DIDS and nifulmic acid did not change the resting membrane potential and frequency of pacemaker potentials in small intestinal ICC. However, DIDS and nifulmic acid hyperpolarized the membrane and decreased the pacemaker potential frequency in colonic ICC. Bars represent the mean ± SE values. Asterisks indicate values that significantly differ from those of the control ($P < 0.05$). RMP: resting membrane potential. NFA: nifulmic acid.

Expression of T-type Ca^{2+} channels in ICC and effects of T-type Ca^{2+} channel inhibitors on pacemaker activities in ICC

To evaluate whether T-type Ca^{2+} channels have function for generating pacemaker activities, we detected T-type Ca^{2+} channels in ICC using RT-PCR and recorded with T-type Ca^{2+} channel inhibitors in both small intestinal and colonic ICC. ANO1-positive cells expressed of α -subunits Cav3.2 (α1H) in small intestinal ICC after PCR assays (Fig. 6A and B). On the other hand, Cav3.2 (α 1G) and Cav3.2 (α 1H) were expressed in colonic ICC (Fig. 6C and D). Next, while recording the pacemaker potentials of ICC, we applied mibefradil and $NiCl_2$, which are T-type Ca^{2+} channel inhibitors. Under control conditions, when $I = 0$, mibefradil (10 μM, $n = 8$) and $NiCl_2$ (30 μM, $n = 7$) had no effects on the generation of pacemaker potentials in small intestinal ICC (Fig. 7A and B). On the contrary, low concentrations of mibefradil (5 μM, $n = 7$) blocked the generation of pacemaker potentials in colonic ICC (Fig. 7C and D) more effectively than those in small intestinal ICC with $NiCl_2$ (30 μM, $n = 7$). The values of the resting membrane potential and the pacemaker potential frequency because of mibefradil and $NiCl_2$ in small intestinal and colonic ICC are summarized in Fig. 7E–H.

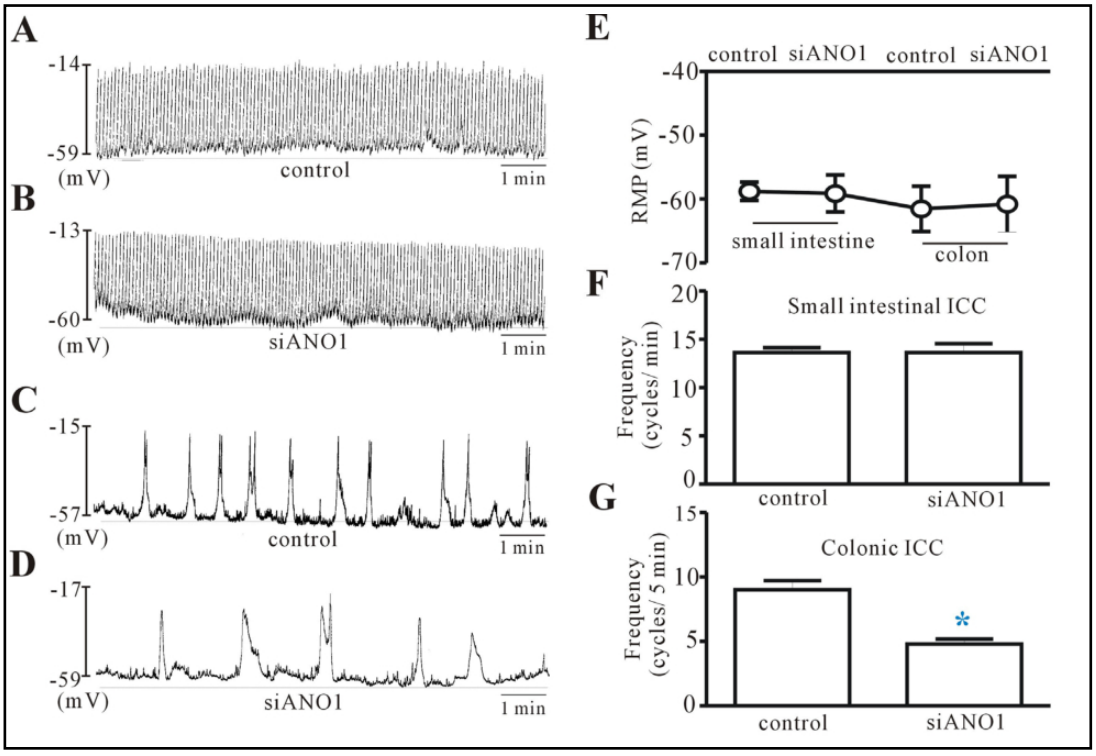

Fig. 5. Effect of the knockdown of ANO1 on pacemaker potentials in cultured ICC. (A, C) The negative control that transfected lipofectamine only in small intestinal and colonic ICC. (B, D) SiRNA ANO1 did not affect the generation of pacemaker potentials in small intestinal ICC, but reduced the pacemaker potential frequency in colonic ICC. (E–G) The summarized data for the effect of siRNA on the resting membrane potential and pacemaker potential frequency in small intestinal and colonic ICC. Bars represent the mean ± SE values. Asterisks indicate values that significantly differ from those of the control (P<0.05).

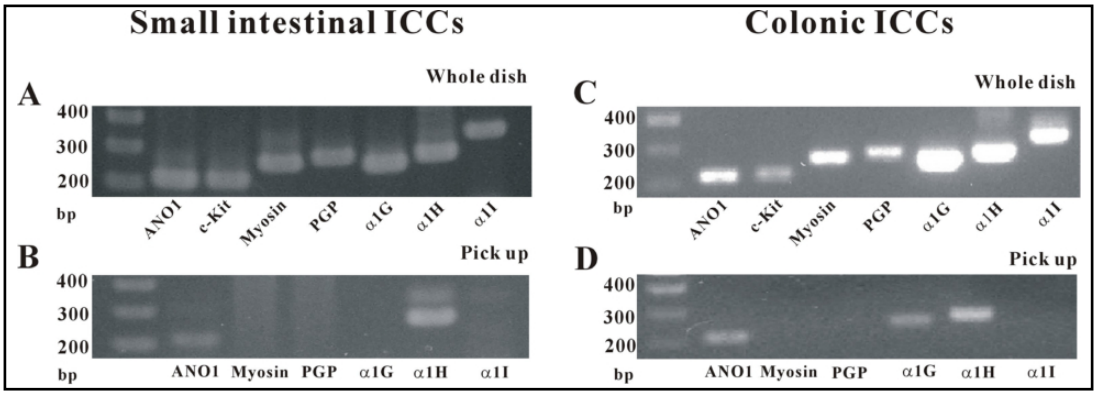

Fig. 6. RT-PCR of ICC isolated from mouse. (A, B) The a1G, a1H, and a1I mRNA transcripts of the T-type Ca^{2+} channel were detected in wholly mounted cultured small intestinal and colonic cells. (C, D) However, the a1H mRNA transcript was expressed only in ANO1- positive small intestinal ICC, and a1G and a1H were expressed in ANO1-positive colonic ICC.

Effects of selective ANO1 inhibitors on intracellular Ca^{2+} oscillation in ICC

Since intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations are known to be a primary pacemaking mechanism of ICC, we examined ANO1 inhibitors to evaluate whether Ca^{2+}-activated Cl^- channels modulate $[Ca^{2+}]_i$ oscillations using a fluorescent Ca^{2+} indicator (Fluo-4) concentrations. Spontaneous $[Ca^{2+}]_i$ oscillations in small intestinal ICC were not changed by application of CaCCinh-A01 (10 μM, n = 7), T16Ainh-A01 (10 μM, n = 8), and MONNA (10

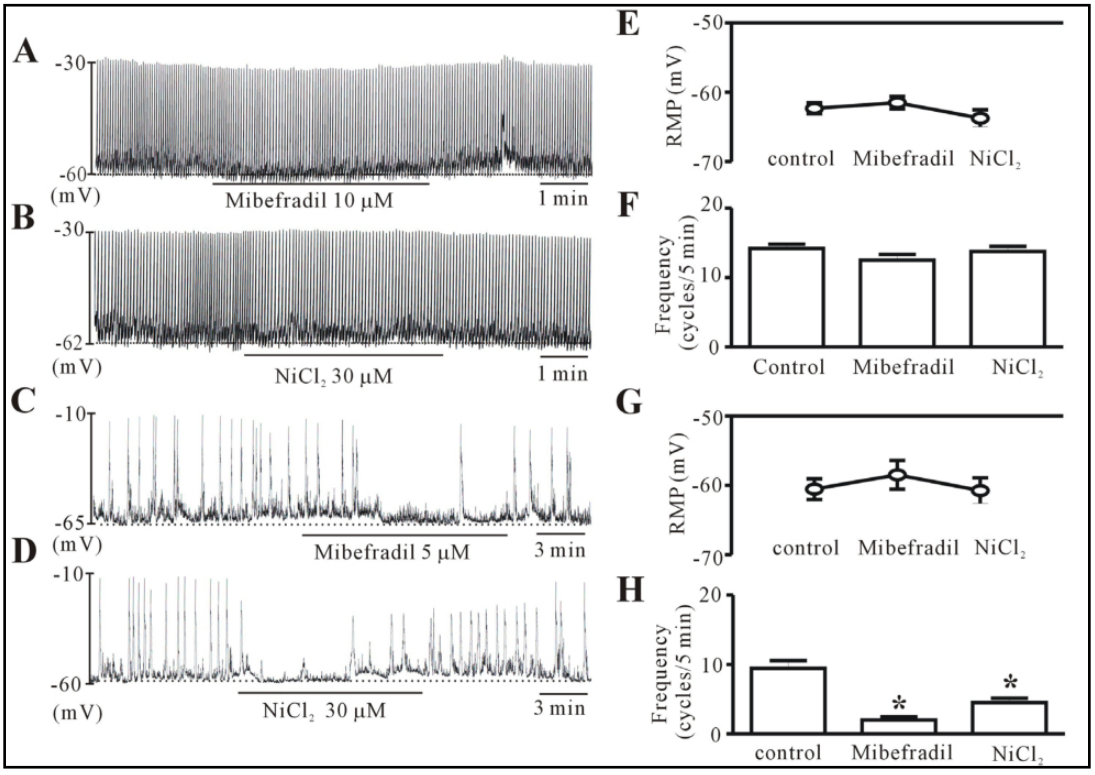

Fig. 7. Effects of T-type Ca2+ channel inhibitors on pacemaker potentials in small intestinal and colonic ICC. (A, B) Mibefradil (10 mM) and NiCl2 (30 mM) showed no effect on the pacemaker potentials in small intestinal ICC. (C, D) However, mibefradil (10 mM) and (D) NiCl2 (30 mM) suppressed the generation of pacemaker potentials in colonic ICC. (E, H) The summarized data for T-type Ca2+ channel inhibitors on pacemaker potentials in ICC. Mibefradil and NiCl2 did not affect the resting membrane potential and frequency of pacemaker potentials of small intestinal ICC. (G, H) However, mibefradil and NiCl2 inhibited the generation of pacemaking potential frequency in colonic ICC. Bars represent the mean ± SE values. Asterisks indicate values that significantly differ from those of the control (P<0.05). RMP: resting membrane potential.

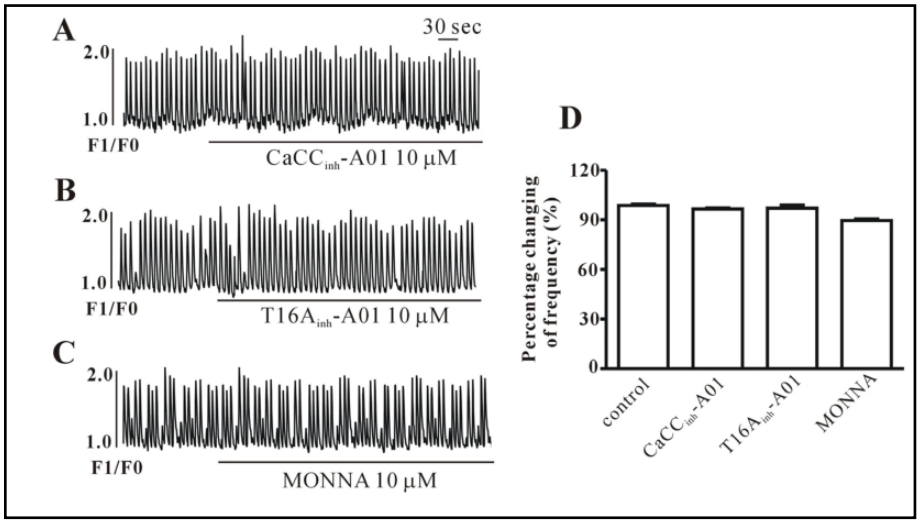

Fig. 8. Effects of selective ANO1 inhibitors on intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in small intestinal ICC. (A–C) At a concentration of 10 mM, CaCCinh-A01, T16Ainh-A01, and MONNA showed no effects on $[Ca^{2+}]_i$ oscillations in ICC. (D) The summarized data for ANO1 Ca^{2+}- activated Cl^- channel inhibitors on ($[Ca^{2+}]_i$) in colonic ICC. CaCCinh-A01, T16Ainh-A01, and MONNA did not change $[Ca^{2+}]_i$ frequency. Bars represent the mean ± SE values.

μM, *n* = 7) (Fig. 8A–D). In contrast, CaCCinh-A01 (5 μM, *n* = 6), T16Ainh-A01 (5 μM, *n* = 7), and MONNA (5 μM, *n* = 7) suppressed spontaneous $[Ca^{2+}]_i$ oscillations in colonic ICC (Fig. 9A–D).

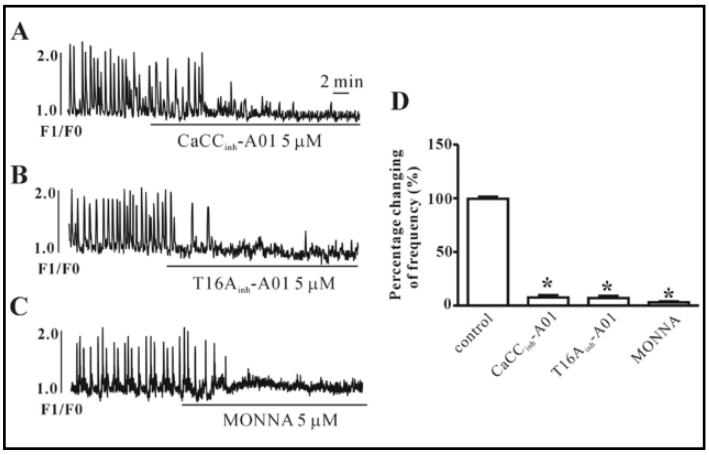

Fig. 9. Effects of selective ANO1 inhibitors on intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in colonic intestinal ICC. (A–C) At a concentration of 5 mM, CaCC$_{inh}$-A01, T16A$_{inh}$-A01, and MONNA suppressed the $[Ca^{2+}]_i$ oscillations in ICC. (D) The summarized data for ANO1 inhibitors on $[Ca^{2+}]_i$ in colonic ICC. CaCC$_{inh}$-A01, T16A-inh-A01, and MONNA inhibited the generation of $[Ca^{2+}]_i$ frequency. Bars represent the mean ± SE values. Asterisks indicate values that significantly differ from those of the control.

Discussion

In the present study, we demonstrated that new selective ANO1 inhibitors and classical Ca^{2+}- activated Cl^- channel inhibitors suppressed the generation of pacemaker potentials in colonic ICC, but not in small intestinal ICC. Therefore, it seems that ANO1differently contribute to pacemaker potentials between small intestinal and colonic ICC.

ANO1 channels are Ca^{2+}-activated Cl^- channels that are distributed in various tissues and perform various physiological functions such as epithelial secretion, smooth muscle contraction, and regulation of neuronal and cardiac excitability [26-29]. ANO1 are selective markers of ICC and are involved in regulating the proliferation of ICC [30]. In addition, ANO1 channels are strongly suggested to function as pacemaker channels of ICC; thus, they are considered as essential components for the regulation of GI motility. However, the classical drugs used to identify Ca^{2+}-activated Cl^- channels in studies of ANO1 in ICC had a very low selectivity. Thus, their effect on ANO1 pacemaker channels in ICC is still unclear. Recently, new selective ANO1 inhibitors (CaCCinh-A01, T16Ainh-A01, and MONNA) have been discovered and used for studying Ca^{2+}-activated Cl^- channels [22-25]. In the present study, it was shown that CaCCinh-A01, T16Ainh-A01, and MONNA had different effects on pacemaker activity in small intestinal and colonic ICC. CaCCinh-A01, T16Ainh-A01, and MONNA had no effects on the generation of pacemaker potentials in small intestinal ICC. These drugs did not change the resting membrane potential, amplitude, and frequency of pacemaker potentials. In contrast, at lower concentrations of CaCCinh-A01, T16Ainh-A01, and MONNA, these inhibitors abolished the generation of pacemaker potentials in colonic ICC, though they did not do so at higher concentrations in small intestinal ICC. Furthermore, classical non-selective Ca^{2+}-activated Cl- channel inhibitors nifulmic acid and DIDS did not suppress the spontaneous pacemaker potentials in small intestinal ICC, whereas they abolished the spontaneous pacemaker potentials in colonic ICC at low concentrations. The study investigating the pacemaking mechanism of ICC has been carried out almost completely in the small intestine. The mechanisms underlying pacemaking in small intestinal ICC are explained as follows: IP_3-dependent $[Ca^{2+}]_i$ release from the endoplasmic reticulum coupled to membrane ANO1 pacemaker channels leads to chloride efflux, resulting in the depolarization of the membrane [16]. In this study, CaCCinh-A01, T16Ainh-A01, and MONNA did not affect the $[Ca^{2+}]_i$ oscillations in small intestinal ICC, but they suppressed the $[Ca^{2+}]_i$ oscillations in colonic ICC. Taken together, pacemaker potentials in small intestinal ICC were unchanged by the knockdown of ANO1, whereas the frequency of pacemaker potentials in colonic ICC was reduced by knockdown of ANO1. Thus, our results provide evidence that ANO1 might not contribute to the generation of pacemaker potentials in small

intestinal ICC in the basal state and might play a different role in small intestinal ICC and colonic ICC, even though ANO1 is present in both the small intestinal and colon. Thus, ANO1 channels may be involved in initiating pacemaking potentials in colonic ICC, but not in small intestinal ICC. Hwang et al. [25] reported that in intact small intestinal tissues of the mouse, slow waves were inhibited by high concentrations of CaCCinh-A01 (30 μM); however, slow waves were still generated in the presence of high concentrations of T16Ainh-A01. They explained that the difference in drug sensitivity might be due to the possibility of different splice variants. However, there is a possibility that high concentrations of CaCCinh-A01 could affect the ionic conductance of smooth muscles. Thus, this aspect needs to be studied further. It has been reported that ANO1 channels were activated by Gq-coupled receptor stimulation, through IP_3-dependent Ca^{2+} release from the stored intracellular Ca^{2+} [31]. Thus, ANO1 in small intestinal ICC might be activated by a receptor rather than be used in the generation of pacemaker potentials. However, further studies are required to elucidate the precise roles of ANO1 channels in small intestinal ICC.

In contrast, from our results, it seems that ANO1 channels might act as pacemaker channels in colonic ICC. However, we could not explain the difference in effects of Ca^{2+}-activated Cl^- channel inhibitors on pacemaker potentials in small intestinal and colonic ICC. The configuration of pacemaker potentials was different in small intestinal and colonic ICC. The generation of pacemaker potentials in small intestinal ICC was more regular and of higher frequency and lower amplitude than those in colonic ICC, suggesting that the ionic mechanisms could be different. We have previously reported that though hyperpolarization-activated cyclic nucleotide-gated (HCN) channels participated in the generation of pacemaker potentials in colonic ICC [32], they did not do so in small intestinal ICC. Moreover, ATP-sensitive K^+ channels that were comprised of the Kir 6.2 and SUR 2B subunits were expressed in ANO1- positive colonic ICC, and maintained the resting membrane potentials by basal activation. However, in ANO1-positive small intestinal ICC, ATP-sensitive K^+ channels were comprised of Kir 6.1 and SUR 2B subunits, and did not represent basal activation [33]. These results showed that despite ATP-sensitive K^+ channels existing in both small intestinal and colonic ICC, ATP-sensitive K^+ channels could play different roles in the small intestine and colon. Thus, we think that the different configuration of pacemaker potentials in the small intestinal and colonic ICC may be due to the difference between their highly specialized functional roles in selective channel subtypes. Therefore, it is suggested that mechanisms underlying pacemaker potential generation and propagation could be different in the small intestinal and colonic ICC, which might explain the difference in pacemaker potential effects observed because of a pharmacological block or knockdown of ANO1 in small intestinal and colonic ICC.

The depolarization of the membrane potential by activation of pacemaker channels leads to an influx of external Ca^{2+} through the activation of T-type Ca^{2+} channels, which were expressed in small intestinal ICC [34, 35]. In the present study, we also analyzed the expression of T-type Ca^{2+} channel subunits in both small intestinal and colonic ICC. However, though the T-type Ca^{2+} channel inhibitors mibefradil and Ni^{2+} suppressed the generation of pacemaker potentials in colonic ICC, they did not do so in small intestinal ICC. We have previously reported that the basal activation of HCN channels was also implicated in the generation of pacemaker potentials in colonic ICC. Thus, we think that the combination of ANO1 Ca^{2+}-activated Cl^- channels, HCN channels, and T-type Ca^{2+} channels generate the pacemaker potentials in colonic ICC.

Conclusion

In conclusion, ANO1 Ca^{2+}-activated Cl^- channels are not active in small intestinal ICC. However, ANO1 Ca^{2+}-activated Cl^- channels show tonic activity in colonic ICC and might play an important role in generating pacemaker potentials with T-type Ca^{2+} channels.

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Disclosure Statement

The authors declare no conflicts of interest.

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