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Role of ATP-dependent K^+ Channels in Interstitial Cells of Cajal

朝鮮大學校 大學院

醫學科

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카할 사이질 세포에서 ATP-의존성 K^+ 통로의 역할

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CONTENTS

LIST OF FIGURES

국문초록

Role of ATP-dependent K^+ channels in interstitial cells of Cajal

: 카할 사이질 세포에서 ATP-의존성 K^+ 통로의 역할

나 지 선

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ATP-의존성 칼륨 통로의 활성화는 신경과 근육에서 세포의 흥분성을 결정 하고 있다. 위장관에 존재하는 카할 사이질 세포 (interstitial cells of Cajal; ICCs)는 자발적인 향도잡이 전압 발생을 통하여 평활근 수축을 조절함으로써 위장관 운동을 조절하고 있다. 본 연구는 대장 ICCs에서 발생되는 향도잡이 전압에 대한 ATP-의존성 칼륨 통로의 역할을 알아보기 위하여, 세포막 전압 기록 법 (patch clamp 기법), 분자 실험 기법 및 세포 내 칼슘 농도 측정을 통하여 다음과 같은 실험 결과들을 얻었다.

1. Pinacidil은 대장 ICCs에서 세포막 전압의 과분극과 함께 자발적으로 발생되는 향도잡이 전압의 발생 빈도를 억제하였다.

2. ATP-의존성 칼륨 통로 길항제인 glibenclamide에 의해 pinacidil에 의해 나타난 효과가 차단되었다.

3. RT-PCR 실험에서 c-kit 양성세포인 대장 ICCs에서 Kir 6.1와 SUR2B의 ATP-의존성 칼륨 통로의 아형이 발현되었다.

4. Glibenclamide 자체는 세포막 전압의 탈분극과 함께 향도잡이 전압의 발생빈도를 증가시켰다.

5. Pinacidil에 의한 효과는 protein kinase C 억제제인 Phorbol 12-myristate 13-acetate에 의해 차단되었다.

6. Glibenclamide 자체는 세포 내 자발적인 칼슘 진동의 빈도를 증가 시켰다.

7. 소장 ICCs에서는 Kir 6.1와 SUR2B의 ATP-의존성 칼륨 통로의 아형이 발현되었으나, 향도잡이 전압에 있어

glibenclamide 자체는 효과가 없었다.

이상의 실험결과들로부터 대장 ICCs에 ATP-의존성 칼륨 통로가 존재하며 정상에서 기초적으로 활성화 되어 안정막 전압을 유지하는 역할을 통하여 향도잡이 전압 발생에 관여하고 있는 것으로 생각된다. 따라서 대장 ICCs의 ATP-의존성 칼륨 통로가 대장성 운동성 질환 치료에 있어 중요한 약물 표적이 될 수 있을 것으로 생각된다.

핵심단어: 카할 사이질 세포, ATP-의존성 칼륨 통로, 향도잡이 전압, 대장

INTRODUCTION

Membrane ion channels contribute to determine the cell excitability in a variety of cells. Among the these ion channels, ATP-dependent K^+ (K_{ATP}) channels play an important role in the maintenance of resting membrane potentials and in the regulation of cell excitability in various active cells (Kuriyama et al., 1998). K_{ATP} channels are inhibited intracellular ATP and were first in identified in heart cells (Noma, 1983) The activation of K_{ATP} channels caused hyperpolarization of resting membrane potential, which leads to vasodilation via closing of voltage dependent Ca^{2+} channels in vascular smooth muscles. In contrast, the inhibition of K_{ATP} channels caused depolarization of resting membrane potential, which leads to vasoconstriction via opening of voltage dependent Ca^{2+} channels (Clapp & Gurney, 1992; Quayle et al., 1997). K_{ATP} channels are opened by K^+ channel openers such as cromakalim, nicorandil, and pinacidil, whereas are blocked by sulfonylurea drugs such as glibenclamide and tolbutamide (Mannhold, 2004; Rodrigo & Standen, 2005). In gastrointestinal tract (GI) tract, K^+ channel openers hyperpolarized the membrane on slow waves and inhibited spontaneous

contractions of stomach, intestine, colon, and gallbladder smooth muscles (Kito & Suzuki, 2003; den Hertog et al., 1989; Koh et al., 1998; Bird et al., 2002). Thus, it has been reported that K_{ATP} channels are targets of enteric neurotransmitters and inflammatory mediators. The excitatory neurotransmitters acetylcholine and substance P inhibit K_{ATP} channels (Hatakeyama et al., 1995; Jun et al., 1998), on the other hand, the inhibitory neurotransmitters nitric oxide (NO) and CGRP (Kasparek et al., 2012; Bird et al., 2002) activate K_{ATP} channels in GI smooth muscles. In addition, the gene expressions of K_{ATP} channels were altered and changed ion channel activity in experimental colitis (Jin et al., 2004). These above findings suggested that K_{ATP} channels contribute to regulate GI motility and can be therapeutic target for altered GI motility disorders.

GI motility is regulated by coordinated activity of smooth muscles, enteric neurons, and interstitial cells of Cajal (ICCs). ICCs are pacemaker cells that generate rhythmical slow waves in smooth muscles and initiate peristalsis by propagating slow waves to anal direction. ICCs also mediate neuronal signals to smooth muscle (Thomsen et al., 1998; Sanders et al., 2006). The abnormality of ICCs was related with diverse GI motility disorders (Farrugia, 2008; Jain et al., 2003). Thus, it is now

recognized that ICCs are the most important components in regulating GI motility. Previously, it has been reported that K^+ channel opener pinacidil hyperpolarized the membrane and suppressed the pacemaker activity in small intestinal ICCs and these effects were blocked by glibenclamide. NO, CGRP, and PGE_2 also showed the mimicked action with pinacidil on pacemaker activity, that effect was blocked by glibenclamide (Park et al., 2007; Choi et al., 2008; Jun et al., 2005). The pacemaker activity of ICCs are dependent on spontaneous intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations (Sanders et al., 2000). Cromakalim inhibited the $[Ca^{2+}]_i$ oscillations and its effect were blocked by glibenclamide in small intestinal ICCs (Nakayama et al, 2005). All above results indicated that K_{ATP} channels exist in small intestinal ICCs and participate in regulation of smooth muscle activity. The configuration of pacemaker activity between small intestinal ICCs and colonic ICCs is different. The frequency of pacemaking potentials in colonic ICCs is lower than small intestinal ICCs, but the amplitude of pacemaking potentials in colonic ICCs is bigger than colonic ICCs, suggesting that different ion channels can be involved in pacemaking mechanisms. Although the existence of K_{ATP} channels and the physiological actions have been reported in small intestinal ICCs, the role in pacemaking activity of K_{ATP} channels has not been reported in

colonic ICCs.

Therefore, the existence of K_{ATP} channels and the functional role in generating pacemaker activity in colonic ICCs were investigated and compared the different role of K_{ATP} channels between small intestinal ICCs and colonic ICCs.

MATERIALS AND METHODS

Preparation of cells

The protocols used, and treatment of animals, in our study were in accordance with the guiding principles approved by the ethics committee of 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.

Electrical activity recording

Current clamp mode of patch clamp was applied to record pacemaker potentials in colonic ICCs that showed a network-like structure in cultures (2–3 days). Pacemaker potentials were amplified using Axopatch 200B (Axon Instruments, Foster, CA, USA). Data were filtered at 5 kHz and displayed on a computer monitor. Results were analyzed using pClamp and GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). All experiments were performed at 30°C.

Separation of ICCs and RT-PCR

Digested muscle by collagenase solution was chopped finely to make a single-cell suspension. The large clumps of cells were removed by spinning down in 3000 rpm for 1 min, and the supernatant containing single cells was transferred to a new tube for the separation. A Robosep Cell separating machine (StemCell Technologies Inc., Vancouver, Canada) was used for this purpose. The cells were incubated with mouse CD117 phycoerythrin (PE) labeling antibody, Magnetic nanoparticles positive selection reagent, and PE selection cocktail according to the protocol stored (manually changed) in the automated machine. After washing with PB, the result was pure, separated ICCs. Total RNA was isolated from c-Kit-positive cells using TRIzol reagent according to manufacturer specifications (Invitrogen). cDNAs were produced from the total RNA using Superscript^{TM} One-Step RT-PCR with Platinum Taq (Invitrogen, Carlsbad, CA, USA). Primers used are shown in Table 1. The thermal cycler was programmed such that cDNA synthesis was followed immediately by PCR amplification automatically. The cDNA synthesis for *Ano-1, Kir 6.1, Kir 6.2, SUR 1, and SUR 2* were carried out at 45°C for 30 min for reverse transcription reaction followed by 94°C for 5 min for the denaturation of cDNA hybrid. The three-step cycling process was carried out for 38 cycles at 94°C for 30 s for denaturation,

59°C for 30 s for annealing, and 72°C for 30 s for the extension. The same PCR protocol was used for myosin and CD14 except the annealing temperature was changed to 55°C. The PCR products were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining.

Table 1. Nucleotide sequences of the primers used for RT-PCR

Gene Name	Sequences	Size (bP)
Ano-1	(Forward) AGG CCA AGT ACA GCA TGG GTA TCA	
	(Reverse) AGT ACA GGC CAA CCT TCT CAC CAA	
Myosin	(Forward) GAGAAAGGAAACACCAAGGTCAAGC	264
	(Reverse) AACAAATGAAGCCTCGTTTCCTCTC	
PGP 9.5	(Forward) GCCAACAACCAAGACAAGCTGGAA	213
	(Reverse) GCCGTCCACGTTGTTGAACAGAAT	
K_{ir} 6.1	(Forward) AGACAGTTACTTGTTGAGTCCTG	201
	(Reverse) ATACAGGAAGTTTTAATGTCTCAAAT	
K_{ir} 6.2	(Forward) ATCATTGTCATCTTGGAAGG	340
	(Reverse) AGCTTCTTGATGACAGGAAA	
SUR 1	(Forward) CTTACGAGAATATGGTAACTGAGAT	113
	(Reverse) TTAGAAGATATTCCACAGCTCTATC	
SUR 2B	(Forward) GGGTTTCTCTGTATAGCCCT	347
	(Reverse) AGCTTCTTGATGACAGGAAA	

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 pinacidil, glibenclamide, 5-hydroxydecanoic acid (5-HD), Phorbol 12-myristate 13-acetate (PMA), SQ 22536 and dideoxyadenosine. All drugs were purchased from Sigma.

Measurement of intracellular Ca^{2+} concentration

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration were monitored using fluo-4/AM pre-dissolved in DMSO and stored at –20°C. The ICCs 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 under a confocal microscope every 0.4 seconds (200×; Fluoviews 300, Olympus, Tokyo, Japan). Excitation

and emission wavelengths of 488 nm 515 nm were used for fluorescence imaging. The variations of $[Ca^{2+}]_i$ fluorescence emission intensity were expressed as F1/F0 where F0 is the intensity of the first imaging. The temperature of the perfusion chamber containing the cultured ICCs was maintained at 30°C.

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

Effects of pinacidil on pacemaker potentials

In the current clamp mode (*I*=0), colonic and small intestinal ICCs generated pacemaker potentials (Fig. 1A and B). In colonic ICCs, the resting membrane potential, the amplitude of pacemaker potential, and the pacemaker potential frequency were –58.7 ± 5.8 mV, 35.3 ± 7.6 mV, and 13.3 ± 2.1 cycles/5 min, respectively (n=18). In small intestinal ICCs, the resting membrane potential, the amplitude of pacemaker potential, and the pacemaker potential frequency were –60.1 ± 3.4 mV, 26.1 ± 3.5 mV, and 65.5 ± 8.7 cycles/5 min, respectively (n=12) (Fig. 2A-C). The addition of 1 μM pinacidil produced hyperpolarization of the resting membrane potentials and decreased the pacemaker potential frequency in colonic ICCs (Fig. 3A). In the presence of pinacidil, the resting membrane was hyperpolarized from -57.3 ± 3.7 mV to –65.2 ± 2.3 mV (n=7, Fig. 3B) and the frequency of pacemaker potentials decreased from 14.1 ± 2.3 cycles/5 min to 1.2 ± 1.0 cycles/5 min (n=7, Fig. 3C). This pinacidil-induced membrane hyperpolarization and the decrease pacemaker potential frequency were blocked by the addition of

glibenclamide, an inhibitor of ATP-dependent K^+ (K_{ATP}) channels (Fig. 3A). The membrane hyperpolarization by pinacidil and the decreased frequency of pacemaker potentials were returned to a control level by the addition of glibenclamide (Fig. 3B and C). These results suggest that K_{ATP} channels exist in ICCs.

The expression of K_{ATP} channel subunits in colonic ICCs.

To support the above functional data, RT-PCR was performed to detect expression of K_{ATP} channels. The K_{ATP} channels are composed by the combination of sulfonylurea receptor (SUR) subunits and subunits of inward rectifier K^+ channels of K_{ir} 6 family. In this study, RT-PCR analysis revealed the mRNA transcripts for K_{ir} 6.1, K_{ir} 6.2, and SUR2 subunits in whole mount cultured colonic cells (n=6, Fig. 4A). However, the mRNA transcripts for only K_{ir} 6.1 and SUR2B were detected in cultured c-kit positive isolated ICCs (n= 8, Fig. 4B).

Basal activation of K_{ATP} channels in colonic ICCs

To know whether K_{ATP} channels in colonic ICCs are active in resting state, the effects of glibenclamide alone on pacemaker potentials were

examined. Glibenclamide (10 μM) itself depolarized the membrane and increased the pacemaker potential frequency (Fig. 5A). In the presence of glibenclamide, the resting membrane was depolarized from -57.8 ± 4.2 mV to –40.1 ± 9.3 mV (n=7, Fig. 5B) and the frequency of pacemaker potentials increased from 13.7 ± 3.2 cycles/5 min to 29.1 ± 4.3 cycles/5 min (n=7, Fig. 5C). To rule out whether the activation of mitochondrial K_{ATP} channels is involved in basal active in resting state on pacemaker potentials, the action of 5-hydroxydecanoic acid (5-HD, a selective mitochondrial K_{ATP} channels blocker) was tested and found that 5-HD (100 μM) had no effect on pacemaker potentials (n=6, Fig. 6A). In the presence of 5-HD, the resting membrane potential and the pacemaker potential frequency were –55.8 ± 5.7 mV, 12.8 ± 4.1 cycles/5 min. These values were not significantly different when compared with the control values obtained in the absence of 5-HD (Fig. 6B and C).

Inhibition of pinacidil-induced actions by protein kinase C

K_{ATP} channels are regulated by dual modulation through cAMP-dependent protein kinase A and phospholipase C-dependent protein kinase C. K_{ATP} channels are activated by cAMP-dependent protein kinase

pathway via activation of adenylate cyclase. In contrast, protein kinase C inhibits K_{ATP} channels. Thus, to investigate whether cAMP or protein kinase C involves in regulating K_{ATP} channels activity, adenylate cyclase inhibitors and protein kinase activator were examined. In the presence of glibenclamide, SQ 22536 (n=5, 100 μM) and dideoxyadenosine (n=6, 100 μM), adenylate cyclase inhibitors, blocked the generation of pacemaker potentials (Fig. 7A and B). These results suggest that cAMP does not involve in opening of K_{ATP} channels in colonic ICCs. However, pinacidil-induced effects on pacemaker potentials were blocked by PMA (100 nM), a protein kinase C activator, like as glibenclamide (n=6, Fig. 8A). PMA returned the hyperpolarization of the resting membrane potential and the decrease the pacemaker potentials by pinacidil to control level (Fig. 8B and C). These results suggesting that protein kinase C inhibits K_{ATP} channels activity in colonic ICCs .

Increase the $[Ca^{2+}]_i$ oscillations by glibenclamide

Because $[Ca^{2+}]_i$ oscillations are considered to be the primary mechanism for the pacemaker activity in ICCs, the effect of glibenclamide on $[Ca^{2+}]_i$ oscillations were examined in colonic ICCs. In this study, spontaneous

$[Ca^{2+}]_i$ oscillations of ICCs which are connected with cell clusters were measured. Spontaneous $[Ca^{2+}]_i$ oscillations observed in colonic ICCs which was loaded with fluo-3. In the presence of glibenclamide (n=6, 10 μM), $[Ca^{2+}]_i$ oscillations were increased (Fig. 9A). The data of $[Ca^{2+}]_i$ oscillations by glibenclamide was summarized in Figure 9B. These results suggest that K_{ATP} channels on ICCs may involve the regulation of spontaneous $[Ca^{2+}]_i$ oscillations.

K_{ATP} channels in small intestinal ICCs

To compare to K_{ATP} channels with colonic ICCs, K_{ATP} channels were examined in small intestinal ICCs. The addition of 1 μM pinacidil produced hyperpolarization of the resting membrane potentials and decreased the pacemaker potential frequency in small intestinal ICCs (n=8, Fig. 10A). However, glibenclamide alone did not change the generation of pacemaker potentials (n=7, Fig. 10B). RT-PCR analysis revealed the mRNA transcripts for K_{ir} 6.2 and SUR2 subunits in cultured Ano-1 positive isolated ICCs (n= 6, Fig. 11).

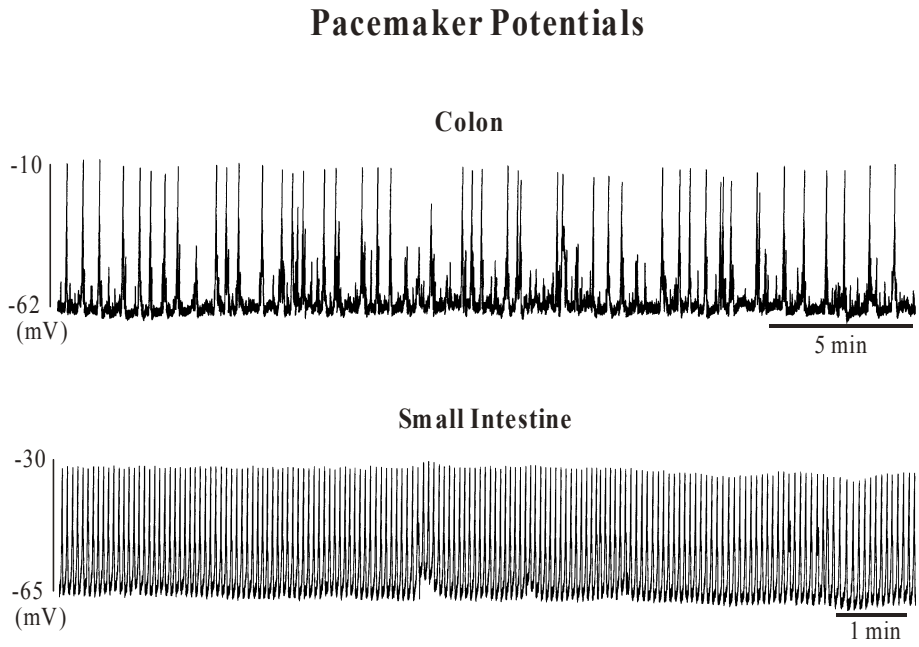

Figure 1. Typical traces of pacemaker potentials in interstitial cells of Cajal (ICCs) from mouse. Spontaneous pacemaker potentials recorded in current clamping mode in colonic ICCs (A) and small intestinal ICCs (B) from mouse.

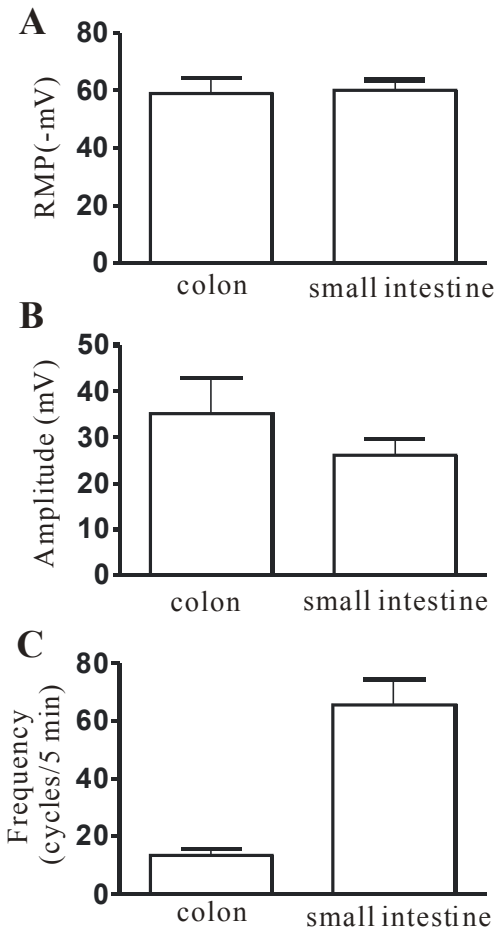

Figure 2. The summarized data on pacemaker potentials in colonic ICCs and small intestinal ICCs. (A), (B), and (C) represent resting membrane potential, amplitude of pacemaker potential, and pacemaker potential frequency, respectively. Bars represent the means±SE values. RMP: resting membrane potential.

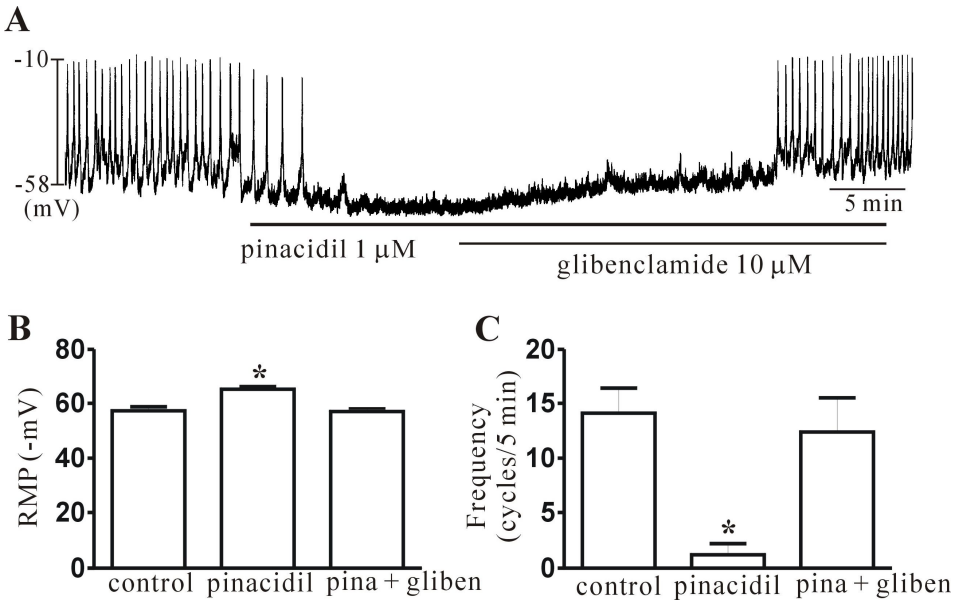

Figure 3. Activation of ATP-dependent K^+ (K_{ATP}) channels by K^+ channel opener and blockade by K_{ATP} channel inhibitor on pacemaker potentials of ICCs from mouse colon. (A) Pinacidil (K^+ channel opener, 1μM) hyperpolarized the resting membrane and decreased the pacemaker potential frequency. Gilbenclamide (a K_{ATP} channel inhibitor, 10 μM) blocked the pinacidil-induced actions in colonic ICCs. (B, C) The summarized effects of pinacidil on pacemaker potentials with glibenclamide in colonic ICCs. Bars represent the means±SE values. *Asterisks indicate a statistically significantly difference from controls ($p<0.05$). Pina; pinacidil, gliben; glibenclamide.

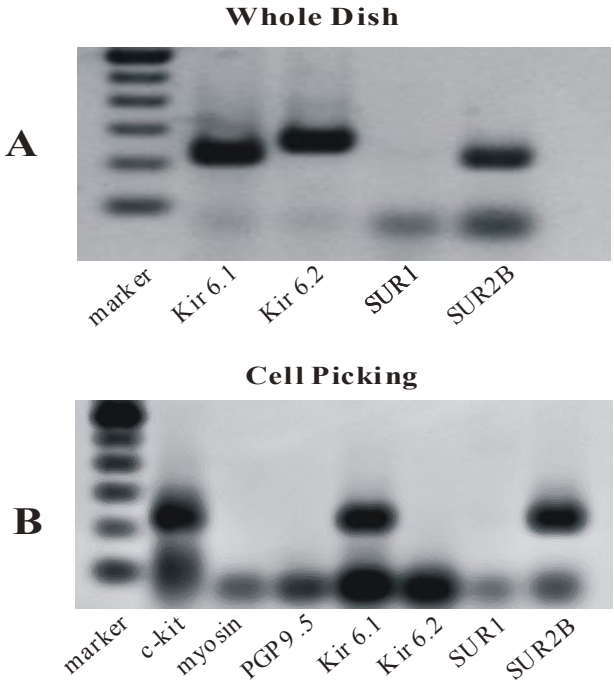

Figure 4. RT-PCR detection and expression of K_{ATP} channel subunits in isolated ICCs from mouse colon. The mRNA transcripts for K_{ir} 6.1, K_{ir} 6.2, and SUR2 subunits are expressed in whole mount cultured colonic cells (A). However, the mRNA transcripts for only K_{ir} 6.1 and SUR2B were detected in cultured c-kit positive isolated colonic ICCs (B).

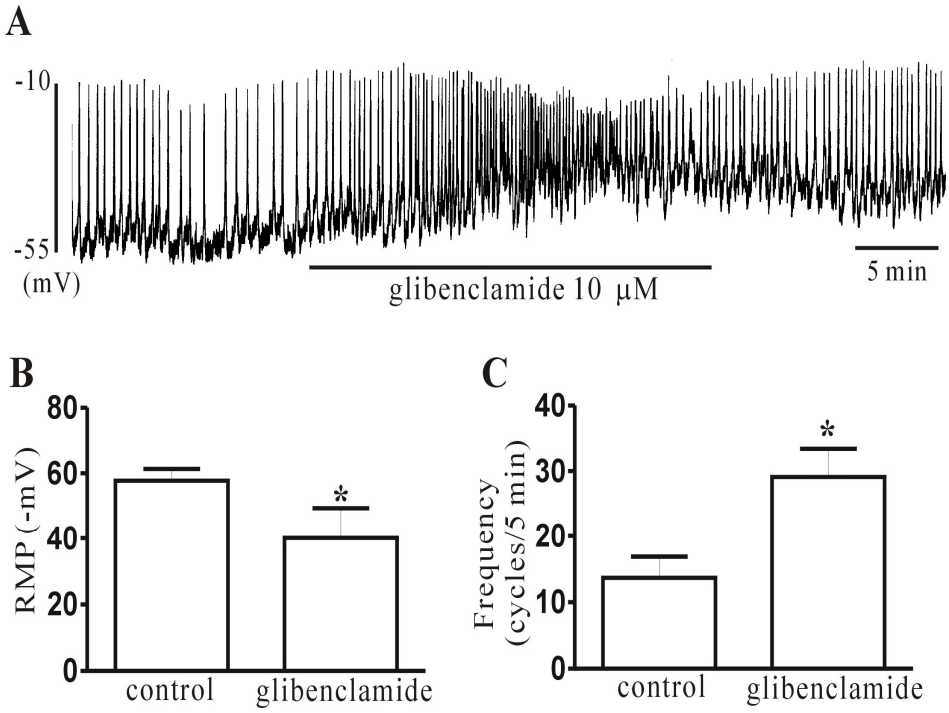

Figure 5. Effects of glibenclamide alone on pacemaker potentials of ICCs from mouse colon. (A) Glibenclamide (10 μM) itself depolarized the resting membrane and increased the pacemaker potential frequency. (B, C) The summarized effects of glibenclamide on pacemaker potentials in colonic ICCs. Bars represent the means±SE values. *Asterisks indicate a statistically significantly difference from controls ($p<0.05$).

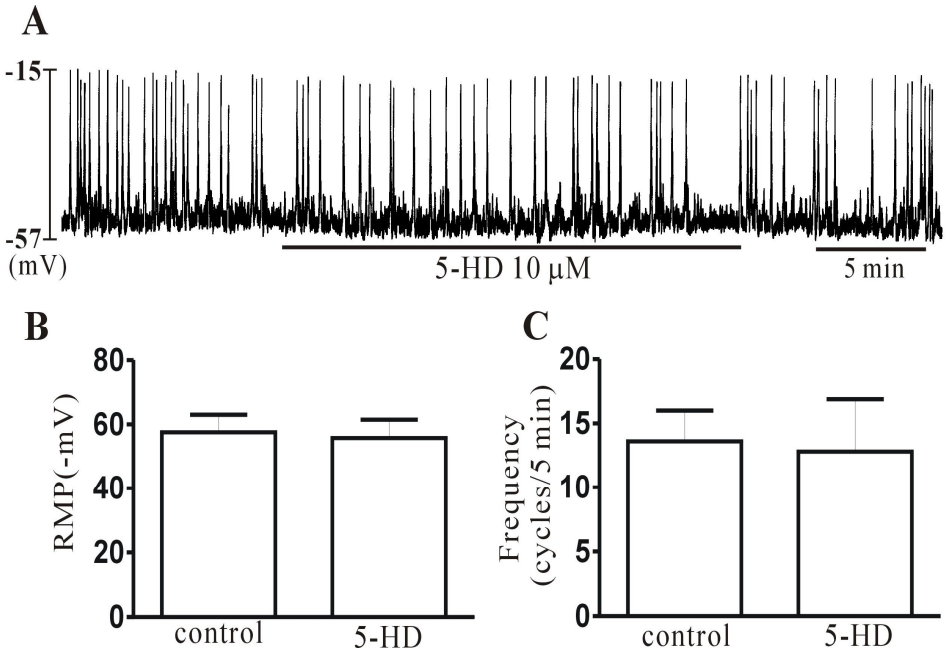

Figure 6. Effects of mitochondrial K_{ATP} channel blocker on pacemaker potentials of ICCs from mouse colon. (A) 5-HD (a mitochondrial K_{ATP} channel blocker, 10 μM) had no effects on the pacemaker potentials in colonic ICCs. (B, C) The summarized effects of 5-hydroxydecanoic acid in colonic ICCs. Bars represent the means±SE values. 5-HD: 5-hydroxydecanoic acid.

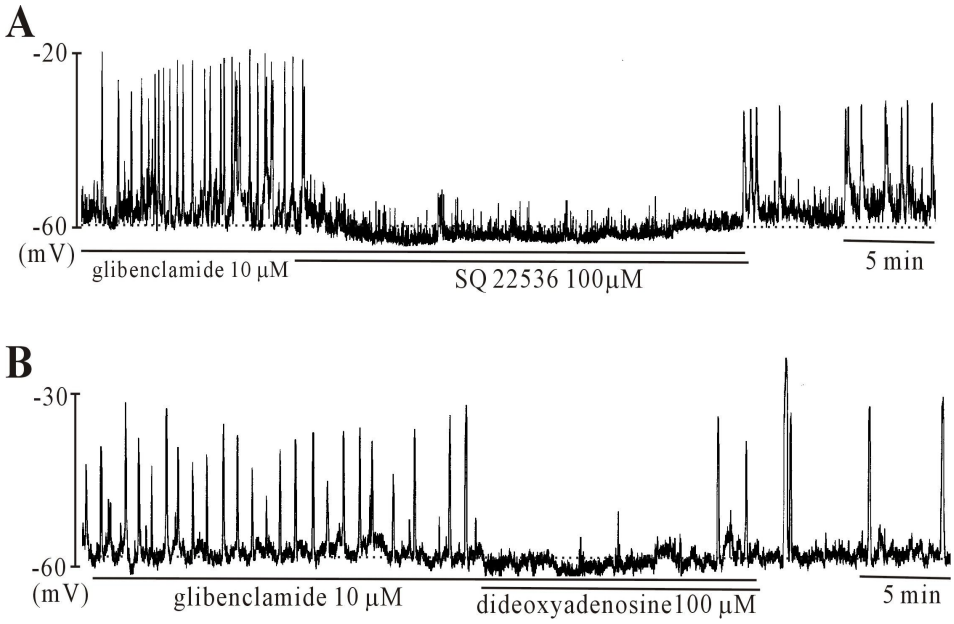

Figure 7. Effects of adenylate cyclase inhibitors on glibenclamide-induced pacemaker potential changes from mouse colonic ICCs. (A) SQ 22536 (100 μM) and (B) dideoxyadenosine (100 μM), adenylate cylase inhibitors, blocked the generation of pacemaker potentials in the presence of glibenclamide (10 μM).

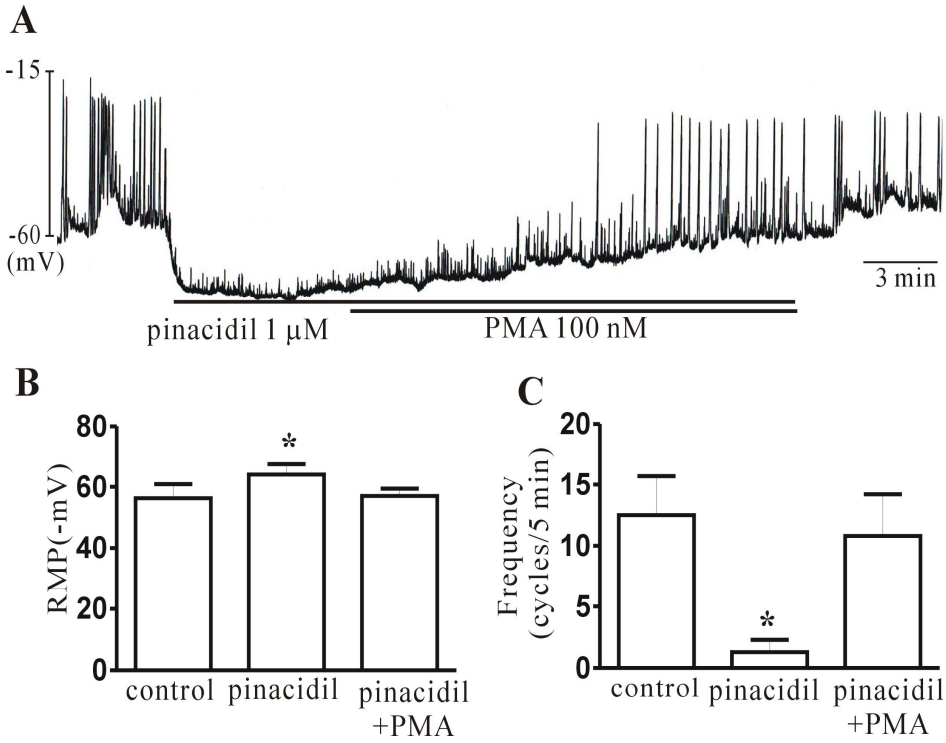

Figure 8. Effects of protein kinase C activator on pinacidil-induced pacemaker potential changes from mouse colonic ICCs. (A) Pinacidil (1μM) hyperpolarized the resting membrane and decreased the pacemaker potential frequency. PMA (a protein kinase C activator, 100 nM) blocked the pinacidil-induced actions in colonic ICCs. (B, C) The summarized effects of pinacidil on pacemaker potentials with PMA colonic ICCs. Bars represent the means±SE values. *Asterisks indicate a statistically significantly difference from controls ($p<0.05$). PMA; Phorbol 12-myristate 13-acetate.

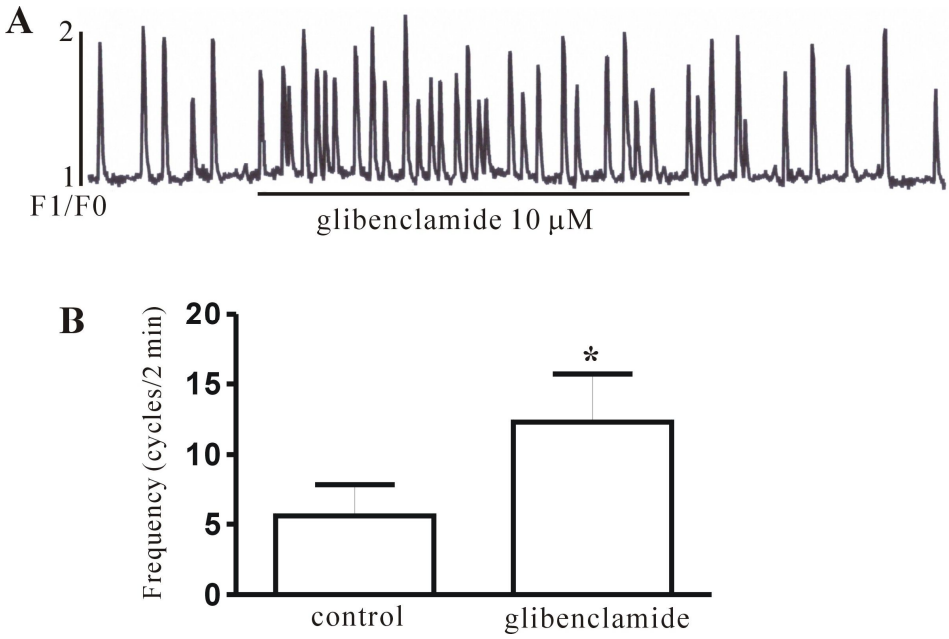

Figure 9. Effects of glibenclamide on intracellular Ca^{2+} ($[Ca^{2+}]_i$)oscillations of ICCs from mouse colon. Series of spontaneous $[Ca^{2+}]_i$ oscillations observed through a time period in colonic ICCs loaded with fluo4-AM. (A) Glibenclamide (10 μM) increased the $[Ca^{2+}]_i$ oscillations. (B) The summarized effects of glibenclamide on the $[Ca^{2+}]_i$ oscillations. Bars represent the means±SE values. *Asterisks indicate a statistically significantly difference from controls ($p<0.05$).

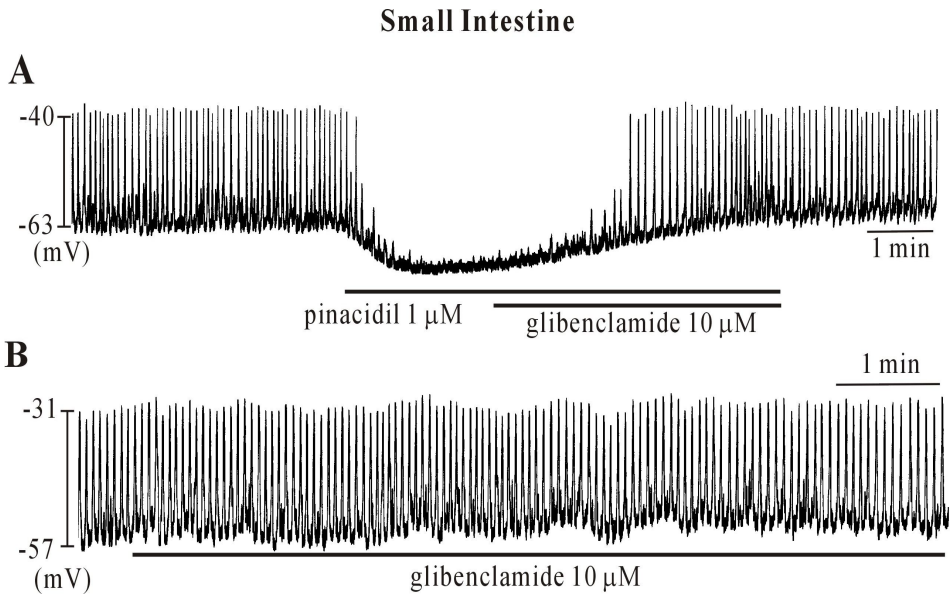

Figure 10. Activation of K_{ATP} by K^+ channel opener and blockade by K_{ATP} channel inhibitor on pacemaker potentials of ICCs from mouse small intestine. (A) Pinacidil (1μM) hyperpolarized the resting membrane and decreased the pacemaker potential frequency. Gilbenclamide (10 μM) blocked the pinacidil-induced actions in ICCs. (B) However, glibenclamide itself had no effects on pacemaker potentials in small intestinal ICCs.

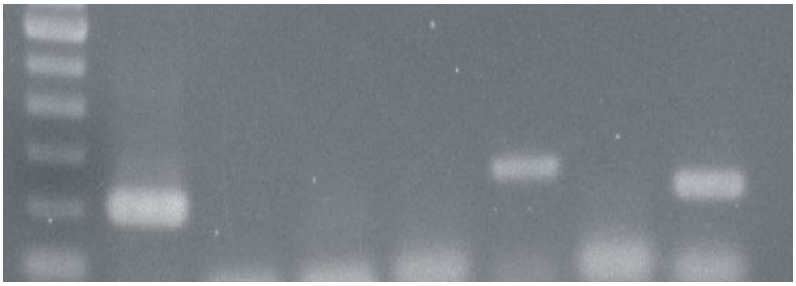

Figure 11. RT-PCR detection and expression of K_{ATP} channel subunits in isolated ICCs from mouse small intestine. The mRNA transcripts for K_{ir} 6.2 and SUR2 subunits were expressed in cultured Ano-1 positive isolated ICCs.

DISCUSSION

In the present study, K_{ATP} channels were expressed and participated in regulating pacemaker activity in colonic ICCs. Furthermore, compared to small intestinal ICCs, K_{ATP} channels are basally activated in resting state and have different molecular identities in colonic ICCs.

K_{ATP} channels maintain the resting membrane potential and determine the cell excitability in various tissues. However, basal activation of K_{ATP} channels is controversial in smooth muscles. Glibenclamide, a blocker of K_{ATP} channels, caused depolarization of resting membrane in pig urethra, guinea pig tania coli, mouse colon, and some vascular smooth muscles (Teramoto et al., 1997; den Hertog et al., 1989; Koh et al., 1998; Nelson et al., 1990) but K_{ATP} channels are not activated under basal conditions in some vascular smooth muscles (Brayden, 2002). In the present study, pinacidil induced the hyperpolarization of resting membrane potential and abolished the generation of pacemaker potentials, and these effects were blocked by glibenclamide in colonic ICCs, implying that the existence of K_{ATP} channels. In addition, glibenclamide itself elicited depolarization of resting membrane potential and increased the

pacemaker potential frequency in colonic ICCs. From these findings, it suggests that K_{ATP} channels are activating tonically and contribute to maintain the resting membrane potential. Basal activation of K_{ATP} channels are reported in murine colonic smooth muscle cells (Koh et al., 1998). However, in small intestinal ICCs, the resting membrane potential and pacemaker frequency were not changed by glibenclamide, indicating that K_{ATP} channels are not active in resting state. In the present study, it could not know the discrepancy of basal activation of K_{ATP} channels between small intestinal ICCs and colonic ICCs. The functional K_{ATP} channels are composed by the combination of sulfonylurea receptor (SUR) subunits and subunits of inward rectifier K^+ channels of K_{ir} 6 family. Two variants of SUR1 and SUR2 and two isoforms of K_{ir} 6.1 and K_{ir} 6.2 have been identified. SUR2 is classified more with SUR2A and SUR2B (Seino & Miki et al., 2003). It is generally recognized that SUR1/Kir 6.2 is pancreatic β-cell K_{ATP} channels and SUR2A/Kir 6.2 is cardiac K_{ATP} channels. On the other hand, in smooth muscles, two types of K_{ATP} channels were identified, namely SUR2B/K_{ir} 6.1 and SUR2B/K_{ir} 6.2 (Teramoto, 2009). The combination of SUR2B and K_{ir} 6.1 is predominant isoforms of vascular K_{ATP} channels. In murine colonic

smooth muscles, mRNA transcripts of SUR2B and K_{ir} 6.2 are detected and showed basal activation of K_{ATP} channels. In the present study, it represented the different molecular identities of K_{ATP} channels between small intestine and colon. SUR2B and K_{ir} 6.2 mRNA transcripts were detected in small intestinal ICCs, whereas SUR2B and K_{ir} 6.1 mRNA transcripts were detected in colonic ICCs. From these findings, although the cause of difference of basal activation between small intestinal ICCs and colonic ICCs could not know, it seems that the difference of molecular identies of K_{ATP} channels could be a possible explanation the disparity of basal activation of K_{ATP} channels. However it requires the further study to find the accurate causes of this difference.

The activities of K_{ATP} channels are regulated by dual modulations in vascular smooth muscles, namely protein kinase A and protein kinase C. Vasodilator CGRP and adenosine activated K_{ATP} channels via cAMP/protein kinase A-dependent pathway by the activation of adenylate cyclase (Quayle et al., 1994; Kleppisch et al., 1995). In contrast, vasoconstrictor angiotensin II and endothelin inhibited K_{ATP} channels via protein kinase C-dependent pathway by the activation of phospholipase C (Kubo et al., 1997; Park et al., 2005). In the present study, PMA, a

protein kinase C activator, suppressed the pinacidil-induced effects. In addition, with PMA alone depolarized the membrane and increased the pacemaker potential frequency, which showed the mimicked effects with glibenclamide. These results suggest that protein kinase C modulate resting membrane potential of pacemaker activity via regulating K_{ATP} channel activity in colonic ICCs. Similar results are reported in murine colon that PDBu, a protein kinase C activator, inhibited the pinacidil-activated currents, and this inhibition was reduced by protein kinase C inhibitor (Jun et al., 2001). On the other hand, cAMP-dependent activation of K_{ATP} channel in colonic ICCs may be not involves in here, due to increase pacemaker potential frequency by basal cAMP. Colonic pacemaker potential frequencies were decreased by adenylate cyclase inhibitor, and increased by cAMP-specific phosphodiesterase inhibitor (Shahi et al., 2014), suggesting that cAMP-dependent protein kinase A does not involve in activating K_{ATP} channel in colonic ICCs.

K_{ATP} channels are also present in mitochondria that have role in cytoprotection from ischemic reperfusion conditions in heart and involved in proliferation of human airway smooth muscle cells (Gross, 2000; Chen et al., 2014). The mitochondrial K_{ATP} channels are opened by

diazoxide and blocked by 5-hydroxydecanoic acid (Costa & Garlid, 2009). However, in the present study, 5-hydroxydecanoic acid alone did not change the generation of pacemaker potentials, implying that mitochondrial K_{ATP} channels does not involve in regulating pacemaker activity in colonic ICCs.

The activation of K_{ATP} channels decreased intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels in some tissues through inhibition of Ca^{2+} influx via voltage dependent Ca^{2+} channels or the inhibition of Ca^{2+} release from intracellular Ca^{2+} release from intracellular Ca^{2+} store (Brayden 2002; Small et al., 1992). The pacemaking mechanism of ICCs is coupled between IP_3-gated Ca^{2+} release from endoplasmic reticulum and reuptake of Ca^{2+} into mitochondria. In small intestinal ICCs, K_{ATP} channel activation by cromakalim attenuated spontaneous intracellular Ca^{2+} oscillations and this effect was prevented by glibenclamide (Nakayama et al., 2005). In the present study, glibenclamide itself increased spontaneous intracellular Ca^{2+} oscillations in colonic ICCs, suggesting that K_{ATP} channels can modulate intracellular Ca^{2+} oscillations that generate pacemaker potentials.

In conclusion, K_{ATP} channels are exist in colonic ICCs that comprised of

SUR2B and Kir6.1 subunits. These channels may have important role in maintaining resting membrane potential by basal activation. And K_{ATP} channels are modulated by protein kinase C. On the other hand, in small intestinal ICCs, K_{ATP} channels are comprised of SUR2B and Kir6.2 subunits, but not activated under basal condition. Thus, K_{ATP} channels in colonic ICCs are can be therapeutic targets for altered colonic motility disorders.

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