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Effects of ANO1 Channel Inhibitors in Interstitial Cells of Cajal

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강 현 구

지도교수: 전 제 열

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Anoctamine1 (ANO1)는 Ca^{2+}에 의해 활성화되는 Cl^- 통로(Ca^{2+}-activated Cl^- channel, ANO1)로 위장관 향도잡이인 카할 사이질 세포(interstitial cells of Cajal; ICC)를 확인하는 선택적 표적이며 자발적인 세포막 전압의 변화를 발생시키는 향도잡이 이온 통로로 제시되고 있다. 최근 선택적으로 ANO1통로를 차단하는 약물이 보고되어 Ca^{2+}에 의해 활성화되는 Cl^- 통로를 연구하는데 사용되고 있다. 본 연구는 소장 및 대장 ICC에서 발생되는 향도잡이 전압에 대한 ANO1 통로 차단제의 효과와 ANO1의 역할을 알아보기 위하여 세포막 전압 기록법 (patch clamp 기법) 및 분자 실

험 기법을 사용하여 다음과 같은 실험 결과들을 얻었다.

1. 소장과 대장 ICC에서 기록된 향도잡이 전압차이는 소장의 경우 발생 빈도수가 매우 규칙적이고 많은 반면, 대장의 경우 빈도수가 상대적으로 적으며 반대로 향도잡이 전압의 크기는 대장에서 더 크게 기록되었다.

2. ANO1 통로 차단제인 $T16A_{inh}$-A01, $CaCC_{inh}$-A01 및 MONNA는 소장 ICC의 향도잡이 전압에는 효과를 나타내지 못한 반면 대장 ICC의 향도잡이 전압 발생을 억제하였다.

3. 비선택성 Ca^{2+}-의존성 Cl^- 통로 차단제인 nifulmic acid와 DIDS 또한 소장 ICC의 향도잡이 전압에는 효과를 나타내지 못한 반면 대장 ICC에서는 향도잡이 전압의 발생을 억제하였다.

4. T-type Ca^{2+} 통로 차단제인 mibefradil과 Ni^{2+}은 소장 ICC의 향도잡이 전압에는 효과를 나타내지 못한 반면 대장 ICC의 향도잡이 전압 발생을 억제하였다.

이상의 실험결과들로부터 소장 ICC에서는 ANO1이 향도잡이 전압의 시작에는 관여하지 않는 것으로 생각되는 반면 대장 ICC에서는 기초적으로 ANO1이 활성화 되어 T-형 Ca^{2+} 통로와 협력하여 자발적인 향도잡이 전압 발생에 관여하는 것으로 생각된다. 따라서 소장과 대장의 향도잡이 전압 발생기전이 서로 차이가 있음을 제시한다.

핵심단어: ANO1, T-형 Ca^{2+} 통로, 카할 사이질 세포, 향도잡이 전압

INTRODUCTION

Gastrointestinal (GI) smooth muscles show phasic spontaneous contractions that were mediated by electrical slow waves [1]. Interstitial cells of Cajal (ICC) are pacemaker cells that generate and propagate slow waves by producing spontaneous electrical activity that called as pacemaker potentials [2-4]. ICC are formed the network each other and with smooth muscle via gap junctions in GI tract. Thus, pacemaker potentials transmitted to smooth muscle through gap junctions followed evoke slow waves [5, 6]. The pacemaking events are due to coupling the release of intracellular Ca^{2+} ($[Ca^{2+}]_i$) from endoplasmic reticulum with the periodic activation of membrane pacemaker ion channels [7,8]. Although non-selective cation channels (NSCCs) or Ca^{2+}-activated Cl^- channels are suggested as pacemaker channels [9-10], Anoctamin-1(ANO1, Ca^{2+}-activated Cl^- channels) are recognized as a major pacemaker channels in now [11,12]. The evidences as pacemaker channels of ANO1 are based on absence of slow waves in ANO1 knockout mice [13] and inhibition of Ca^{2+}-activated Cl^- currents by Ca^{2+}-activated Cl^- channel blockers in ICC [14]. However, it has been known

that ANO1 knockout mice have low survival rates [14]. Besides, the used Ca^{2+}-activated Cl^- channel blockers like as nifulmic acid and 4,40-diisothiocyanatostilbene-2,20-disulfonic acid (DIDS) are not specific drugs on Ca^{2+}-activated Cl^- channels. DIDS inhibited NSCCs in small intestinal ICC [9] and vascular smooth muscle [15], inhibited L-type Ca^{2+} channels and delayed rectifier K^+ channels in canine colonic smooth muscle cells [16]. Nifulmic acid also inhibited NSCCs in small intestinal ICC [9], pancreatic cells [17], and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in neuronal cells [18]. Recently the new selective inhibitors of ANO1 Ca^{2+}-activated Cl^- channels, $T16A_{inh}$-A01, $CaCC_{inh}$-A01 and MONNA are reported and used in diverse tissues [19-21]. But the effects of new selective inhibitors of ANO1 Ca^{2+}-activated Cl^- channels on pacemaker potentials in ICC were not reported until now. Moreover, the functional study of ANO1 in ICC was almost carried out in small intestine. Therefore, the functional roles of ANO1 Ca^{2+}-activated Cl^- channels in generating pacemaker activity were investigated by using new selective ANO1 blockers in cultured small intestinal and colonic ICC.

MATERIALS AND METHODS

Preparation of cells

The protocols and animal care used in these experiments were in accordance with the guiding principles approved by the ethics committee in Chosun University and the National Institutes of Health Guide, South Korea for the Care and Use of Laboratory Animals. Mice had free access to water, and they 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 euthanized by cervical dislocation. The small intestine from 1 cm below the pyloric ring to the cecum was removed from each mouse and opened along the mesenteric border. The colon from below the cecum to the rectum was removed, and the middle portion of the colon was used. The colon was opened along the mesenteric border. The luminal contents were washed with Krebs–Ringer bicarbonate solution. Tissues were pinned to the base of a Sylgard dish and the mucosa was removed by dissection. Small strips of the colonic muscle were equilibrated in Ca^{2+}-free Hank's solution for 30 min. Cells

were dispersed with an enzyme solution comprising 1.3 mg/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 μg/ml Falcon/BD) in 35-mm culture dishes. Cells were cultured 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) at 37°C/5% CO_2.

Patch clamp recording

The patch-clamp technique was performed using ICC that showed the network-like structures in culture (2 – 3 days). The whole-cell configuration of the patch clamp technique was used to record membrane potentials (current clamp). Membrane potentials were amplified by 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 GraphPad Prism software (version 2.01, GraphPad Software Inc, San Diego, CA, USA). All experiments pertaining to patch clamp were performed at 30°C.

Reagents

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. 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. The drugs used were $T16A_{inh}$-A01 (2-[(5-ethyl-1,6-dihydro-4-methyl-6-oxo-2-pyrimidinyl) thio]-N-[4-(4-methoxyphenyl)-2-thiazolyl]acetamide), $CaCC_{inh}$-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) which were purchased from Tocris (Abingdon, UK). Nifulmic acid, DIDS, mibefradil, and $NiCl_2$ were purchased Sigma Chemicals.

Statistical analysis

Data are expressed as the means ± standard errors (SE). Differences in the data were evaluated using the Student *t* test. A P-value < 0.05 was 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 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 and high frequency than those of colonic ICC. In contrast, the amplitude of pacemaker potentials was bigger in colonic ICC than in small intestinal ICC. Under control conditions, the resting membrane potential was –61.7 ± 4.1 mV, and the amplitude of pacemaker potential was 25.4 ± 3.9 mV, and the pacemaker potential frequency was 68 ± 5.7 cycles/ 5 min in small intestinal ICC (n = 38). In colonic ICC, the resting membrane potential was –56.7 ± 6.3 mV, and the amplitude of pacemaker potential was 36.6 ± 6.4 mV, and the pacemaker potential frequency was 14.2 ± 3.3 cycles/5 min (n = 18, Fig. 2A-C).

Effects of ANO1 channel inhibitors in ICC

To determine whether specific ANO1 channel inhibitors can modulate

pacemaker activities of ICC, the pacemaker potentials were recorded and tested with $CaCC_{inh}$-A01, $T16A_{inh}$-A01, and MONNA. Under control conditions at $I = 0$, $CaCC_{inh}$-A01 (10 μM, $n = 7$), $T16A_{inh}$-A01 (10 μM, $n = 8$), and MONNA (10 μM, $n = 7$) all had no effects on the generation of pacemaker potentials in small intestinal ICC (Fig. 3A-C). The values of the resting membrane potential, amplitude, and frequency of pacemaker potential by ANO1 channel inhibitors were not significantly different from control values in the absence of ANO1 channel inhibitors (Fig. 4A and B). However, $CaCC_{inh}$-A01 (5 μM, $n = 7$), $T16A_{inh}$-A01 (5 μM, $n = 7$), and MONNA (5 μM, $n = 8$) all induced hyperpolarization of the resting membrane potentials and inhibited the generation of pacemaker potentials in colonic ICC (Fig. 5A-C). The values of the resting membrane potential and frequency of pacemaker potential by ANO1 blockers were significantly different from control values in the absence of ANO1 blockers (Fig. 6A and B).

Effects of nifulmic acid and DIDS in ICC

To compare effects of classical nonspecific Ca^{2+}-activated Cl^- channel blockers with ANO1 blockers, nifulmic acid and DIDS were tested on the

pacemaker potentials of ICC. Under control conditions at $I = 0$, nifulmic acid (10 μM, $n = 8$) and DIDS (10 μM, $n = 7$) had no effects on the generation of pacemaker potentials in small intestinal ICC (Fig. 7A and B). The values of the resting membrane potential, amplitude, and frequency of pacemaker potential by DIDS were summarized in Figure 8A and B). However, in colonic ICC, nifulmic acid (10 μM, $n = 8$) and DIDS (10 μM, $n = 8$) markedly inhibited the generation of pacemaker potentials (Fig. 9A and B). The values of the resting membrane potential and frequency of pacemaker potential by nifulmic acid and DIDS were significantly different from control values in the absence of nifulmic acid and DIDS in colonic ICC (Fig. 10A and B).

Effects of T-type Ca^{2+} channel blockers on pacemaker activities of ICC

To evaluate whether T-type Ca^{2+} channels are involve in generating pacemaker activities, T-type Ca^{2+} channels blockers were tested on the pacemaker potentials in both small intestinal and colonic ICC. During the record of pacemaker potentials of ICC, mibefradil and low concentration of Ni^{2+}, T-type Ca^{2+} channel blockers, were treated. Under control conditions at $I = 0$, mibefradil (10 μM, $n = 8$) and Ni^{2+} (30 μM, $n = 7$)

had no effects on the generation of pacemaker potentials in small intestinal ICC (Fig. 11A and B). The values of the resting membrane potential, amplitude, and frequency of pacemaker potential by mibefradil and Ni^{2+} in small intestinal ICC were summarized in Figure 12A and B. On the contrary, low concentrations of mibefradil (5 μM, n = 7) and Ni^{2+} (30 μM, n = 7) inhibited the generation of pacemaker potentials in colonic ICC (Fig. 13A and B). The values of the resting membrane potential, amplitude, and frequency of pacemaker potential by mibefradil and Ni^{2+} were summarized in Figure 14A and B.

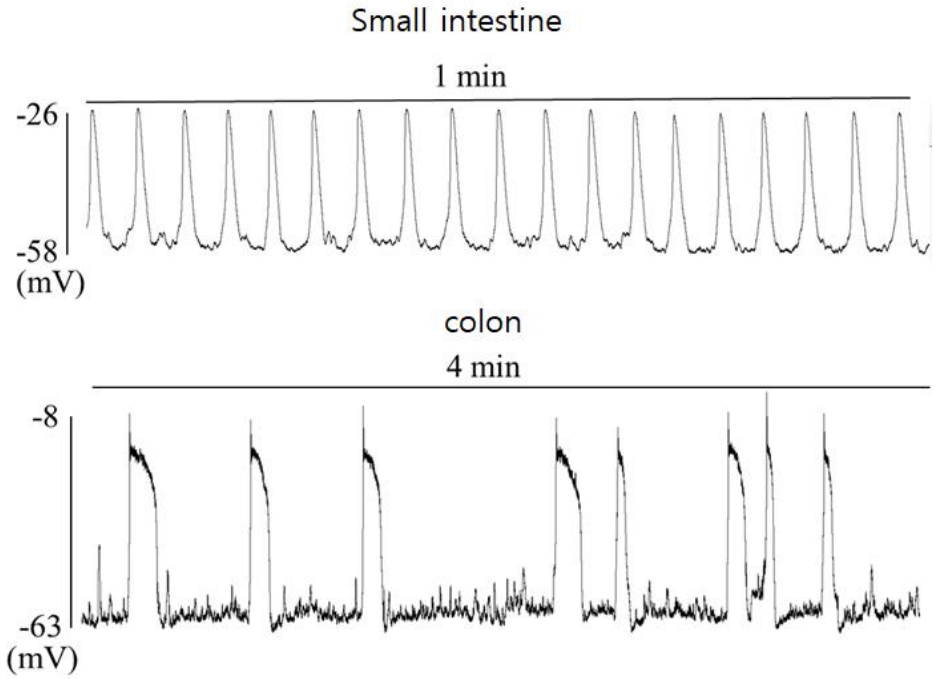

Figure 1. Pacemaker potentials recorded in cultured interstitial cells of Cajal (ICC) from mouse. Spontaneous pacemaker potentials recorded in current clamping mode in small intestinal ICC (A) and colonic ICC (B).

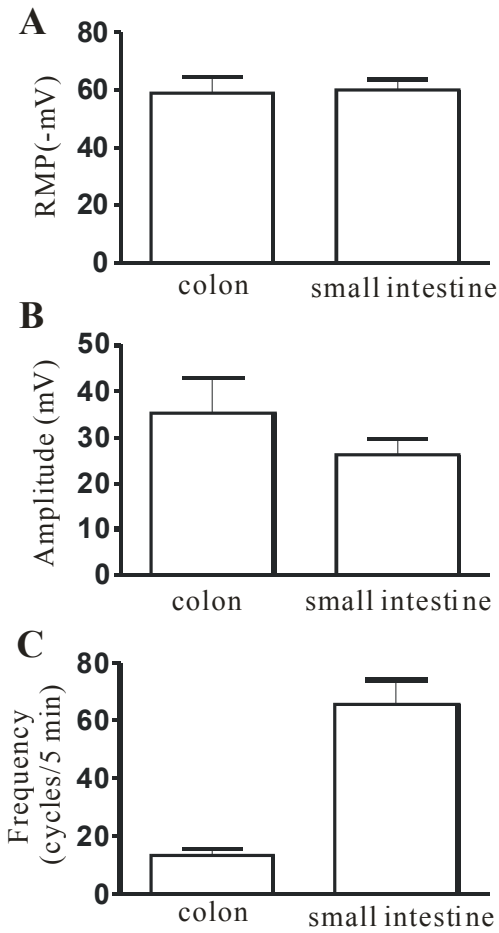

Figure 2. The summarized data on pacemaker potentials in colonic ICC and small intestinal ICC. (A), (B), and (C) represent resting membrane potential, amplitude of pacemaker potential, and pacemaker potential frequency, respectively. Bars represent the means±SE values. RMP: resting membrane potential.

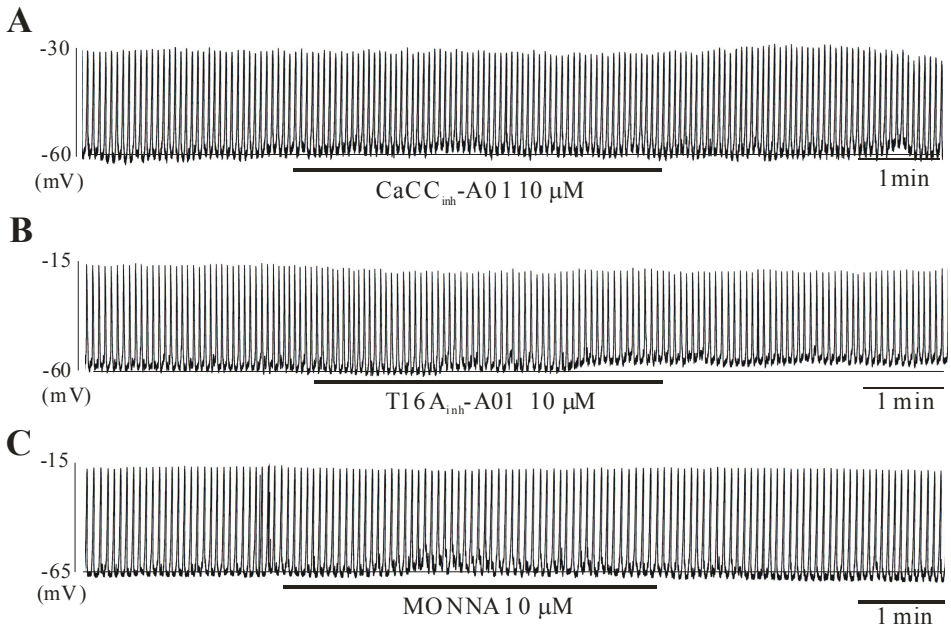

Figure 3. Effects of ANO1 Ca^{2+}-activated Cl^- channel inhibitors on pacemaker potentials in small intestinal ICC. (A) $CaCC_{inh}$-A01, (B) $T16A_{inh}$-A01 and (C) MONNA at 10 μM had no effects on pacemaker potentials in small intestinal ICC, respectively.

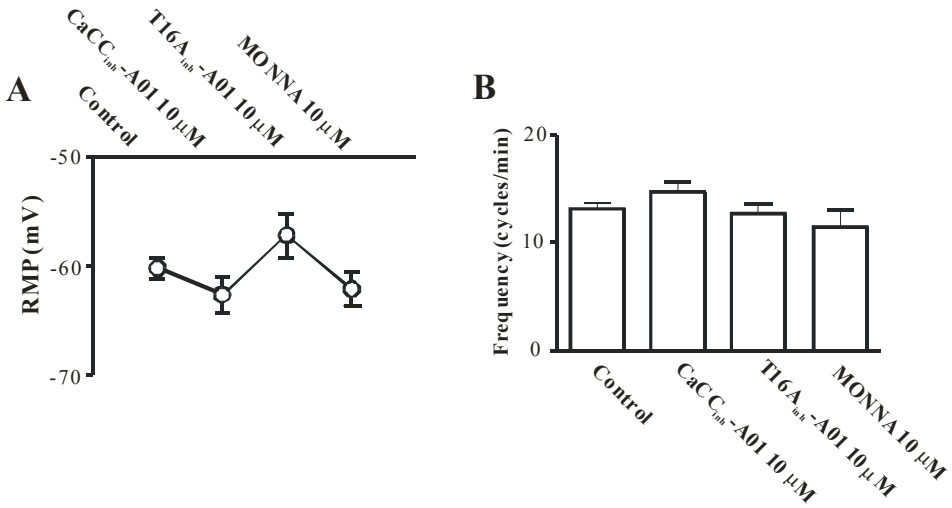

Figure 4. The summarized data of ANO1 Ca^{2+}-activated Cl^{-} channel inhibitors on pacemaker potentials in small intestinal ICC. $CaCC_{inh}$-A01, T16A-inh-A01 and MONNA did not change resting membrane potential (A) and frequency (B) of pacemaker potentials. Bars represent the means ± SE values. RMP: resting membrane potential.

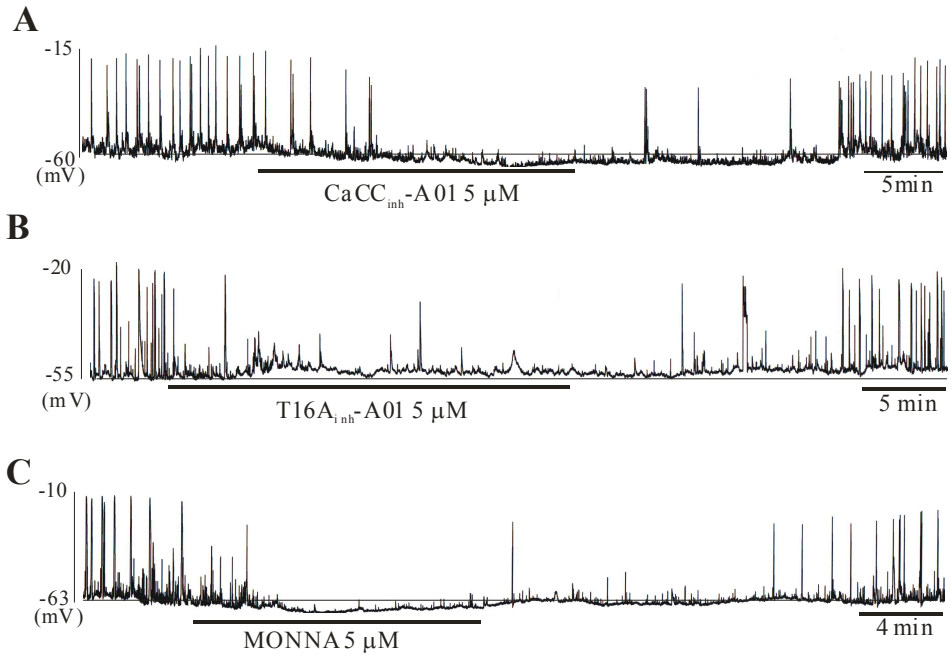

Figure 5. Effects of ANO1 Ca^{2+}-activated Cl^{-} channel inhibitors on pacemaker potentials in colonic ICC. (A) $CaCC_{inh}$-A01, (B) T16A-inh-A01 and (C) MONNA at 5 μM all blocked the generation of pacemaker potentials in colonic ICC.

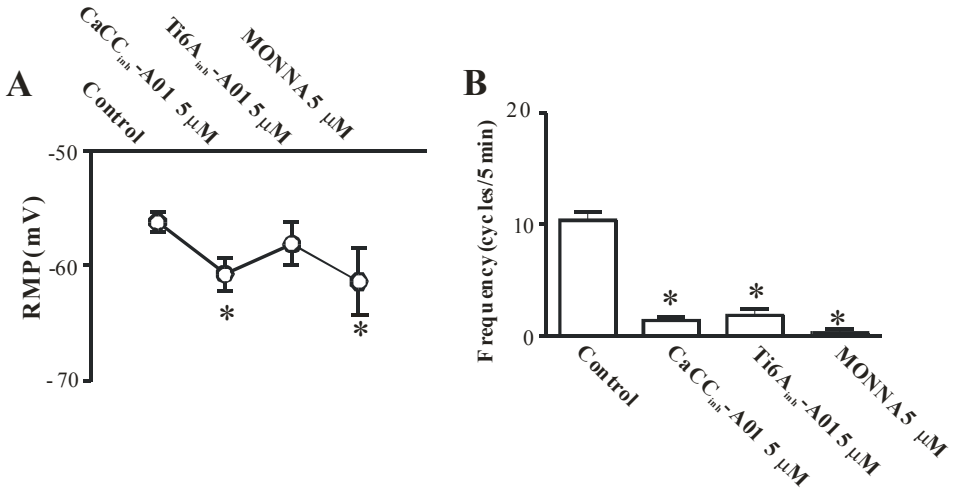

Figure 6. The summarized data of ANO1 Ca^{2+}-activated Cl^{-} channel inhibitors on pacemaker potentials in colonic ICC. $CaCC_{inh}$-A01 and MONNA hyperpolarized the resting membrane potentials (A) and reduced frequency (B) of pacemaker potentials. Bars represent the means ± SE values. RMP: resting membrane potential. *$P < 0.05$; significantly different from control: one way ANOVA.

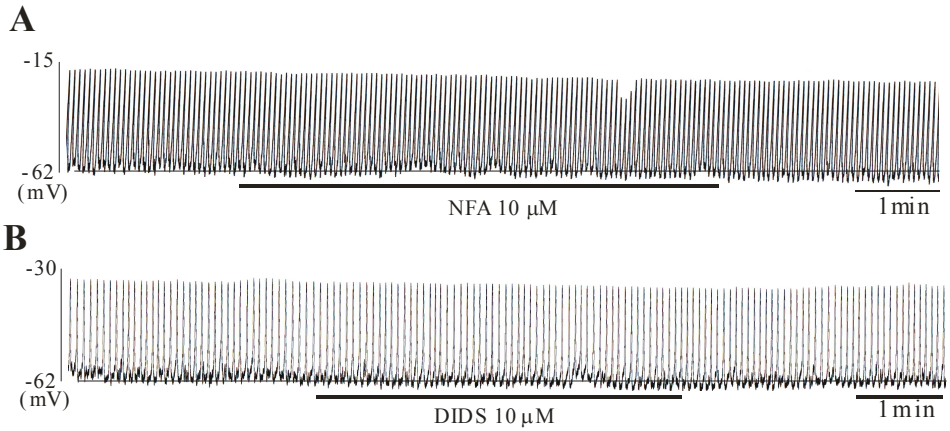

Figure 7. Effects of classical Ca^{2+}-activated Cl^{-} channel blockers on pacemaker potentials in small intestinal ICC. (A) Nifulmic acid (10 μM) and (B) DIDS (10 μM) had no effects on pacemaker potentials in small intestinal ICC. NFA: nifulmic acid.

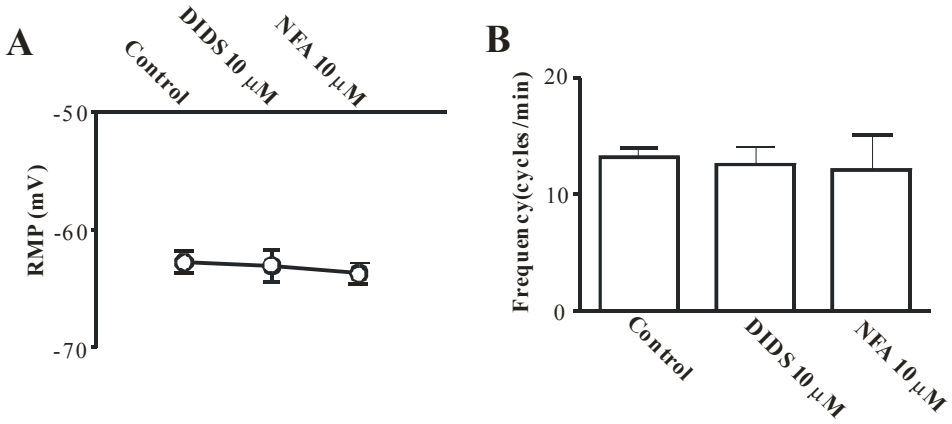

Figure 8. The summarized data of classical Ca^{2+}-activated Cl^{-} channel blockers on pacemaker potentials in small intestinal ICC. Nifulmic acid and DIDS at 10 μM did not change resting membrane potential (A) and frequency (B) of pacemaker potentials. Bars represent the means ± SE values. RMP: resting membrane potential. NFA: nifulmic acid.

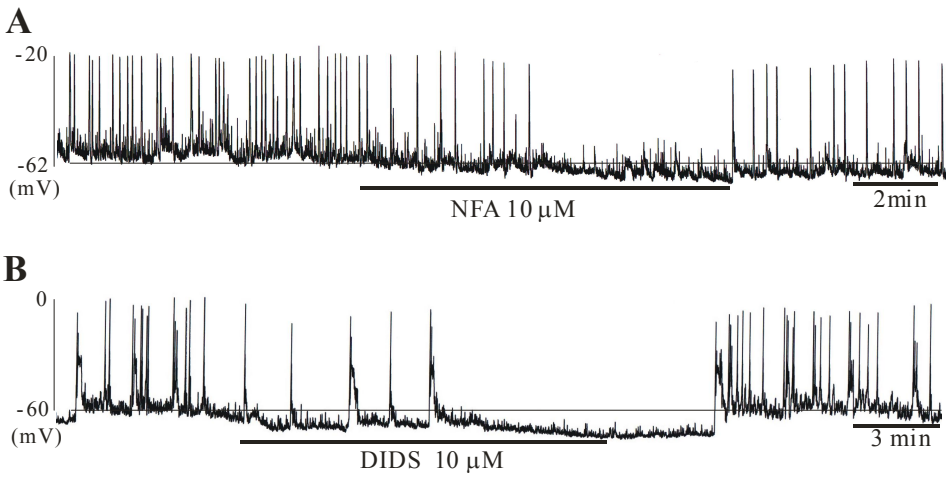

Figure 9. Effects of classical Ca^{2+}-activated Cl^{-} channel blockers on pacemaker potentials in colonic ICC. (A) Nifulmic acid (10 μM) and (B) DIDS (10 μM) blocked the generation of pacemaker potentials in colonic ICC. NFA: nifulmic acid.

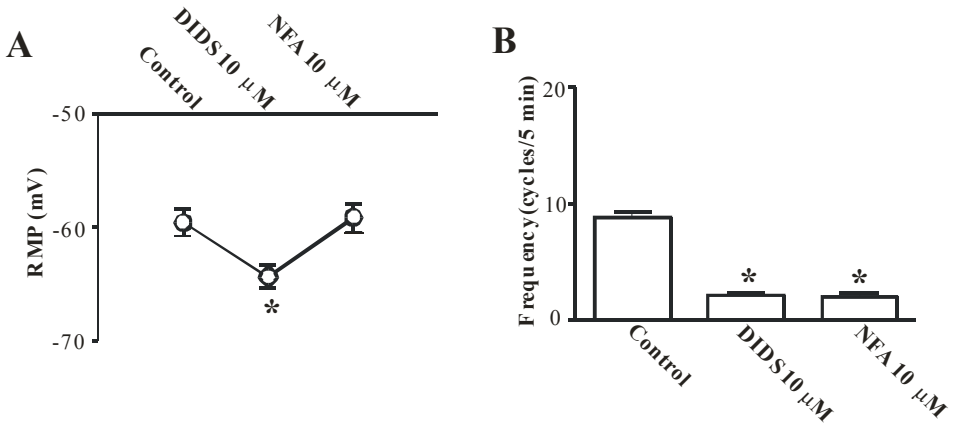

Figure 10. The summarized data of classical Ca^{2+}-activated Cl^{-} channel blockers on pacemaker potentials in colonic ICC. Nifulmic acid and DIDS hyperpolarized the resting membrane potentials (A) and reduced frequency (B) of pacemaker potentials. Bars represent the means ± SE values. RMP: resting membrane potential. *P < 0.05; significantly different from control: one way ANOVA. NFA: nifulmic acid.

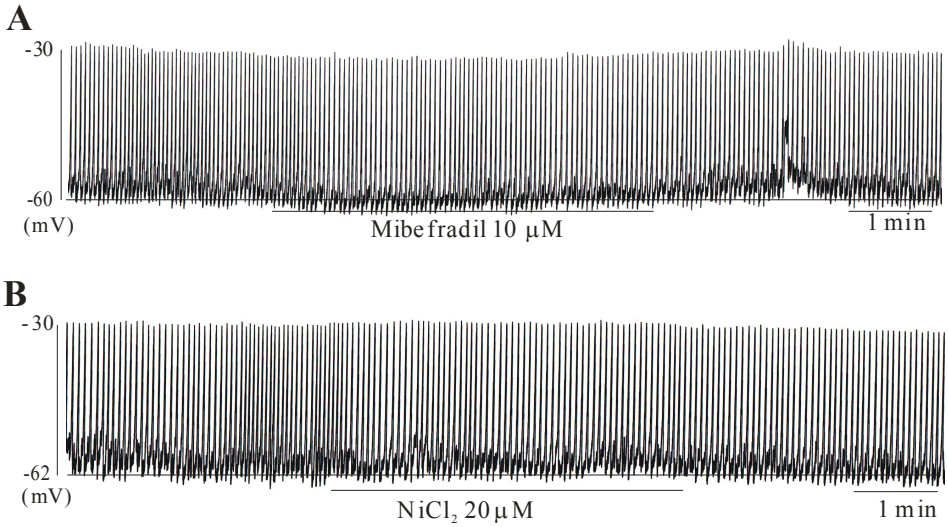

Figure 11. Effects of T-type Ca^{2+} channel blockers on pacemaker potentials in small intestinal ICC. (A) Mibefradil (10 μM) and (B) $NiCl_2$ (30 μM) had no effects on pacemaker potentials in small intestinal ICC.

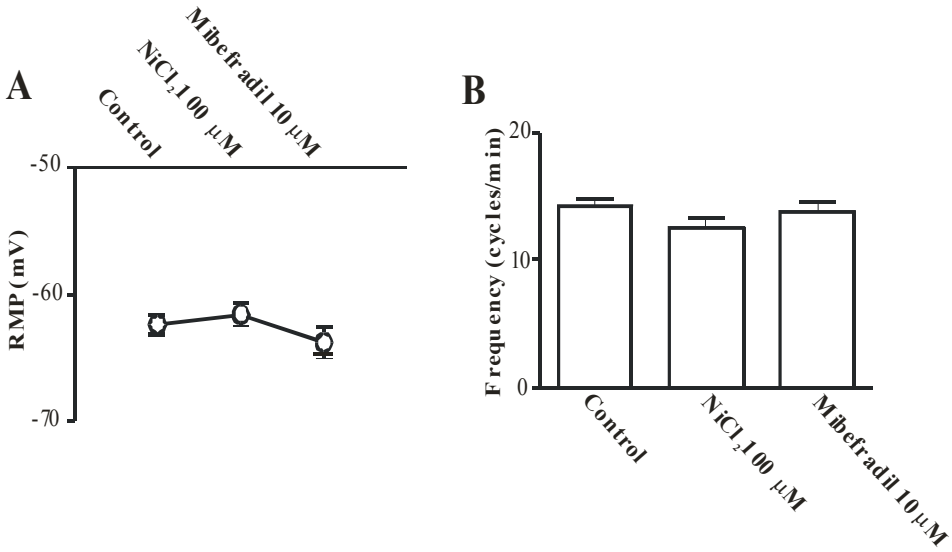

Figure 12. The summarized data of T-type Ca^{2+} channel blockers on pacemaker potentials in small intestinal ICC. Mibefradil and $NiCl_2$ did not change resting membrane potential (A) and frequency (B) of pacemaker potentials. Bars represent the means ± SE values. RMP: resting membrane potential.

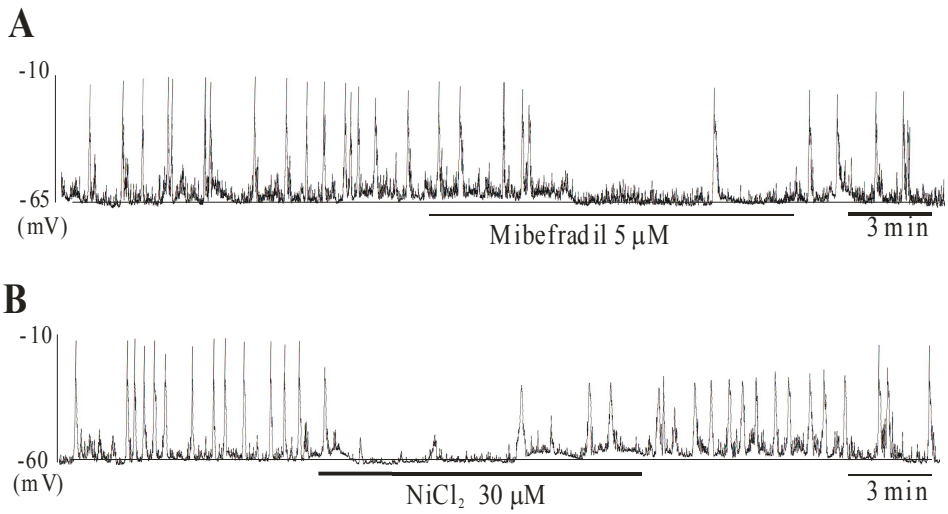

Figure 13. Effects of T-type Ca^{2+} channel blockers on pacemaker potentials in colonic ICC. (A) Mibefradil (5 μM) and (B) $NiCl_2$ (30 μM) blocked the generation of pacemaker potentials in colonic ICC.

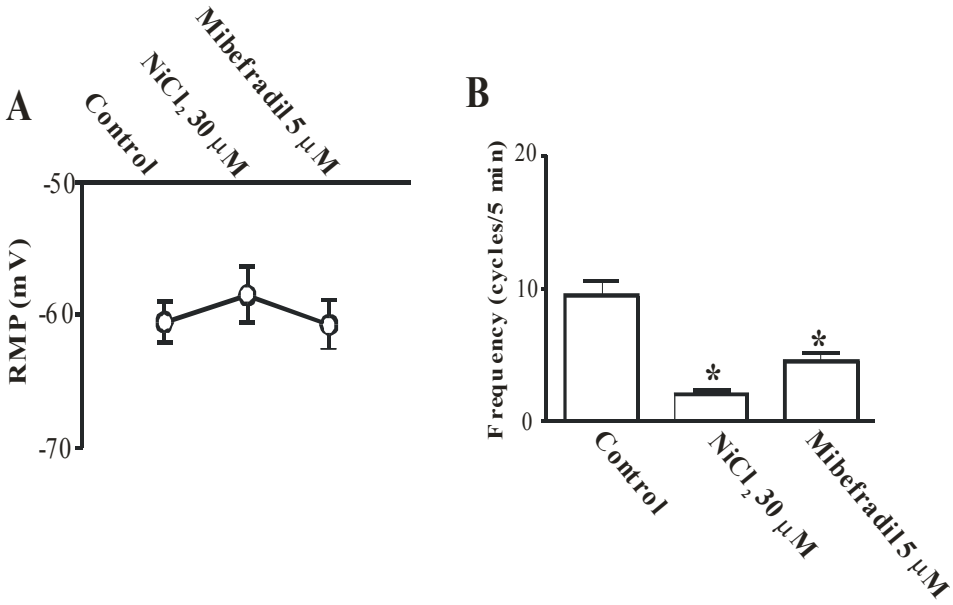

Figure 14. The summarized data of T-type Ca^{2+} channel blockers on pacemaker potentials in colonic ICC. Mibefradil and $NiCl_2$ did not change the resting membrane potential (A), but reduced the frequency (B) of pacemaker potentials. Bars represent the means ± SE values. RMP: resting membrane potential. *P < 0.05; significantly different from control: one way ANOVA.

DISCUSSION

Gastrointestinal ICC are spontaneous active cells that determine the smooth muscle contractions by generating electrical pacemaker potentials. A variety of motility disorders are related with loss of ICC functions and reduction of ICC numbers [22-25]. Thus, understanding of underlying pacemaking mechanism of ICC is very important in GI motility research. Many studies candidate non-selective cation channel (NSCCs) or Ca^{2+}-activated Cl^- channels as pacemaker ion channel in ICC [9-10]. Nevertheless, it is still controversial which ion channel is responsible for the initiation of pacemaking activity in ICC.

Anoctamine1 (ANO1) are Ca^{2+}-activated Cl^- channels that distributed in diverse tissues and performed various physiological functions like as epithelial secretion, smooth muscle contraction, regulation of neuronal and cardiac excitability [26-28]. ANO1 are selective marker of ICC in GI tract and involved in regulating proliferation of ICC [29, 30]. In now, ANO1 are strongly suggested as pacemaker channels in ICC. However, the used drugs to identify Ca^{2+}-activated Cl^- channels in studies of ANO1 from ICC were very low specificity. Thus, the role as pacemaker

channels of ANO1 in ICC is still unclear. Recently, new selective ANO1 blockers ($T16A_{inh}$-A01, $CaCC_{inh}$-A01, and MONNA) are found and used on study of Ca^{2+}-activated Cl^- channels [19-21].

In the present study, it showed that $T16A_{inh}$-A01, $CaCC_{inh}$-A01, and MONNA had different effects on pacemaker activity between small intestinal ICC and colonic ICC. $T16A_{inh}$-A01, $CaCC_{inh}$-A01, and MONNA all 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 low concentrations of $T16A_{inh}$-A01, $CaCC_{inh}$-A01, and MONNA than those of small intestinal ICC all suppressed and abolished the generation of pacemaker potentials in colonic ICC. Taken together, classical non-specific Ca^{2+}-activated Cl^- channel blockers 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. The study of underlying pacemaking mechanism has been carried out almost in small intestine until now. The underlying pacemaking mechanisms of small intestinal ICC are explained as follows; inositol triphosphate dependent $[Ca^{2+}]_i$ release from endoplasmic

reticulum coupled with membrane ANO1 pacemaker channels, leading to chloride efflux, results in depolarization of the membrane [31]. However, our results provide evidence that ANO1 may do not contribute to generate pacemaker potentials in small intestinal ICC in basal state and may play a different role between small intestinal ICC and colonic ICC even though presence of ANO1 in both small intestinal and colon. Namely, ANO1 may be involved in initiating pacemaking potentials in colonic ICC, but not in small intestinal ICC. In intact small intestinal tissues of mouse, slow waves were still generated in the presence of $T16A_{inh}$-A01 and $CaCC_{inh}$-A01 [32], which are consistent with our data. It has been reported that ANO1 were activated by Gq-coupled receptor stimulation through IP_3-dependent Ca^{2+} release from intracellular Ca^{2+} store [33]. This it seems that ANO1 in small intestinal ICC may can be activated by receptor activation rather than initiate to generate pacemaker potentials. However, the further study is required to elucidate the precise roles of ANO1 in small intestinal ICC.

In contrast, from our results, it seems that ANO1 may be act as pacemaker channels in colonic ICC. However, we could not explain the difference of drug effects by Ca^{2+} activated Cl^- channel blockers on

pacemaker potentials between small intestinal ICC and colonic ICC. The configuration of pacemaker potentials was different in small intestinal ICC and colonic ICC. The generations of pacemaker potential in small intestinal ICC are more regular, high frequency and low amplitude compared with colonic ICC, suggesting that the ionic mechanisms may can be different. Actually it has been reported that hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are participate in generating pacemaker potentials in colonic ICC [34], but not in small intestinal ICC. Moreover, ATP-sensitive K^+ channels that comprised with Kir 6.2 and SUR 2B subunits are expressed in ANO1 positive colonic ICC and maintain the resting membrane potentials by basal activation. However, in ANO1-positive small intestinal ICC, ATP-sensitive K^+ channels are comprised with Kir 6.1 and SUR 2B subunits and did not represent basal activation [35]. These results showed that even though ATP-sensitive K^+ channels are exist in both small intestinal ICC and colonic ICC, ATP-sensitive K^+ channels can be play different roles between in small intestine and in colon. Thus we think that this different ion channel contributions or different ion channel subunits may be one possible explanation about the difference effects by ANO1 blockers in small intestinal and colonic ICC.

The depolarization of the membrane potential by activation of pacemaker channels leads to influx of external Ca^{2+} through activation of T-type Ca^{2+} channels, which were expressed in ICC [36-37]. In the present study, T-type Ca^{2+} channel blockers mibefradil and Ni^{2+} suppressed the generation of pacemaker potentials in colonic ICC but not in small intestinal ICC. Together with basal activation of HCN channels also implicated in generating pacemaker potentials of colonic ICC [34]. Thus, we think that the combination of ANO1, HCN channels, and T-type Ca^{2+} channels comprise of generating pacemaker potentials in colonic ICC.

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

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