생쥐 소장의 interstitial cells of Cajal에서 기록된 향도잡이 전류에 대한 Ginseng Total Saponin의 효과

The Effects of Ginseng Total Saponin on Pacemaker Currents in Cultured Interstitial Cells of Cajal Isolated from Murine Small Intestine

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국문 초록

생쥐 소장의 interstitial cells of Cajal에서 기록된 향도잡이 전류에 대한 Ginseng Total Saponin의 효과

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인삼(*Panax* ginseng C.A. Meyer)은 오래 전부터 우리나라를 중심으로 중국 및 일본에서 신체 기능을 강화시켜주는 강장제(tonic agent)로서 민속의약으로 혹은 한방 처방의 한 성분으로 동양에서 널리 이용되어 왔다. 현재에는 전 세계적으로 인삼 시장의 확대와 수요가 증가하는 추세에 있다. 최근에 인삼의 여러 성분 중에 인삼 사포닌 (혹은 진세노사이드)이 인삼의 작용을 발휘하는 성분이라는 많은 증거들이 보고되고 있다. 진세노사이드는 화학 구조의 골격에 있어서 dammarane 계통의 화합물과 유사하나 여러 종류의 糖이 side chain에 붙어 있는 관계로 지용성 및 친수성을 모두 가지는 성질을 지닌 것으로 생각되고 있다. 진세노사이드의 종류는 약 30여종이 있는 것으로 알려져 있으며, 당의 부착위치에 따라 주

로 protopanaxadiol (PD) 혹은 protopanaxatriol (PT) 계통의 진세노사이드로 분류된다. 지금까지 진세노사이드는 신경계 또는 비신경계에서 신경전달물질 및 내인성 물질과 같은 기능을 세포수준에서 보고되어 있으며, 특히 이온통로와 수용체에 대한 많은 연구들이 보고되었다.

Interstitial cells of Cajal (ICC)은 위장관 향도잡이 세포로 전기적 현상인 서파를 발생시켜 위장관 평활근의 자발적 수축을 야기시킨다. 서파는 주기적으로 활성화되는 자발적 내향성 전류에 기인하며 향도잡이 전류(pacemaker currents)라 일컫는다.

본 연구는 생쥐 소장에서 배양한 Cajal세포에서 세포막 전압 고정법(whole-cell patch clamp technique)을 사용하여 향도잡이 전류를 기록하고 이 전류에 대한 진세노사이드의 효과를 알아보고자 하였다.

향도잡이 전류는 총 진세노사이드에 의해 막전압의 저분극을 유발하였으며, 향도잡이 전류에 대한 발생빈도의 감소와 더불어 지속적인 내향성 전류가 발생되었다. 이와 더불어 그동안의 연구보고에서 total 진세노사이드는 크게 PD와 PT 계열로 크게 나누어져 있다. 향도잡이 전류에 대한 PD와 PT의 효과는 총 진세노사이드의 효과와 비슷한 결과를 보였으나, 그 효과 정도는 총 진세노사이드보다 적은 효과를 보였고, 단일 진세노사이

드의 처리시 같은 결과를 보여주었다.

이와 같은 결과는 진세노사이드가 ICC를 통하여 소장의 운동성을 조절할 수 있음을 시사하며 그 효과는 단일 진세노사이드가 아닌 전체적인 진세노사이드의 효과의 결과로 사료된다.

중심단어: Interstitial cells of Cajal, Pacemaker currents, Ginseng total saponin

Introduction

Ginseng, the root of *Panax* ginseng C.A. Meyer, is a well-known folk medicine and has been used as a tonic agent. The main molecular components responsible for the actions of ginseng sare ginsenosides, which are also known as ginseng saponins. Ginsenoside is one of the derivatives of triterpenoid dammarane consisting of thirty carbon atoms[1]. Ginsenoside has a four-ring, steroid-like structure with sugar moieties attached, and about 30 different forms have been isolated and identified from the root of *Panax* ginseng.

Several reports demonstrated the action of ginsenosides on ion channel in neuronal or non-neuronal cell. For example, ginsenosides inhibited voltage-dependent Ca^{2+} channels in sensory neurons via pertussis toxin (PTX)-sensitive G protein[2]. Ginsenoside were also found to inhiibit voltage-dependent Ca^{2+} channels in rat chromaffin cells[3]. Ginsenosides attenuate acetylcholine (ACh)-stimulated catecholamine release in bovine chromaffin cells via inhibition of Na^{+} influx through nicotinic receptor-gated cation channels[4, 5].

Ginsenosides have also shown to induce an increase of intracellular Ca^{2+}

in non-neuronal cells such as macrophages or NIH3T3 cells[6,7]. However, relatively little is known about the action of gastrointestinal tract by ginsenosides. Especially, it is still unclear in smooth musle cells how ginsenosides are coupled effector systems to produce second messengers and to exert their final pharmacology or physiological responses.

Interstitial cells of Cajal (ICC) are the pacemaking cells in gastrointestinal (GI) muscles that generate the rhythmic oscillations in membrane potential known as slow waves[8,9]. Slow waves propagate within ICC networks, conduct into smooth muscle cells via gap junctions, and initiate phasic contractions via activation of Ca^{2+} entry through L-type Ca^{2+} channels. Ablation of ICC networks by genetic means[10] or through inactivation of Kit receptors with neutralizing antibodies[11] results in elimination of slow wave activity and alterations in GI motility. The pacemaker mechanism has been shown to involve rhythmic oscillations in intracellular calcium concentration in a compartment near the plasma membrane that controls the open probability of channels responsible for pacemaker currents. This mechanisminvolves Ca^{2+} release from D-myo-inositol 1,4,5-trisphosphate (IP_3) receptor-operated stores and uptake of Ca^{2+} uptake activates voltage-independent, Ca^{2+}-inhibited, nonselective cation channels with a

unitary conductance of 13pS[12]. The molecular species responsible for the pacemaker conductance has not been identified.

Although the mechanism underlying pacemaker activity, and the current responsible for slow waves may be common in different organs of the GI tract, slow waves occur at a wide range of frequencies[13]. Endogenous agents such as neurotransmitters, hormones and paracrine substances can alter slow-wave frequency. Namely, because ICC spontaneously generate the pacemaker currents having activity of channels and also regulate by endogenous agents, I thought that ginsenosides having diverse function on many channels and acting like as endogeneous agents may have action on pacemaker currents in ICC.

In present study, I examined the effects of ginseng total saponins (GTS) and each single ginsenoside on pacemaker currents in ICC isolated from murine small intestine.

Materials and Methods

Materials - Figure 1 shows the structures of the five representative ginsenosides. Ginsenosides and its individual ginsenosides were obtained from Korea Ginseng and Tabacco Research Institute (Taejon, Korea). Ginsenosides used in this study include Rb1 (17.1%), Rb2 (9.07%), Rc (9.65%), Rd (8.26%), Re (9%), Rf (3%), Rg2 (6.4%), Rg2 (4.2%), Rg3 (3.8%), Ro (3.8%), Ra (2,91%), and other minor ginsenosides. Forstock solutions, all drugs were dimethylsulfoxide (DMSO) and stored at -20 °C.

Preparation of cells and tissues - Balb/C mice(8-13 days old) of either sex were anethetized with ether and sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. Luminal contents were washed away with Krebs-Ringer bicarbonate solution and the tissues were pinned to the base of Sylgard dish and the mucosa was removed by sharp dissection. Small tissue stripes of intestinal muscle (contained both circular and longitudinal muscles) were equilibrated for 30 min in Ca^{2+}-free Hanks solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM

glucose, 2.9 mM sucrose and 11 mM HEPES. Then,cells were dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co, Lakewood, NJ, USA) 1.3 mg/ml, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) 2 mg/ml, trypsin inhibitor (Sigma) 2 mg/ml and ATP 0.27 mg/ml. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in 35 mm culture dish. The cells were then cultured at 37 °C in a 95 % O_2-5 % CO_2 incubator in SMGM (smooth muscle growth medium, Clonetics Corp., San Diego, CA, USA) supplemented with 2 % antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5 ng/ml, sigma). Interstitial cells of Cajal (ICCs) were indentified immunologically with a monoclonal antibody for Kit protein (ACK2) labelled with Alexa Fluor 488 (molecular prove, Eugene, OR, USA). Morphologies of ICC are distinct from other cell types in the culture, therefore it was possible to identify the cells with phase contrast microscopy, when the cells were once verified with ACK2-Alexa Fluor 488 labeling.

Patch clamp experiments - The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and potentials (current clamp) in cultured ICC, and Axopatch 1-D (Axon

Instruments, Foster, CA, USA) amplified membrane currents and potentials. Command pulse was applied using IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor and a pen recorder (Gould 2200, Gould, Vally view, OF, USA). The cells were bathed in a solution containing 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$ and 10 mM HEPES adjusted to pH 7.2 with Tris. The pipette solution contained 140 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM Na_2GTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, 0.1 mM EGTA adjusted to pH 7.2 with Tris. Results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments were performed at 30 °C.

Statistical analysis - Data were expressed as means ± standard errors. Differences in the data were evaluated by Student's t test. A P values less than 0.05 were taken as a statistically significant difference. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

Results

Spontaneous inward currents and depolarizations in ICCs

Under a voltage clamp at a holding potential of -70 mV, ICCs showed spontaneous inward currents, which is referred to as pacemaker current (Fig. 2A). The frequency of the pacemaker currents was 14 ± 1.6 cycles/min and the amplitude and resting current level were -420 ± 57 pA and -22 ± 18 pA, respectively (n = 8; bar graph not shown). Converting the amplifier to current clamp mode, spontaneous depolarization was generated in ICCs (Fig. 2B). The spontaneous depolarizations recorded from cultured ICC were similar in waveform to events recorded from intact strips of antral muscle. In the remainder of the experiments, I used a constant holding potential of -70 mV.

Effects of ginseng total saponins (GTS) on pacemaker currents activity in ICC

First, I tested the effect of GTS on pacemaker currents activity in ICC. As shown in figure 3A, the addition of GTS (10 μg/ml) to the bathing solution increased tonic inward currents and decreased frequency of pacemaker currents. Under control conditions, the frequency pacemaker currents was 16 ± 2 (n=8) (Fig. 3B). In the presence of GTS (10 μg/ml), The frequency

pacemaker currents was 2 ± 0.4 (n=8). The amplitude of tonic inward currents generated by GTS was 27 ± 9 (n=8) (Fig. 3C). Thus, these results suggest the possibility that GTS may regulated the frequency and amplitude of pacemaker current in ICC.

Dose-dependent effect of GTS on pacemaker currents in ICC

In dose-dependent experiments with GTS, treatment with GTS (5, 10 and 20 μg/ml) had effects on pacemaker currents in a dose-dependent manner in ICC (Fig. 4). The ED_{50} of GTS action on pacemaker currents in case of the amplitude was 5.48 ± 1.47 μg/ml in ICC (n=4-6).

Effects of Protopanaxdiol (PD) and Protopanaxtriol (PT) on pacemaker currents in ICC

In previous reports, the main molecular components responsible for the action of ginseng are ginsenosides, which are also known as ginseng saponins. Ginsenosides largely subdivide by the position of sugar moieties, Protopanaxdiol (PD) and Protopanaxtriol (PT). In present, I checked the effects of PD and PT on pacemaker currents in ICC and also showed which components have actions on pacemaker currents. In figure 5A, I could see

the effects of PD (10 μg/ml) on pacemaker currents same as GTS action but the inhibitiory intensity was not large than the GTS action. Also, in figure 5B, PT (10 μg/ml) had the inhibitory action on pacemaker currents and the action amplitude was same as the action of PD. These results suggest that PD and PT have inhibitory actions on pacemaker currents same as GTS but this actions is not large than GTS actions on pacemaker currents.

Effects of single Protopanaxdiol (PD) ginsenosides on pacemaker currents in ICC

Protopanaxdiol (PD) constitute with Ra1, Ra2, Ra3, Rb2, Rb2, Rb3, Rc, Rd and so on. In present study, I wanted to show that what kinds of PD ginsenosides have action on pacemaker current in ICC. I tested the PD ginsenosides action, Ra1, Rb1, Rb2, Rc, Rd, on pacemaker currents. Figure 6 showed that, as representative ginsenosides, under control conditions the treatment of Rb1, Rc (100 μM) had slightly inhibitory effects on pacemaker currents. Also, other PD ginsenosides had shown like this (data not shown). The inhibited amplitude and frequency of pacemaker currents by treatments of each ginsenosides showed in figure 7 and 8. These results suggested that single PD ginsenosides have inhibitory effects on pacemaker currents in ICC and PD

action on pacemaker currents may have by co-effects of each single ginsenoside.

Effects of single Protopanaxtriol (PT) ginsenosides on pacemaker currents in ICC

Up to date, single Protopanaxtriol (PT) ginsenosides reported 14 ginsenosides, Re, Rf, Rg1, Rg2 and so on. Especially, PT ginsenosides was reported that the actions of PT ginsenosides on neuronal and non-neuronal cells have diverse effects and this action is like to endogenous agents or neurotransmitters. So, in this study, I checked which single PT ginsenosides have actions on pacemaker currents or not.

As shown in figure 9, the addition of Rf (100 μM) to the bathing solution induced slightly inhibitory effects on pacemaker currents. Rf slightly increased tonic inward currents and decreased frequency of pacemaker currents in ICC. Also, Rg2 (100 μM) showed similar effects like Rf on pacemaker currents. In the presence of Rf and Rg2, the frequency and amplitude of pacemaker currents slightly inhibited (Fig. 10 and 11). This results suggested that nevertheless several reports about the actions of PT on neuronal and non-neuronal cells, single PT ginsenosides had not shown

strong effects on pacemaker currents in ICC.

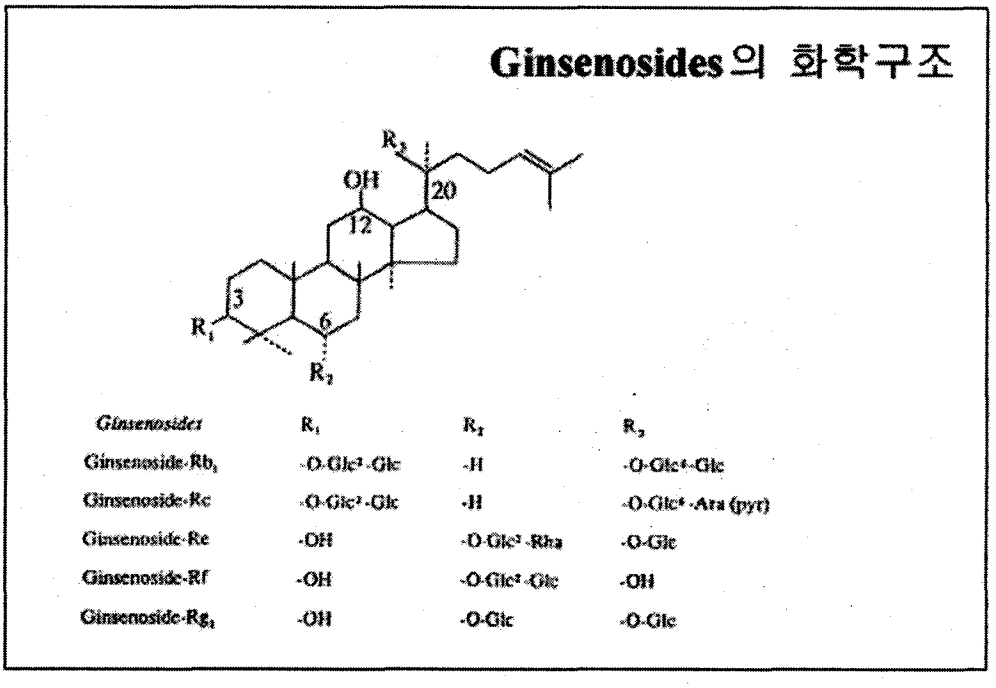

Fig. 1. The structure of ginsenosides

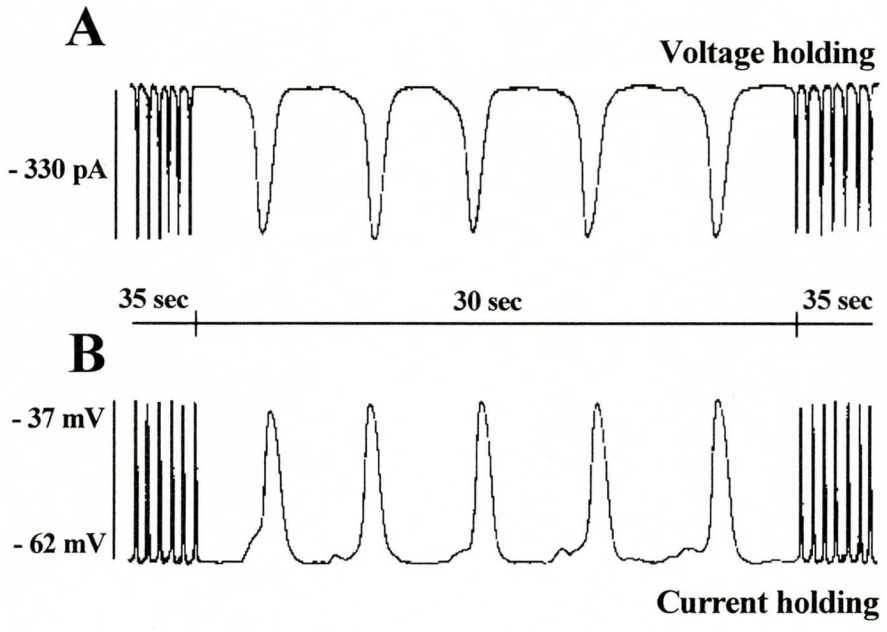

Fig. 2. Spontaneous inward currents and depolarizations in cultured ICCs of the murine small intestine. (A) Under a voltage clamp at a holding potential of -70 mV, ICCs showed spontaneous inward currents oscillations, called pacemaker currents. (B) Under a currents clamp mode, spontaneous depolarization was generated from the same cell.

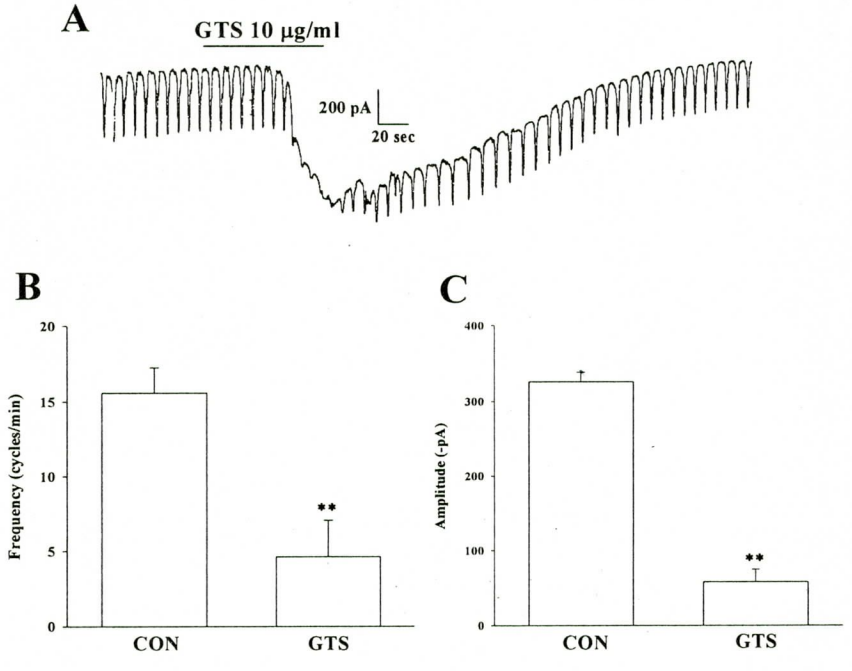

Fig. 3. Effects of GTS on pacemaker currents. Under control conditions at a holding potential of -70 mV, (A) GTS (10 μg/ml) inhibited the amplitude and the frequency of pacemaker currents in ICCs. (B) and (C) summarize the inhibitory effects of pinacidil on pacemaker currents. Each bar represents the mean ± SE. (n = 6/group). Those noted with * were significantly different from the control ($p < 0.05$).

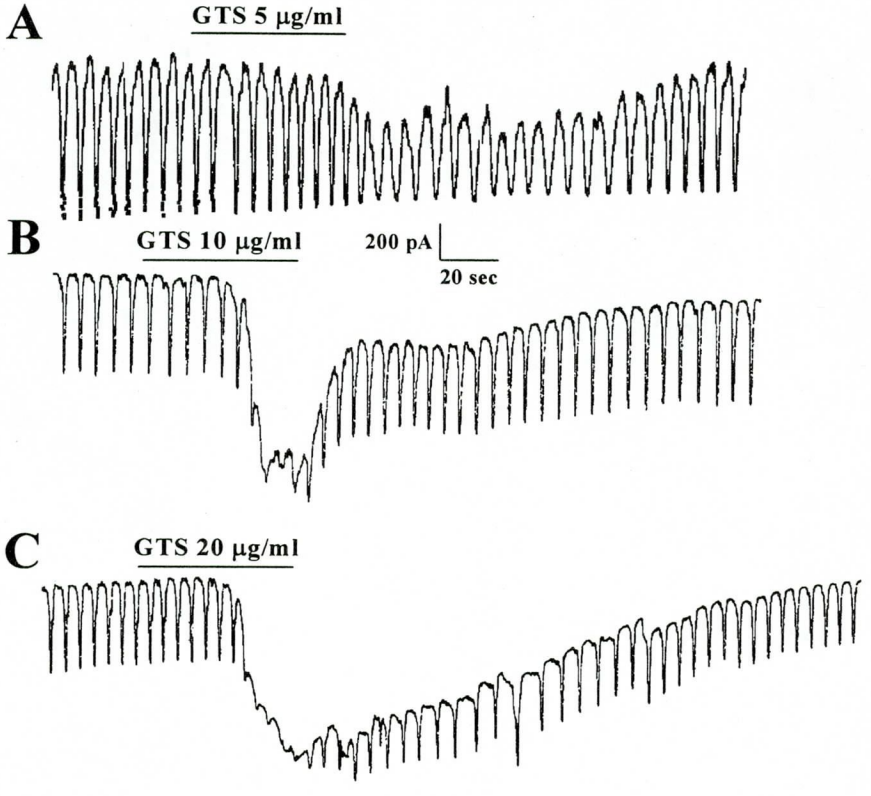

Fig. 4. Dose-dependent effects of GTS on pacemaker currents in cultured ICCs of the murine small intestine. (A), (B) and (C) show the pacemaker currents of ICCs exposed to GTS (5, 10 and 20 μg/ml) at a holding potential of -70 mV.

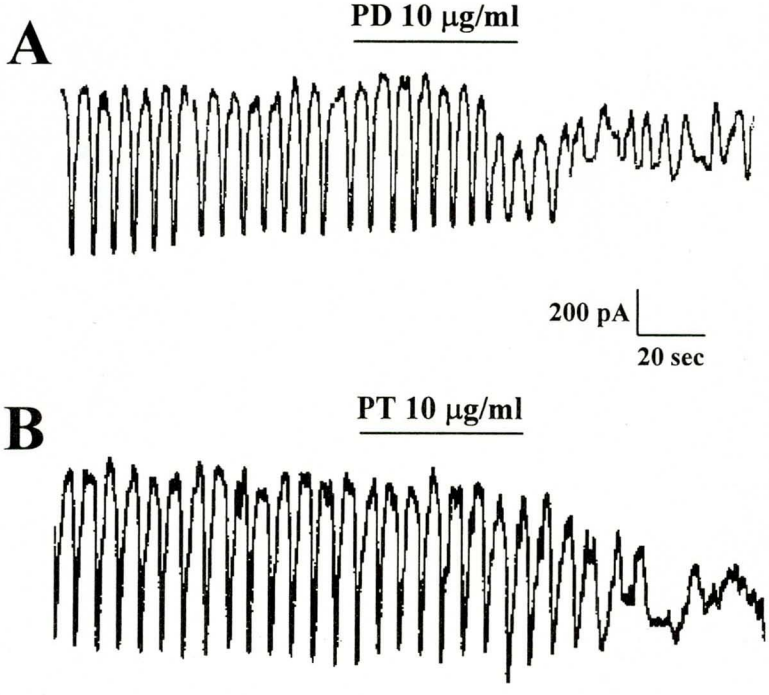

Fig. 5. Effects of PD and PT on pacemaker currents. Under control conditions at a holding potential of -70 mV, (A) PD (10 μg/ml) inhibited the amplitude and the frequency of pacemaker currents in ICCs. Also, (B) PT (10 μg/ml) inhibited the amplitude and the frequency of pacemaker currents in ICCs.

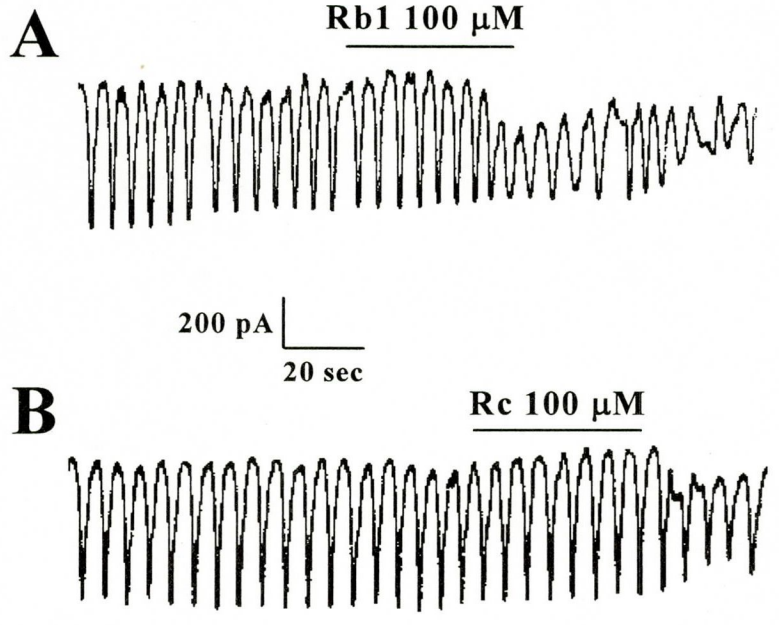

Fig. 6. Effects of Rb1 and Rc on pacemaker currents. Under control conditions at a holding potential of -70 mV, (A) Rb1 (100 μM) inhibited the amplitude and the frequency of pacemaker currents in ICCs. Also, (B) Rc (100 μM) inhibited the amplitude and the frequency of pacemaker currents in ICCs.

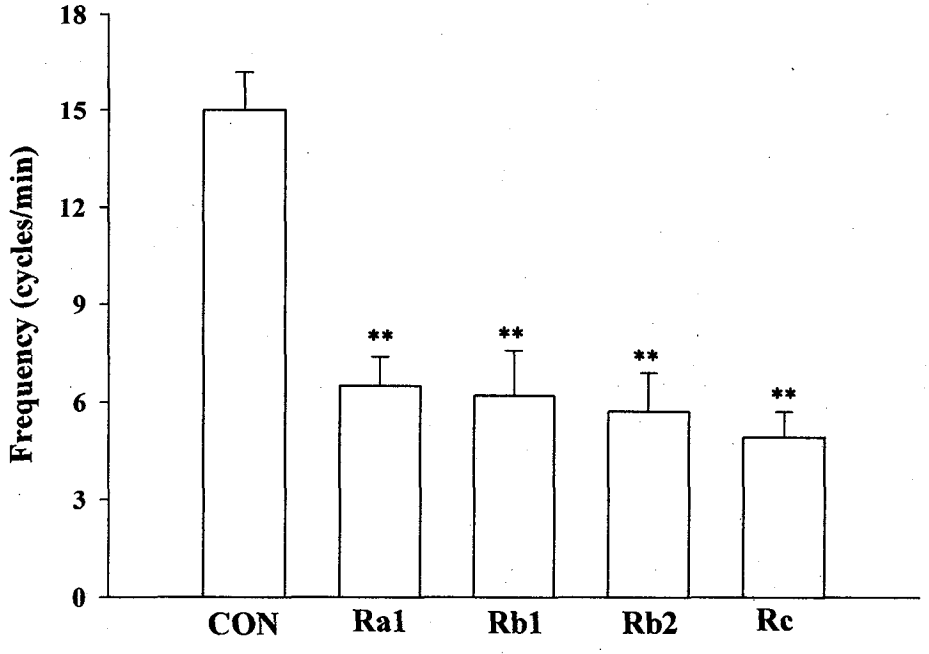

Fig. 7. The effects of Ra1, Rb1, Rb2 and Rc on the frequency of pacemaker currents in cultured ICCs of the murine small intestine. Figure shows the summarized frequency of ICCs exposed to Ra1, Rb1, Rb2 and Rc (100 μM) at a holding potential of −70 mV. Those noted with * were significantly different from the controls ($p < 0.05$).

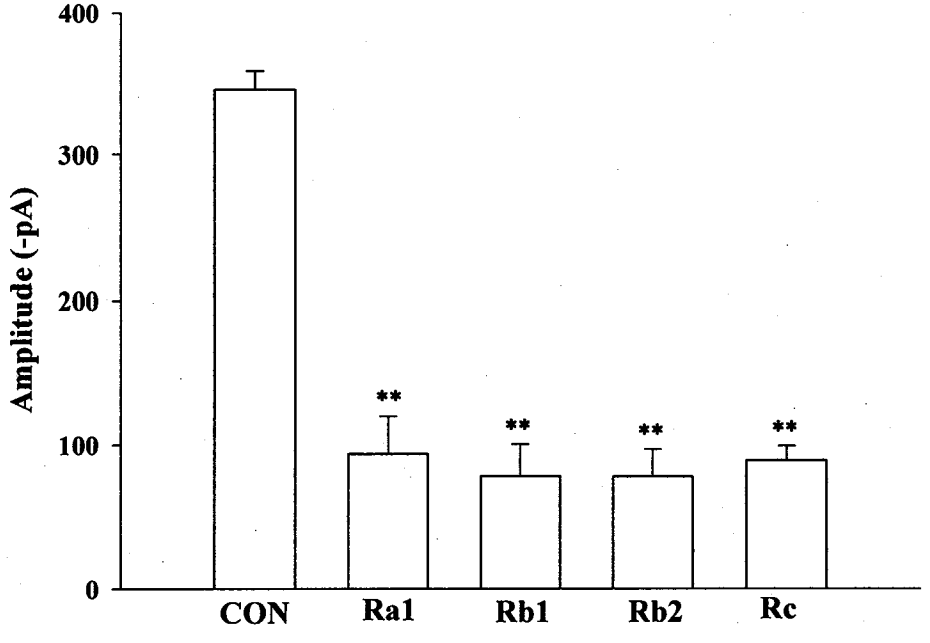

Fig. 8. The effects of Ra1, Rb1, Rb2 and Rc on the amplitude of pacemaker currents in cultured ICCs of the murine small intestine. Figure shows the summarized the amplitude of ICCs exposed to Ra1, Rb1, Rb2 and Rc (100 μM) at a holding potential of -70 mV. Those noted with * were significantly different from the controls ($p < 0.05$).

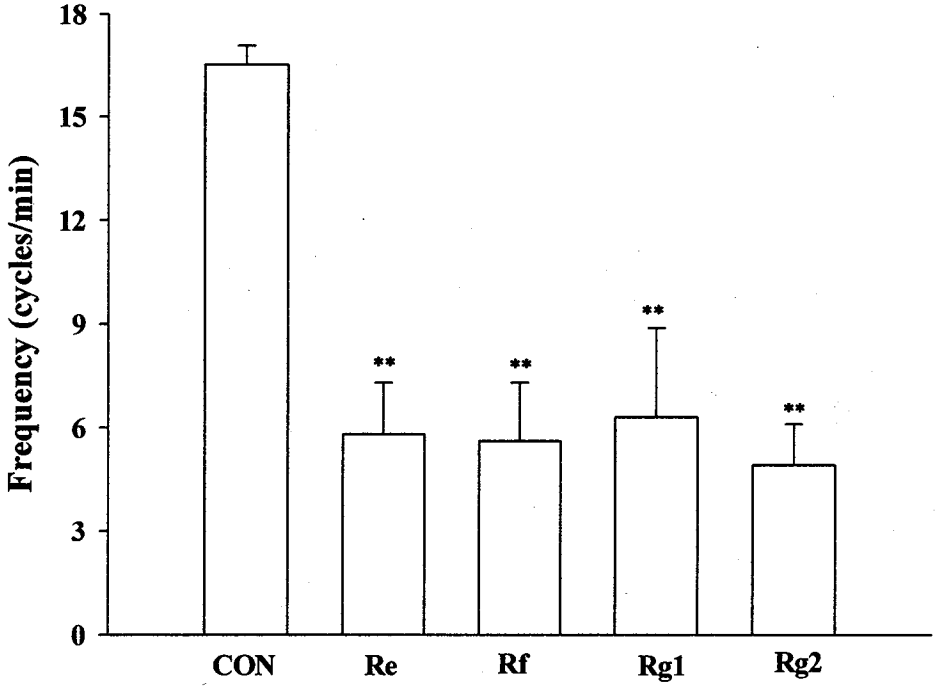

Fig. 10. The effects of Re, Rf, Rg1 and Rg2 on the frequency of pacemaker currents in cultured ICCs of the murine small intestine. Figure shows the summarized frequency of ICCs exposed to Re, Rf, Rg1 and Rg2 (100 μM) at a holding potential of −70 mV. Those noted with * were significantly different from the controls ($p < 0.05$).

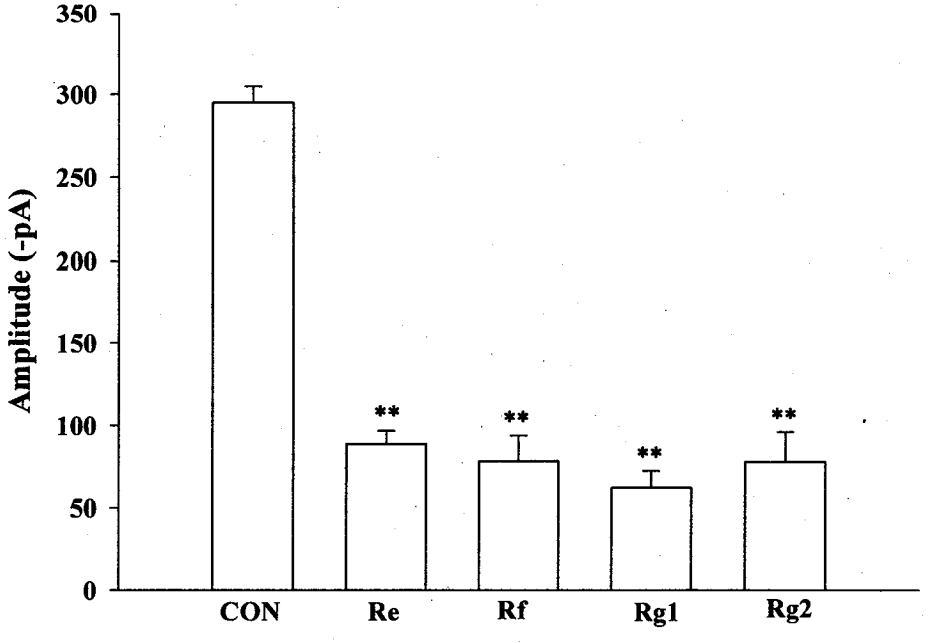

Fig. 11. The effects of Re, Rf, Rg1 and Rg2 on the amplitude of pacemaker currents in cultured ICCs of the murine small intestine. Figure shows the summarized the amplitude of ICCs exposed to Re, Rf, Rg1 and Rg2 (100 μM) at a holding potential of −70 mV. Those noted with * were significantly different from the controls ($p < 0.05$).

Discussion

Ginseng is usually mild and subtle in its efficacy compared to other medicines. Ginsenosides are known to represent a variety of physiological or pharmacological effects of ginseng in non-neuronal cells[1]. However, nevertheless the diverse function of ginsenosides, the action on gastrointestinal tract are not well studies. Here, I demonstrate that ginsenosides have effects on motility of small intestine through modulation of ICC in cellular basis. In addition to, I further showed the co-modulation of ginsenosides on pacemaker currents in ICC not single one.

ICCs generate spontaneous pacemaker inward currents that depolarize membrane, this spread to smooth muscle via gap junctions resulting in depolarization of membrane in smooth muscle leads to contraction by generating acting potential through voltage dependent Ca^{2+} channel activation. It has been suggested that pacemaker currents of ICCs are mediated by the activation of voltage-independent nonselective cation channels[14,15]. And pacemaker currents of ICCs are regulated by endogenous agents or neurotransmitters. Namely, because actions of ginsenosides on diverse channels and functions as like neurotransmitters of ginsenosides, I

thought that ginsenosides may have functions on pacemaker currents in ICCs. In this study, I could see that GTS have effects on pacemaker currents in ICCs. GTS depolarized memebrane in ICCs by increasing inward currents suggesting GTS regulates intestinal motility indirectly via acting on ICCs. Similarly cholinergic stimulation produced same responses in gastric ICCs of murine. This means that GTS also may have action on pacemaker currents like as neurotransmitters in small intestine. Also, the effects on GTS on pacemaker currents was shown dose-dependent manner. Namely, GTS may regulate the channels or receptors in ICC.

Using purified compounds of ginsenosides, it has been reported that ginsenoside Rb1 protected hippocampal neurons against ischemia[16]. It has been also shown that ginsenosides Rb1 and Rg3 protected cultured cortical neurons from glutamate-induced neurotoxicity[17]. Recently, Liao *et al*[18] also reported that ginsenosides Rb1 and Rg1 protect spinal neurons from excitotoxicity induced by glutamte or KA. That is individual ginsenosides have different effects in cellular levels. So in this study, I first tested that the effect PD and PT on pacemaker currents in ICC. PD and PT all have effects on pacemaker currents in ICC but the intensity of effects not more potent than that of GTS. In generally, PD have been reported to have an effect on

cultured cancer cells by reducing cell proliferation, blocking cell cycle, inducing apoptosis and PT reported an effect on neuronal cells by regulating channels and receptors. I thought that because the action of PT on channels and receptors, PT may have influence on pacemaker currents in ICC. But, in ICC from small intestine, PD and PT showed the same effects on pacemaker currents. Namely, the action of GTS on pacemaker currents in ICC may co-working of PD and PT ginsenosides.

Especially, individual ginsenoside each alone have been shown to reported many action on channels and receptor. In present study, I found each individual ginsenosides showed similar action on pacemaker currents in ICC. The effects of single ginsenoside generated tonic inward currents. The generation of pacemaker currents is initiated by the release of Ca^{2+} from endoplasmic reticulum. Cyclopiazonic acid, a Ca^{2+} ATPase inhibitor in endoplasmic reticulum or xestospongin C, an inhibitor of inositol (1,4,5)-triphosphate (IP_3) receptor in endoplasmic reticulum abolished the generation of pacemaker currents whereas ryanodine, a Ca^{2+}-induced Ca^{2+} release blocker in endoplasmic reticulum, did not affect on generating pacemaker currents. These mean suggesting IP_3-mediated Ca^{2+} release from endoplasmic reticulum is essential for generation pacemaker currents. In

lately, Nah *et al*[19] reported that GTS utilize a well-known signal pathway of the G protein coupled PLC activation and IP_3 mediated intracellular Ca^{2+} release to activate Ca^{2+}-activated Cl^- channels in native *Xenopus* oocytes. This means that the action of ginsenosides on pacemaker currents in ICC may influence by intracellular Ca^{2+} mobilization and this effects show by co-working all ginsenosides not alone ginsenoside.

In conclusion, this study provides evidence that ginsenosides may have possibility of action on pacemaker current in ICC of murine small intestine. Ginsenosides depolarized the membrane with increased inward pacemaker currents via Ca^{2+} mobilization. Thus, ginsenosides may play a very important role in the regulating intestinal smooth muscle by acting on ICC indirectly.

Summary

Several reports demonstrated the diverse action of ginseng total saponins (GTS) at the level of many tissues or single cell. Especially, many ginsenosides showed several effects in neuronal and non-neuronal systems.

The functional study was investigated to determine whether ginseng total saponins (GTS) modulates pacemaker currents generated in interstitial cells of Cajal (ICC) of murine small intestine by using a whole cell patch clamp techniques at 30 °C.

ICC generated spontaneous inward currents (pacemaker currents) at a -70 mV of holding potential. Under control conditions, the frequency pacemaker currents was 16 ± 2 (n=8). In the presence of GTS (10 μg/ml), The frequency pacemaker currents was 2 ± 0.4 (n=8). The amplitude of tonic inward currents generated by GTS was 27 ± 9 (n=8). These effects of GTS showed dose dependent manner and reversible. GTS largely divided by the position of sugar in structure, Protopanaxdiol (PD) and Protopanaxtiol (PT). I further checked the effects of PD and PT on pacemaker currents in ICC. When treatment of PD and PT (100 μg/ml) in ICC, pacemaker currents also

generated the tonic inward inward currents and inhibited the frequency same as GTS. But the action of PD and PT on pacemaker currents in ICC not potent than that of GTS.

Many reports suggested that ginsenosides alone have large and different effects on cellular levels. In present study, individual PD, Rb1, Rb2, Rc (100 μM) had slightly inhibitory effects on pacemaker currents. Also, other PD ginsenosides had shown like this (data not shown). The addition of Rf, Rg2 and Re (PT ginsenosides, 100 μM) to the bathing solution induced slightly inhibitory effects on pacemaker currents. Rf slightly increased tonic inward currents and decreased frequency of pacemaker currents in ICC.

Our result demonstrate that GTS may modulate intestinal motility acting on ICC by co-effects of total ginsenosides not single ginsenosides via the Ca^{2+} mobilization.

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