Effects of Samchulkunbi-tang in Cultured Interstitial Cells of Cajal of Murine Small Intestine

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We studied the modulation of pacemaker activities by Samchulkunbi-tang (SCKB) in cultured interstitial cells of Cajal (ICC) from murine small intestine with the whole-cell patch-clamp technique. Externally applied SCKB produced membrane depolarization in the current-clamp mode. The pretreatment with Ca^{2+}-free solution and thapsigargin, a Ca^{2+}-ATPase inhibitor in endoplasmic reticulum, abolished the generation of pacemaker potentials and suppressed the SCKB-induced action. The application of flufenamic acid (a nonselective cation channel blocker) abolished the generation of pacemaker potentials by SCKB. However, the application of niflumic acid (a chloride channel blocker) did not inhibit the generation of pacemaker potentials by SCKB. In addition, the membrane depolarizations were inhibited by not only GDP-β-S, which permanently binds G-binding proteins, but also U-73122, an active phospholipase C inhibitor. These results suggest that SCKB modulates the pacemaker activities by nonselective cation channels and external Ca^{2+} influx and internal Ca^{2+} release via G-protein and phospholipase C-dependent mechanism. Therefore, the ICC are targets for SCKB and their interaction can affect intestinal motility.

Key words : Samchulkunbi-tang, Interstitial Cells of Cajal, whole-cell patch-clamp technique

Introduction

Samchulkunbi-tang (SCKB) is composed of Ginseng Radix, Atractylodis Rhizoma, Hoelen, Magnoliae Cortex, Citri Pericarpium, Crataegi Fructus, Ponciri Fructus, Paeoniae Radix, Amomi Semen, Massa Medicata Fermentata, Hordei Fructus Germinatus, Glycyrrhizae Radix, Zingiberis Rhizoma, and Zizyphi Fructus[1]. It is a traditional herbal medicine widely used for the treatment of chronic gastritis, gastric ulcers, gastroptosis, indigestion, diarrhea, and emesis[2]. However it is little known about the effects on how SCKB works in gastrointestinal (GI) motility.

Many regions of the tunica muscularis of the gastrointestinal tract display spontaneous contractions. These are mediated by the periodic generation of electrical slow waves[3]. Recent studies have shown that the interstitial cells of Cajal (ICCs) act as the pacemakers and conductors of the electrical slow waves in gastrointestinal smooth muscles[4-8]. However there is no evidence how traditional herbal medicine modulate gastrointestinal tract motility by influencing the ICCs.

In this study, therefore, we investigated the possibility that SCKB affects the electrical properties of cultured ICC cells.

Materials and Methods

1. Preparation of cells and cell cultures

Balb/c mice (3-7 days old) of either sex were anesthetized with ether and killed 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 removed by washing with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa removed by sharp dissection. Small tissue strips of the intestine muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution (containing in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min. Then, the cells were dispersed using 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 cover slips coated

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with murine collagen (2.5 μg/mL, Falcon/BD, Franklin Lakes, NJ, USA) in a 35-mm culture dish and then cultured at 37℃ in a 95% O_2, 50 mL/L CO_2 incubator in a smooth muscle growth medium (Clonetics Corp., SanDiego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (SCF, 5ng/mL, Sigma). ICCs were identified immunologically with anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, SanDiego, CA, USA) at a dilution of 1:50 for 20min[9]. ICCs were morphologically distinct from other cell types in the culture and thus it was possible to identify the cells by phase contrast microscopy once they had been verified with anti c-kit antibody.

2. Patch-clamp experiments

The whole-cell patch-clamp configuration was used to record membrane potentials (current clamp) from cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, USA) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). Data obtained were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and using a pen recorder (Gould 2200, Gould, Valley View, OH, USA). Results were analyzed using pClamp and Origin (version6.0) software. All experiments were performed at 30-32℃.

Table 1. Amount and Composition of SCKB

Herb	Scientific Name	Amount (g)
In Sam	*Ginseng Radix*	0.65
Baek Chul	*Atractylodis Rhizoma*	1
Baek Bok Ryung	*Hoelen*	0.05
Hu Bak	*Magnoliae Cortex*	0.22
Jin Pi	*Citri Pericarpium*	0.86
San Sa Yuk	*Crataegi Fructus*	0.95
Ji Sil	*Ponciri Fructus*	0.58
Baek Jak Yak	*Paeoniae Radix*	0.54
Sa In	*Amomi Semen*	0.16
Sin Kok	*Massa Medicata Fermentata*	0.65
Maek A	*Hordei Fructus Germinatus*	0.33
Kam Cho	*Glycyrrhizae Radix*	0.35
Saeng Kang	*Zingiberis Rhizoma*	0.26
Dae Chu	*Zizyphi Fructus*	4.15
Total amount		10.75g

3. Solutions and drugs

The physiological salt solution used to bathe cells (Na^+-Tyrode) contained (mmol/L) : KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH7.4 with NaOH. The pipette solution contained (mmol/L): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH7.2 with KOH. SCKB was purchased from I-WORLD Pharmaceuticals (South Korea). SCKB is composed of Ginseng Radix, Atractylodis Rhizoma, Hoelen, Magnoliae Cortex, Citri Pericarpium, Crataegi Fructus, Ponciri Fructus, Paeoniae Radix, Amomi Semen, Massa Medicata Fermentata, Hordei Fructus Germinatus, Glycyrrhizae Radix, Zingiberis Rhizoma, and Zizyphi Fructus(Table 1). The dosage for adult is 10–15 g (crude materials) per day. More information about SCKB can be found in I-WORLD Pharmaceuticals Homepage (http://i-pharm.koreasme.com). The SCKB was dissolved with distilled water at the concentration of 0.5 g (crude drug)/ml and stored in refrigerator. All other drugs were obtained from Sigma (Sigma Chemical Co., USA). Drugs were dissolved in distilled water, and added to bath solution to make the desired concentrations, just prior to use. Addition of these chemicals to bath solution did not alter the pH of the solution. Thapsigargin, U-73122, and U-73343 were dissolved in dimethyl sulfoxide (DMSO) for 50 or 100 mmol/L stock solution and added (1000 times dilution) to the bathing solution at the day of the experiment. The final concentration of DMSO in the bath solution was always <0.1%, and we confirmed that this concentration of DMSO did not affect the results that were recorded.

4. Statistics

All data are expressed as mean ± S.E. Student's t-test for unpaired data was used to compare control and experimental groups. The P value of less than 0.05 was considered statistically significant.

Results

1. Effect of SCKB on pacemaker potentials in cultured ICCs

Isolated ICC, which express c-kit receptor tyrosine kinase[10], were identified with the Kit immunofluoescence (Fig. 1). Kit-positive cells had a distinctive morphology that was easily recognized in cultures. Recording from cultured ICCs under current clamp mode (I = 0) showed spontaneous pacemaker potentials. The resting membrane potential was -52 ± 3 mV and the amplitude was 21 ± 3 mV. In the presence of SCKB (1-50 mg/ml), the membrane potentials were depolarized to 0 ± 0.2 mV at 1 mg/ml, 15.6 ± 1.2 mV at 10 mg/ml, 21.3 ± 1.5 mV at 30 mg/ml, and 24.3 ± 0.5 mV at 50 mg/ml (n = 4 ; Fig. 2). The summarized values and bar graph of the SCKB effects on pacemaker potentials are indicated in Fig. 2E. Because 30 mg/ml SCKB was shown the good and safe effects, we decided to apply 30 mg/ml SCKB in cultured ICCs.

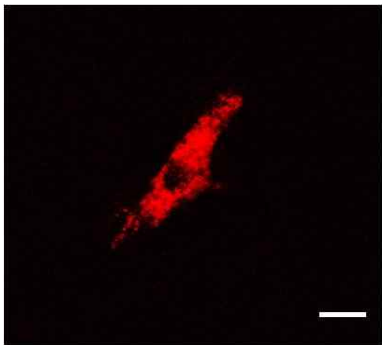

Fig. 1. Cultured interstitial cells of Cajal of the murine small intestine. We identified the cells as ICC with c-kit immunoreactivity in a single cultured ICC grown for 1 day. Scale bars : 10 m

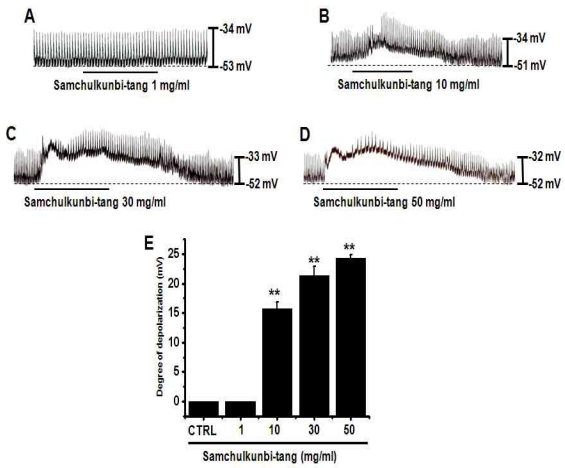

Fig. 2. Effects of SCKB on pacemaker potentials in cultured ICCs from murine small intestine. It shows the pacemaker potentials of the ICC exposed to SCKB (1-50 mg/ml) in the current clamping mode (I = 0) (A-D). Responses to SCKB are summarized in (E). Bars represent mean values ± SE. **(P < 0.01) Significantly different from the untreated control. CTRL, Control.

2. Effects of external Ca^{2+}-free solution and Ca^{2+}-ATPase inhibitor of endoplasmic reticulum in SCKB-induced pacemaker potentials in cultured ICCs

The external Ca^{2+} influx is necessary for GI smooth muscle contractions and is essential for generating pacemaker potentials in the ICCs. The generation of pacemaker potentials was dependent upon intracellular Ca^{2+} oscillation[11]. To investigate the role of external Ca^{2+} or internal Ca^{2+}, SCKB was tested under external Ca^{2+}-free conditions and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum[12]. The pacemaker potentials were completely abolished by an external Ca^{2+}-free solution. In this condition, SCKB-induced membrane depolarizations were blocked to 2 ± 0.5 mV at 30 mg/ml (n = 5; Fig. 3A). Under external Ca^{2+}-free conditions, the value of membrane depolarizations (2 ± 0.5 mV) with SCKB (30 mg/ml) was significantly different when compared with SCKB (30 mg/ml) in normal Ca^{2+} solution (21.2 ± 0.6 mV) (Fig. 3C). In addition, SCKB-induced membrane depolarizations were inhibited to 1 ± 0.3 mV by pretreatment of thapsigargin (Fig. 3B). In the presence of thapsigargin (5 μM), the value of membrane depolarizations (1 ± 0.3 mV) with SCKB was significantly different when compared to SCKB in the absence of thapsigargin (21.2 ± 0.6 mV) (n = 4, Fig. 3C).

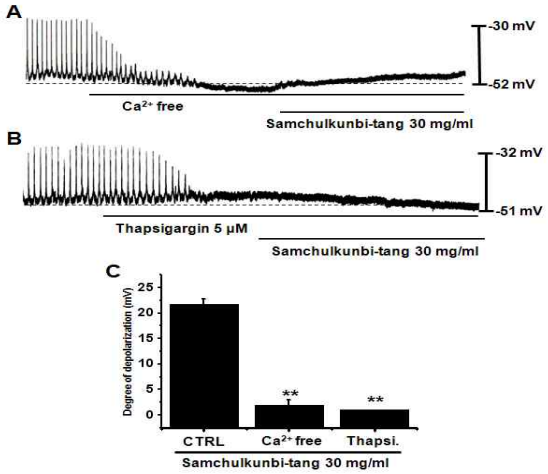

Fig. 3. Effects of an external Ca^{2+}-free solution or thapsigargin (a Ca^{2+}-ATPase inhibitor in endoplasmic reticulum) on SCKB-induced pacemaker potentials in cultured ICCs (A) External Ca^{2+}-free solution abolished the generation of pacemaker potentials, and inhibited SCKB-induced (30 mg/ml) membrane depolarization. (B) Thapsigargin (5 μM) abolished the generation of pacemaker potentials, and blocked SCKB-induced (30 mg/ml) membrane depolarization. (C) Responses to SCKB in external Ca^{2+}-free solution or in the presence of thapsigargin are summarized in C. Bars represent mean values ± SE. **(P < 0.01) Significantly different from untreated controls. CTRL: Control.

3. Effects of non-selective cation channel blocker or Cl- channel blocker in SCKB-induced pacemaker potentials in cultured ICCs

To determine the characteristics of the membrane depolarizations produced by SCKB, flufenamic acid (a non-selective cation channel blocker)[13] or niflumic acid (a Cl^- channel blocker)[14] were tested. In the presence of flufenamic acid (5 μM), the pacemaker potentials were abolished and then the application of SCKB (30 mg/ml) did not produce membrane depolarizations (1 ± 0.2 mV) (Fig. 4A). The value of membrane depolarizations (1 ± 0.2 mV) produced by SCKB in the presence of flufenamic acid was significantly different when compared to the control condition (21.6 ± 0.6 mV) (n = 4; Fig. 4C). In the presence of the application of niflumic acid (5 μM), the pacemaker potentials also were abolished. In this condition, SCKB still produced membrane depolarizations to 19.2 ± 1.1 mV at 30 mg/ml (Fig. 4B). In the presence of niflumic acid, the value of membrane depolarizations (19.2 ± 1.1 mV) produced by SCKB were not significantly different when compared with control values obtained in the absence of niflumic acid (21.6 ± 0.6 mV) (n = 4; Fig. 4C).

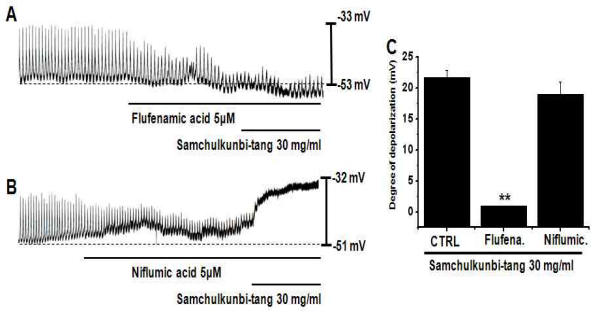

Fig. 4. Effects of flufenamic acid (a nonselective cation channel blocker) or niflumic acid (a Cl^- channel blocker) on SCKB-induced pacemaker potentials in cultured ICCs from murine small intestine. (A) The application of flufenamic acid (5 μM) abolished the generation of the pacemaker potentials, and in the presence of flufenamic acid, SCKB (30 mg/ml) did not cause membrane depolarization. (B) On the other hand, though niflumic acid (5 μM) abolished the generation of pacemaker potentials, it did not block SCKB-induced (30 mg/ml) membrane depolarization. (C) Responses to SCKB in the presence of flufenamic acid or niflumic acid are summarized in C. Bars represent mean values ± SE. **(P < 0.01) Significantly different from untreated controls. CTRL: Control.

4. Involvement of G proteins on SCKB pacemaker potentials in cultured ICCs

The effects of GDP-β-S, a non-hydrolysable guanosine 5′-diphosphate analogue which permanently inactivates G protein binding proteins[15], were examined to determine whether the G protein is involved in the effects of SCKB in the ICC. When GDP-β-S (1 mM) was in the pipette, SCKB (30 mg/ml) did not show membrane depolarizations (Fig. 5A). In the presence of GDP-β-S in the pipette, the membrane depolarizations were 8.1 ± 1.4 mV (n = 4; Fig. 5B).

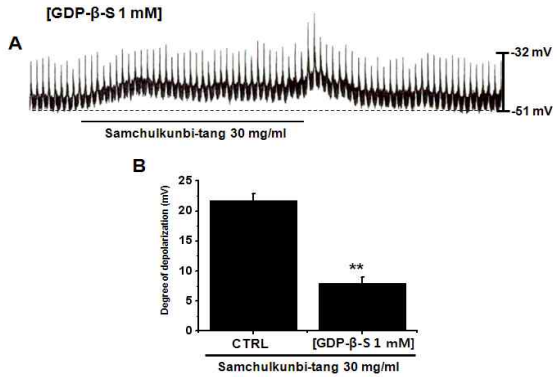

Fig. 5. Effects of GDP-β-S in the pipette on SCKB-induced pacemaker potentials in cultured ICCs of murine small intestine. A: Pacemaker potentials of ICCs exposed to SCKB (30 mg/ml) in the presence of GDP-β-S (1 mM) in the pipette. Under these conditions, SCKB (30 mg/ml) did not cause membrane depolarization. (B) Responses to SCKB in the presence of GDP-β-S in the pipette are summarized in B. Bars represent mean values ± SE. **(P < 0.01) Significantly different from untreated controls. CTRL: Control.

5. Effects of phospholipase C inhibitor on SCKB-induced pacemaker potentials in cultured ICCs

Since the membrane depolarizations by SCKB was related to intracellular Ca^{2+} mobilization, we examined whether the effects on pacemaker potentials require PLC activation. To test this possibility, SCKB-induced membrane depolarizations were measured in the absence and presence of U-73122, an active PLC inhibitor[16]. The pacemaker potentials were completely abolished by application of U-73122 (5 μM) and under these conditions, SCKB-induced (30 mg/ml) membrane depolarizations were suppressed (n = 4; Fig. 6A). In the presence of U-73122, the membrane depolarizations produced by SCKB were 7.6 ± 1.5 mV. The value of the membrane depolarizations (7.6 ± 1.5 mV) by SCKB was significantly different when compared with SCKB in the absence of U-73122 (21.6 ± 0.6 mV) (n = 4, Fig. 6C). The treatment of U-73343 (5 μM), an inactive analog of U-73122, had no influence on the SCKB-induced pacemaker potentials and under these conditions, SCKB-induced (30 mg/ml) membrane depolarizations were not suppressed by U-73343 to 21.6 ± 0.8 mV (Fig. 6B and C).

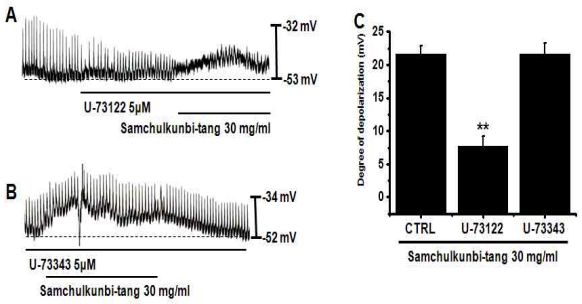

Fig. 6. Effects of SCKB on phospholipase C inhibitors in cultured ICCs (A) U-73122 (5 μM; a phospholipase C inhibitor) abolished the generation of pacemaker potentials, and blocked SCKB-induced (30 mg/ml) membrane depolarization. (B) The application of U-73343 (5 μM) did not influence the generation of pacemaker potentials or block SCKB-induced (30 mg/ml) membrane depolarization. (C) Responses to SCKB in the presence of phospholipase C inhibitors are summarized in C. Bars represent mean values ± SE. **(P < 0.01) Significantly different from untreated controls. CTRL: Control.

Discussion

ICCs generate spontaneous pacemaker currents that depolarize membrane, which then spreads to smooth muscle via gap junctions and results in the depolarization of smooth muscle cell membrane. This membrane depolarization leads to smooth muscle contraction by generating an action potential via the activation of voltage dependent Ca^{2+} channels. It has been suggested that the pacemaker currents of interstitial cells of Cajal are mediated by the activation of voltage-independent nonselective cation channels or chloride channels[17,18], allowing net inward current predominantly by Na^+ under physiological condition and leading to excitatory action in gastrointestinal smooth muscles.

To date, pharmacological studies of SCKB have been

confined to its roles in gastroprotection and immune regulation[2]. In previous studies showed that hesperidin and glycyrrhizin have anti-oxidant[19], anti-inflammatory[20], and immunomodulatory effects[21]. Liquiritin has been shown to possess the neuroprotective and anti-depressant activities[22]. Albiflorin and paeoniflorin, the major active constituents of Paeoniae Radix, has been reported to exhibit anti-coagulant[23], neuromuscular blocking[24], immunoregulatory[25] and anti-hyperglycemic effects[26]. Additionally, poncirin and naringin, the major active compounds of Ponciri Fructus, have been found to have anti-Helicobacter pylori[27], anti-inflammatory[28], and anti-atherogenic effects[29].

In this study, SCKB produced membrane depolarization in the current-clamp mode. Pretreatment with a Ca^{2+}-free solution and a thapsigargin, a Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum, abolished the generation of pacemaker potentials. In Ca^{2+}-free solution conditions, SCKB did not show the membrane depolarizations. Also, the application of flufenamic acid (a nonselective cation channel blocker) abolished the generation of pacemaker potentials by SCKB. However, the application of niflumic acid (a chloride channel blocker) did not inhibit the generation of pacemaker potentials by SCKB. Furthermore, the pacemaker membrane depolarizations were inhibited by U-73122, an active phospholipase C (PLC) inhibitor. These results suggest that SCKB might affect GI motility by the modulation of pacemaker activity in the ICC, and the activation is associated with nonselective cation channel, PLC activation, and Ca^{2+} release from external or internal storage and chloride channel-independent manner.

Taken together, our data suggest that the gastroprokinetic effects of SCKB might be mediated by the induction of pacemaker potentials in the ICC. Considering the effects of this drug on the ICC, further research, including finding active compound(s) and examining their action mechanisms, are clearly needed.

Acknowledgements

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