Effect of the ginsenoside Rb1 on the spontaneous contraction of intestinal smooth muscle in mice

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Abstract

AIM: To investigate the effect and the possible mechanism of ginsenoside Rb1 on small intestinal smooth muscle motility in mice.

METHODS: Intestinal smooth muscle strips were isolated from male ICR mice (5 wk old), and the effect of ginsenoside Rb1 on spontaneous contraction was recorded with an electrophysiological method. The effect of ginsenoside Rb1 on ion channel currents, including the voltage-gated K⁺ channel current (IKV), calcium-activated potassium channel currents (IKCa), spontaneous transient outward currents and ATP-sensitive potassium channel current (IKATP), was recorded on freshly isolated single cells using the whole-cell patch clamp technique.

RESULTS: Ginsenoside Rb1 dose-dependently inhibited the spontaneous contraction of intestinal smooth muscle by 21.15% ± 3.31%, 42.03% ± 8.23% and 67.23% ± 5.63% at concentrations of 25 μmol/L, 50 μmol/L and 100 μmol/L, respectively (n = 5, P < 0.05). The inhibitory effect of ginsenoside Rb1 on spontaneous contraction was significantly but incompletely blocked by 10 mmol/L tetraethylammonium or 0.5 mmol/L 4-aminopyridine, respectively (n = 5, P < 0.05). However, the inhibitory effect of ginsenoside Rb1 on spontaneous contraction was not affected by 10 μmol/L glibenclamide or 0.4 μmol/L tetrodotoxin. At the cell level, ginsenoside Rb1 increased outward potassium currents, and IKV was enhanced from 1137.71 ± 171.62 pA to 1449.73 ± 162.39 pA by 50 μmol/L Rb1 at +60 mV (n = 6, P < 0.05). Ginsenoside Rb1 increased IKCa and enhanced the amplitudes of spontaneous transient outward currents from 582.77 ± 179.09 mV to 788.12 ± 278.34 mV (n = 5, P < 0.05). However, ginsenoside Rb1 (50 μmol/L) had no significant effect on IKATP (n = 3, P < 0.05).

CONCLUSION: These results suggest that ginsenoside Rb1 has an inhibitory effect on the spontaneous contraction of mouse intestinal smooth muscle mediated by the activation of IKV and IKCa, but the IKATP channel was not involved in this effect.

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Key words: Ginsenoside Rb1; Intestinal smooth muscle; Intestinal smooth muscle cell; Potassium channel; Spontaneous contraction; Whole-cell patch clamp technique

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INTRODUCTION

Sijunzi decoction (SJZD) is one of the most famous and widely used traditional prescriptions. This prescription contains four common herbs, including Panax ginseng, Poria cocos, Atractylodes macrocephala and Glycyrrhiza uralensis, and it has been used either alone to replenish or invigorate intestinal and stomach function or as a complement to other herbs during treatment of other diseases, such as poor health and cancer. Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), is a principal component of SJZD. Ginsenoside, a component of ginseng, has a four-ring steroid-like structure with attached sugar moieties. Recently, ginseng’s chemical and pharmacological properties have been reported by many investigators. Approximately 30 ginsenosides have been isolated and identified from the Panax ginseng root. These ginsenosides appear to be responsible for most of the pharmacological effects of ginseng. Many reports have shown that ginseng saponins, or ginsenosides, have various effects on gastrointestinal motility. Ginsenosides modulate the pacemaker activities of the interstitial cells of Cajal (ICCs), making the ICCs targets for ginsenosides, and their interaction can affect intestinal motility. The aqueous extract of Ginseng Radix possesses ameliorative properties and improves carbachol-induced accelerated small intestinal transit, and Rb1 contributed to the suppressive effects of Ginseng Radix on intestinal motility. Rb1 is one representative of the compounds contained in Ginseng Radix that is capable of ameliorating the accelerated transit of the small intestine. However, the mechanism of Rb1 modulation of gastrointestinal motility has not been clearly demonstrated. Based on the studies cited above, it could be deduced that ICCs and gastrointestinal smooth muscle cells might be targets for Rb1. In this study, we attempted to determine the effect of ginsenoside Rb1 on the motility of intestinal smooth muscle and determine its mechanism.

MATERIALS AND METHODS

Preparation of intestinal smooth muscle and isometric measurement

Five-week-old male ICR mice (provided by the Experimental Animal Centre of the Chinese Academy of Sciences, Shanghai) weighing approximately 30 g were sacrificed by cervical dislocation. The small intestines were excised by cervical dislocation. The small intestines were excised, Shanghai) weighing approximately 30 g were sacrificed by cervical dislocation. The small intestines were excised, and the mucosal and submucosal layers, single circular muscle bundles with the attached longitudinal muscle layer were removed and kept in Krebs solution. After removing the mucosal and submucosal layers, single circular muscle bundles with the attached longitudinal muscle layer were removed and kept in Krebs solution. Approximately 2 mm × 6 mm muscle strips were fixed in a vertical chamber (5-mL capacity containing 5 mL CO₂/bicarbonate-buffered Krebs solution bubbled with 5% CO₂/95% O₂). The chamber was maintained at 37 °C using a water jacket. One end of the chamber was attached to an isometric force transducer (RM6240C, Chengdu Instrument Factory, China) to record the contraction. The muscle strip was incubated at the appropriate tension.

Cell preparation and electrophysiological recording

Intestinal smooth muscle cells were freshly isolated from mice. The intestine was rapidly cut, and the mucosal layer was separated from the muscle layers in a Ca²⁺-free physiological salt solution (Ca²⁺-free PSS). The circular muscle layer was dissected from the longitudinal layer using fine scissors and was cut into small segments (2 mm × 3 mm). These segments were incubated in a medium modified from Kraft-Bruhe (K-B) solution for 30 min at 4 °C. The segments were subsequently incubated for 10-12 min at 36 °C in Ca²⁺-free PSS digestion medium containing collagenase (0.5 mg/mL, Worthington), DTI [0.5 mg/mL, Sigma Aldrich (St. Louis, MO, United States)], papain [1.5 mg/mL, Sigma Aldrich (St. Louis, MO, United States)] and bovine serum albumin (4 mg/mL, Biotech Grade). After digestion, the supernatant was discarded, and the softened muscle segments were transferred into the modified K-B solution. The single cells were dispersed by gentle trituration using a wide-bore fire-polished glass pipette. The isolated intestinal smooth muscle cells were incubated in a modified K-B solution at 4 °C until use on the same day. Several drops of the cell suspension were dropped into a perfusion bath, which was fixed on the stage of an inverted phase-contrast microscope for 15-20 min before the experiments. Next, the cells were perfused with PSS at a rate of 1-1.5 mL/min. A single 4-channel perfusion system (BPS-4, ALA, United States) was used to exchange the solution.

A conventional whole-cell patch clamp configuration was used to record the K⁺ATP current (IK⁺ATP), the spontaneous transient outward currents (STOC) and the voltage-gated K⁺ channel current (IKVT). To record IK⁺ATP, the membrane potential was clamped at -60 mV. The pipette solution consisted of the following (mmol/L): KCl 107, KOH 33, Hepes 10, MgCl₂ 1, Na₂ATP 0.1, NaADP 0.1, and GTP 0.3, adjusted to a pH of 7.2 with NaOH. To observe the effect of Rb1 on IKVT, we applied a depolarising step pulse to the cells, and the membrane potential was clamped at -60 mV. The pipettes were filled with solution containing the following (mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, disodium creatine phosphate 2.5, MgATP 5, Hepes 5, MgCl₂ 1.0, and EGTA 10, adjusted to a pH of 7.3 with KOH. To record STOC, the holding potential was clamped at -20 mV. The pipettes were filled with a solution containing the following (mmol/L): KCl 140, MgCl₂ 5, K₃ATP 2.7, NaGTP 0.1, disodium salt 2.5, Hepes 5, and EGTA 0.1, adjusted to a pH of 7.2 with Tris. The patch pipettes were pulled from borosilicate glass capillaries using a pipette puller (PC-10, Narishige Group, Japan). The current was amplified with an EPC-10 patch clamp.
clamp amplifier (HEKA Instruments, Germany) and digitised with a PCI-16 A/D converter (HEKA Instrument). All pipettes had a resistance of 3-5 MΩ [7,9,10].

All experimental protocols included in this manuscript were approved by the local animal care committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of the PRC (STCC Publication No. 2, revised 1988).

Solutions and drugs

Chemicals used included Ginsenoside Rb1 (purchased from Sichuan Weiqi Biological Technology CO., Ltd.), Gilbenclamide [a K_{ATP} channel blocker, purchased from Tocris (Ellisville, Missouri, United States)], tetrodylammonium (TEA, a non-selective potassium channel blocker), 4-aminopyridine (4-AP, a voltage-gated K⁺ channel blocker), and tetrodotoxin (TTX, a blocker of voltage-dependent Na⁺ channels) purchased from Sigma Aldrich (St. Louis, MO, United States). Ginsenoside Rb1 was dissolved first in dimethyl sulphoxide (DMSO) at a concentration of 200 mmol. For the intestinal smooth muscle isometric measurements, all chemicals were further diluted with Krebs solution to prepare the desired concentrations before use. In the electrophysiological recording experiment, Ginsenoside Rb1 was diluted with PSS to the final concentration immediately before use.

The ionic composition of the Krebs solution was as follows (in mmol/L): Na⁺ 137.4, K⁺ 5.9, Ca²⁺ 2.5, Mg²⁺ 1.2, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134, and glucose 11.5. The solution was aerated with O₂ containing 5% CO₂, and the pH was maintained at 7.2-7.3. The composition of Kraft-Bruhe (K-B) solution was as follows (in mmol/L): EGTA 0.5, Hepes 10, MgCl₂ 5, KCl 50, glucose 10, KH₂PO₄ 20, Taurine 20, and L-Glutamic acid 50, adjusted to a pH of 7.4 with KOH. The composition of Ca²⁺-free PSS was as follows (in mmol/L): NaCl 134.8, KCl 10, MgCl₂ 1, and glucose 10, adjusted to a pH of 7.4 with Tris. The composition of PSS was as follows (in mmol/L): NaCl 134.8, KCl 10, MgCl₂ 1, glucose 10, and CaCl₂ 2, adjusted to pH 7.4 with Tris. The pipette solution for recording the K_{ATP} channel current contained the following (mmol/L): KCl 107, KOH 33, Hepes 10, MgCl₂ 1, Na₂ATP 0.1, NaADP 0.1, and GTP 0.3, adjusted to a pH of 7.2 with NaOH. The pipettes were filled with a solution for IKᵥ containing the following (in mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, disodium-creatine phosphate 2.5, MgATP.

Figure 1  Effect of Rb1 on spontaneous contraction of intestinal smooth muscle. A: The representative effects of Rb1 on spontaneous contraction of intestinal smooth muscle in a dose-dependent manner; B, C: The amplitude of spontaneous contraction was decreased after Rb1 administration, but the frequency was unaffected. Values are expressed as means ± SE. n = 5, *P < 0.05 vs control group.
RESULTS

Effect of Rb1 on the spontaneous contraction of intestinal smooth muscle

In this study, Rb1 exhibited an inhibitory effect on the spontaneous contraction of intestinal smooth muscle strips in a dose-dependent manner (Figure 1). Rb1 suppressed spontaneous contraction by 21.15% ± 3.31%,
Xu L et al. Rb1 and spontaneous contraction of ISM

![Summary of effect of Rb1 on spontaneous contraction.](image)

**Figure 3** Summary in effect of Rb1 on spontaneous contraction of normal intestinal smooth muscles and those pretreated with TEA (10 μmol/L). 4-AP (0.5 μmol/L), Glibenclamide (10 μmol/L) and TTX (0.4 μmol/L) respectively. Values are expressed as means ± SE; n = 5, *P < 0.05 vs control group; *P < 0.05 TEA + Rb1 or 4-AP + Rb1 vs Rb1 group. TEA: Tetraethylammonium; 4-AP: 4-aminopyridine; TTX: Tetrodotoxin.

42.03% ± 8.23% and 67.23% ± 5.63% (Figure 1B, n = 5, P < 0.05) at concentrations of 25 μmol/L, 50 μmol/L, and 100 μmol/L, respectively. Rb1-induced inhibition of spontaneous contraction appeared to decrease the amplitude of spontaneous contractions (Figure 1B), but the frequency was not changed (Figure 1C).

The Rb1-induced inhibitory effect on spontaneous contractions was almost completely abolished by 10 mmol/L TEA (a non-selective potassium channel blocker) and 0.5 mmol/L 4-AP (Figure 2B, C). The inhibitory percentage of Rb1 decreased from 42.03% ± 8.23% to 9.17% ± 3.54%, and the inhibitory percentage decreased from 10.90% ± 5.19% with TEA and 4-AP, respectively (Figure 3, n = 5, P < 0.05). After pre-treatment with 0.4 μmol/L TTX and 10 μmol/L glibenclamide, the inhibitory effect of Rb1 on spontaneous contraction was stable (Figure 2D, E). The inhibition percentages of Rb1 were 42.03% ± 8.23%, 46.12% ± 5.66% and 47.16% ± 3.99% in the control, TTX and glibenclamide groups, respectively (Figure 3, n = 5, P > 0.05).

**Effect of Rb1 on voltage-gated K+ channel current of intestinal smooth muscle cells**

Previous experiments demonstrated that both TEA, a non-specific potassium channel blocker, and 4-AP, a specific delayed potassium channel blocker, significantly suppressed the inhibitory effect of Rb1 on the spontaneous contraction of intestinal smooth muscle strips. These results indicate that Rb1-induced inhibition might be mediated by calcium-activated potassium channels and delayed repolarisation of the potassium channel. The effect of Rb1 on the IKv in intestinal smooth cells was observed in succession using the conventional whole-cell patch clamp technique. IKv was elicited by a step voltage command pulse from -40 mV to +100 mV at 20-mV increments for 400 ms at 10 s intervals. The membrane potential was clamped at -60 mV. Rb1 significantly increased IKv elicited by the step voltage command pulse (Figure 4A). Furthermore, based on the I-V relation curve, Rb1 increased IKv at all command potentials from +20 mV to +100 mV (Figure 4B). The IKv at +60 mV increased from 1137.71 ± 171.62 pA to 1449.73 ± 162.39 pA, which represented 132.11% ± 7.77% of the level in the control concentration (100%) of 50 μmol/L Rb1 (Figure 4C, n = 6, P < 0.05).

**Effect of Rb1 on the Ca2+-sensitive K+ channel current of intestinal smooth muscle cells**

IKCa is activated by intracellular Ca2+ and can be monitored by spontaneous transient outward currents (STOCs). STOCs are believed to represent the spontaneous, sporadic release of Ca2+ from storage sites in the cell in relation to Ca2+-sensitive K+ channels[8,11]. In this study, we observed that Rb1 (50 μmol/L) enhanced the amplitude of STOCs from 582.77 ± 179.09 nA to 788.12 ± 278.34 nA, which represented a 137.76% ± 11.95% increase from the control level (100%) (Figure 5A, C, n = 5, P < 0.05) without changing the frequency.

**DISCUSSION**

Sijunzi decoction (SJZD) is widely used as a regular decoction in Chinese Traditional Medicine that can invigorate Pi viscera and replenish Qi. Conventionally, SJZD is useful for treating hypofunction of the spleen, a symptom that is partially equivalent to that of gastrointestinal motility disorders (e.g., abdominal distension and dyspepsia). The mechanism by which SJZD improves gastrointestinal disorder symptoms may relate to gastrointestinal hormones and motility. SJZD could correct deficiencies of the spleen and stomach, which are caused by digestive dysfunction to some extent[4]. Symptoms of rat models with Pi-deficiency could be significantly corrected to the normal level by SJZD treatment[4]. External nutrition plus SJZD treatment can improve and optimise cellular immune function and nutritional status in post-operative gastric cancer patients[12]. Recently, the major active components of SJZD, including ginsenoside, flavonoid, and triterpenoid, have been identified using LC/MS/MS[13]. Kim et al[4] reported that ginsenosides modulate the pase-
maker activities of the ICCs. The ICCs can be targets for ginsenosides, and their interaction can affect intestinal motility. The ICCs and smooth muscle cells (SMCs) are coupled electrically, forming a multicellular syncytium. Activation of depolarising or hyperpolarizing ionic conductances in either cell type affects the total input resistance and excitability of the syncytium. For example, activation of K\textsuperscript{+} channels in ICCs reduces excitability of coupled SMCs and reduces the likelihood of reaching the action potential threshold. Responses to other stimuli, such as hormones and paracrine substances, are likely to target both ICCs and SMCs, depending upon the expression of appropriate receptors and second-messenger pathways\textsuperscript{[15]}.

Hashimoto \textit{et al}\textsuperscript{[16]} reported that Rb1 was one representative of the compounds contained in Ginseng Radix that were capable of ameliorating the accelerated transit of the small intestine. To date, the mechanism of ginsenoside action on gastrointestinal (GI) smooth muscle has not been fully studied.

In this study, we found that ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscles in mice by decreasing the amplitude of spontaneous contractions in a dose-dependent manner (Figure 1). The presence of TEA (10 mmol), a non-selective potassium channel blocker, partially blocked the inhibitory effect of Rb1 on spontaneous contraction (Figure 2B). This finding suggested that the inhibitory effect of ginsenoside Rb1 on the spontaneous contraction of intestinal smooth muscle in mice might be associated with K\textsuperscript{+} channels; importantly, at least 20 species of potassium channel types are expressed by SMCs of the GI tract\textsuperscript{[15,17]}. These species include voltage-gated K\textsuperscript{+} channels, ATP-dependent K\textsuperscript{+} channels, and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels. We evaluated 4-AP, a voltage-gated K\textsuperscript{+} channel blocker, which partially blocked the inhibitory effect of Rb1 on spontaneous contraction (Figure 2C). In contrast, glibenclamide, an ATP-dependent K\textsuperscript{+} channel blocker, did not influence the inhibitory effect of Rb1 on spontaneous contraction (Figure 2E). In addition, the presence of TTX, a blocker of voltage-dependent Na\textsuperscript{+} channels that can block enteric nerves, did not affect the inhibitory effect of Rb1 on spontaneous contraction (Figure 2D). Thus, the results indicated that the inhibitory effect of Rb1 on spontaneous contraction was associated with activation of K\textsuperscript{+} channels in intestinal smooth muscle cells. A conventional whole-cell patch clamp configuration showed that Rb1 activated IK\textsubscript{V} and IK\textsubscript{Ca} (Figures 4, 5) without any influence on IK\textsubscript{ATP} (Figure 5B, D). We concluded that Rb1 inhibited the spontaneous contraction of intestinal smooth muscles via increased Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channel currents and voltage-dependent K\textsuperscript{+} channel currents. However, enteric nerves and K\textsubscript{ATP} channels were not involved in this process. Next, to determine the Rb1-

\begin{figure}[h]
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\caption{Effect of Rb1 on the voltage-gated K\textsuperscript{+} channel current of intestinal smooth muscle cell in mouse. A: The raw trace; B: The I-V relation curve; C: Summary in effect of Rb1 (50 \textmu mol/L) on the voltage-gated K\textsuperscript{+} channel current at +60 mV. Values are expressed as means ± SE. \textit{n} = 6, \textit{P} < 0.05 vs control group.}
\end{figure}
induced inhibitory effect on the spontaneous contraction of intestinal smooth muscle, the effect of Rb1 on the slow wave contraction of intestinal smooth muscle was observed. However, the amplitude and frequency of slow wave contraction was not affected by Rb1 (50 μmol/L, 100 μmol/L or 200 μmol/L, data not shown). The results indicated that the inhibitory effect of Rb1 on spontaneous contraction relies on the direct action of the compound with smooth muscle and not the ICCs themselves.

The broad ranges of resting potentials and electrical patterns of GI muscles are partly a function of the variable expression of K+ channels in SMCs. At least 20 species of K+ channels are expressed by SMCs in the GI tract[15]. The activation of potassium channels is the main determinant of cell membrane potential. Therefore, potassium channels participate in the regulation of smooth muscle tone. Activation of K+ channels in the cell membrane allows K+ efflux, causing a decrease in membrane potential and hyperpolarization. As a consequence, voltage-gated calcium channels in the cell membrane close, and the smooth muscle relaxes[18].

It has been reported that ginsenosides, including Rb1, regulate Ca2+ channels in chromaffin cells[19], sensory neurons[20] and ventricular myocytes[21]. Rb1 can alleviate cardiac hypertrophy in vitro, mediated by an inhibitive effect on elevated [Ca2+]i[18]. The ginsenoside Rb1 suppressed ventricular myocyte shortening and intracellular Ca2+ in isolated cardiac myocytes[20]. These results indicate that the primary physiological or pharmacological targets of ginsenosides are Ca2+ channels. Li et al[23] reported that ginsenosides increased IKca activity in endothelial cells. The modulation of IKca activity stimulated by ginsenosides was inhibited by 0.5 mmol TEA but not by 0.5 mmol glibenclamide. In our study, we first discovered that potassium channels, especially the Ca2+-dependent K+ channels and voltage-dependent K+ channels, were involved with the effects of Rb1 on the spontaneous contraction of intestinal smooth muscles in mice. This result is partially in accordance with the report of the action of ginsenosides by Li et al[23] and Kang et al[24].

In conclusion, ginsenoside Rb1 exerted an inhibitory effect on the spontaneous contraction of intestinal smooth muscles in mice by decreasing the amplitude of spontaneous contractions in a dose-dependent manner. The inhibitory effect of Rb1 is mediated by potentiating IKv and IKca channel currents.

Figure 5  Effect of Rb1 on spontaneous transient outward currents and ATP sensitive potassium channel current of intestinal smooth muscle cell in mouse (n = 5). A: The raw trace of Rb1-induced effect on STOC of intestinal smooth muscle cell; B: The raw trace of Rb1-induced effect on IKATP of intestinal smooth muscle cell; C: Relative current evoked by Rb1 (50 μmol/L) on STOC, n = 5, P < 0.05 vs control group; D: Relative current evoked by Rb1 (50 μmol/L) on IKATP comparing with control group. Values are expressed as means ± SE, n = 3. IKATP: ATP sensitive potassium channel current; STOC: Spontaneous transient outward currents.
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