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[Original Article]

THE INHIBITORY EFFECT OF SHAKUYAKUKANZOTO ON K⁺ CURRENT IN H9c2 CELLS

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Abstract : Shakuyakukanzoto (shao-yao-gan-cao-tang) is a commonly used Chinese traditional herbal medicine for the treatment of acute pain with muscle cramp. However, its mechanism of action is unclear. We previously reported that a low concentration of Kanzo (licorice) and isoliquiritigenin, a component of licorice, inhibited the potassium (K⁺) current in H9c2 cells. Therefore, in the present study, we examined the effects of Shakuyakukanzoto, Shakuyaku or Kanzo on the K⁺ current (I_{Kur}) in H9c2 cells. Shakuyakukanzoto inhibited I_{Kur} in a concentration-dependent manner. The half-maximal concentration of Shakuyakukanzoto was approximately 1.3 mg/mL and the Hill coefficient was 1.2. The order of potency of inhibiting I_{Kur} was Kanzo > Shakuyakukanzoto > Shakuyaku. Glycyrrhizin, a major component of licorice, had no inhibitory effect on I_{Kur}. A small interfering RNA experiment indicated that I_{Kur} was most likely to be Kv2.1 in H9c2 cells. Our results suggest that Shakuyakukanzoto may normalize intracellular and extracellular K⁺ balance by inhibiting I_{Kur} and reducing K⁺ efflux, while the Na⁺-K⁺ pump promotes K⁺ influx into myofibers. Consequently, excess K⁺ may be reduced from external space of myofibers. This may be a part of the Shakuyakukanzoto mechanism for improving muscle pain.

Key words : K⁺ channel, Kv2.1, Shakuyakukanzoto, H9c2 cell, whole-cell clamp

INTRODUCTION

Shakuyakukanzoto is a popular Chinese traditional herbal medicine used for the treatment of acute pain associated with muscle cramp and disc herniation^{1,2)}. Patients with hemodialysis often experience severe leg cramps, and Shakuyakukanzoto is a drug of choice for these patients³⁻⁵⁾. More recently, Shakuyakukanzoto has been clinically recognized as being effective for myalgia and neuralgia caused by anticancer drugs including paclitaxel⁶⁻⁹⁾.

Using rat diaphragm, Kimura *et al.*¹⁰⁾ reported that either paeoniflorin, a main component of Shakuyaku (paeoniae radix), or glycyrrhizin, a main component of Kanzo (licorice), had only a weak

effect of muscle relaxation. However, the combination of the two components had a strong effect of muscle relaxation¹⁰⁾, although its mechanism of action is not clear.

Muscle cramp frequently develops under abnormal serum K⁺ concentrations^{4,11)} during exercise-induced dehydration¹²⁾ and hemodialysis^{3,4)}. These indicate the possible involvement of intra- and extracellular K⁺ concentration change in muscle cramps. Since Shakuyakukanzoto has an immediate relieving effect on muscle cramp, its mechanism of action might be on ion channels.

H9c2 cells are originated from rat embryonic cardiac ventricles¹³⁾. However, since they can be differentiated not only into cardiac myocytes but

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also into skeletal myofibers, they have been used as a skeletal muscle cell model. H9c2 cells have an ultrarapid delayed rectifier K⁺ current (IKur)¹⁴, whose gene was reported as Kv2.1¹⁵. Kv2.1 is expressed in neurons, cardiac muscle, smooth muscle, and skeletal muscle^{16,17}.

Shakuyakukanzoto consists of two herbal components, i.e. 50% Shakuyaku and 50% Kanzo. To clarify the mechanism of action of Shakuyakukanzoto, we examined the effects of Shakuyakukanzoto and Shakuyaku on IKur in H9c2 cells and compared them with those of Kanzo, on which we reported previously¹⁸.

MATERIALS AND METHODS

The detailed method has been described in previous papers from this laboratory^{14,18}.

Cell culture

H9c2 cells (Dai Nippon Seiyaku, Osaka) were maintained in Dulbecco's modified Eagle's medium (Wako, Osaka), containing penicillin G (Banyu Pharmaceutical, Tokyo), and streptomycin (Meiji Seika, Tokyo) supplemented with 10% fetal bovine serum in a humidified atmosphere of 10% CO₂ and 90% air at 37°C. Cells were used after 4–11 days of subculture. For electrophysiological recordings, cells were separated from the culture dish using a Ca²⁺-free solution containing 0.02% trypsin and then placed in a recording chamber.

Whole-cell patch-clamp recording

Membrane currents were recorded by the whole-cell patch clamp using an inverted microscope (Model 80121; Nikon, Tokyo) and a patch-clamp amplifier (TM-1000; Act ME, Tokyo). Current signals were stored online and analyzed using pClamp Version 9 (Axon Instruments, Union City, CA, USA).

Solutions

The pipette solution contained 100 mM KOH, 30 mM KCl, 3 mM MgCl₂, 5 mM MgATP, 10 mM BAPTA (1,2-bis[2-aminophenoxy]-ethane-N, N, N', N'-tetraacetic acid), 10 mM HEPES (4-[2-hydroxyethyl]-1-piperazine-ethanesulphonic acid), and 50 mM aspartic acid (adjusted to pH 7.2 with aspartic acid). The external Tyrode solution contained 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 0.33 mM NaH₂PO₄, 5 mM HEPES, and 5.5 mM glucose (pH 7.4 with NaOH).

Determination of membrane capacitance

The membrane capacitance of the H9c2 cells was measured at the beginning and at the end of each experiment. If the membrane capacitance at the end was changed by more than 10% of the initial value, the data were excluded. The average membrane capacitance of the H9c2 cells used in this study was 120 ± 16 pF (*n* = 30).

RNA interference

To confirm that IKur in H9c2 cells was Kv2.1, we transfected small interfering RNA (siRNA) to H9c2 cells to knockdown Kv2.1. After 48–72 hours from the transfection, currents were recorded from the cells. Transfection was repeated three times.

Reverse-transcription polymerase chain reaction (RT-PCR)

To identify the expression of Kv2.1 mRNA, the total RNA was extracted from rat skeletal muscle and from H9c2 cells using the acid guanidium thiocyanate phenol-chloroform method¹⁹. The details of this method are the same as those described previously by Suzuki *et al.*¹⁴.

Drugs

Shakuyakukanzoto (the powder composed of 50% paeoniae radix and 50% licorice), Shakuyaku (paeoniae radix, root of *Paeoniae lactiflora* Pallas, Ranunculaceae), and Kanzo (glycyrrhizae radix, root and stolon of *Glycyrrhizae uralensis* fisher, Fabaceae) were purchased from Tsumura Co. (Tokyo). Each powder was dissolved in Tyrode solution, filtrated and the supernatant was frozen until use. The supernatant was diluted with Tyrode solution to obtain the desired concentration. Glycyrrhizin (SIGMA-Aldrich Japan K.K., Tokyo) was also dissolved in Tyrode solution.

Data analyses

All the values were presented as mean ± standard deviation. The percent inhibition of the current was measured at the peak of the current at 60 mV at various concentrations of Shakuyakukanzoto. The median inhibitory concentration (IC₅₀) and the Hill coefficient values were obtained using Origin version 6.1 (OriginLab, Northampton, USA). The concentration-response curve was fitted using the following equation :

$$I = I_{\min} + (I_{\max} - I_{\min}) / [1 + ([SKT] / IC_{50})^{nH}]$$

In this equation, [SKT] indicates Shakuyaku-

kanzoto concentration, IC_{50} is the median inhibitory concentration of Shakuyakukanzoto, and nH is an empirical parameter describing the steepness of the fit, which has the same meaning as the Hill coefficient. Statistical significance between the two groups was evaluated using Student's *t*-test after the *F*-test. *P*-value < 0.05 was considered statistically significant.

RESULTS

K⁺ current of H9c2 cells

In H9c2 cells, an ultra-rapidly activating slowly inactivating delayed rectifier K^+ current (IK_{ur}) was elicited by a series of depolarizing square voltage pulses with 200-ms duration from the holding potential (HP) of -60 mV to a voltage range between -40 and 100 mV (Fig. 1A). The pulse was given every 10 s. The current was measured at the peak and just before the end of a 200-ms pulse at each voltage, and a current-voltage relationship was plotted (Fig. 1B). Figs. 1A and B are typical examples of the currents and their I-V curves obtained from an H9c2 cell.

Effects of Shakuyakukanzoto on K^+ current

If we employed the protocol in Fig. 1 with 15 voltage pulses, it would take time and the current

run down may occur. Therefore, we examined the effects of Shakuyakukanzoto by repeating a single voltage pulse to shorten the time of the study. A square voltage pulse was given every 10 s from the HP of -60 mV to 60 mV with 200-ms duration. After recording a control current, we perfused 10 mg/ml Shakuyakukanzoto in the external solution, and the typical data are shown in Fig. 2. Shakuyakukanzoto at 10 mg/ml suppressed IK_{ur} to 25.0% of the control. In three different cells, 10 mg/ml Shakuyakukanzoto suppressed the current to $28.2 \pm 3.1\%$ of the control. As shown in Fig. 2 (A-D), various concentrations of Shakuyakukanzoto (0.3 – 10 mg/ml) were applied, and a concentration-response curve was plotted (Fig. 3). Shakuyakukanzoto inhibited IK_{ur} in a concentration-dependent manner. The half-maximal concentration (IC_{50}) of Shakuyakukanzoto was approximately 1.3 mg/ml and the Hill coefficient was 1.2 .

Effects of Shakuyaku on K^+ current

Shakuyakukanzoto contains 50% Shakuyaku and 50% Kanzoto. Therefore, we examined the effect of Shakuyaku alone on the K^+ current. Shakuyaku was also perfused in Tyrode solution. Typical data are shown in Fig. 4. Shakuyaku at 10 mg/ml suppressed the peak current to $69.6 \pm 8.0\%$ ($n=3$) of the control (Fig. 3).

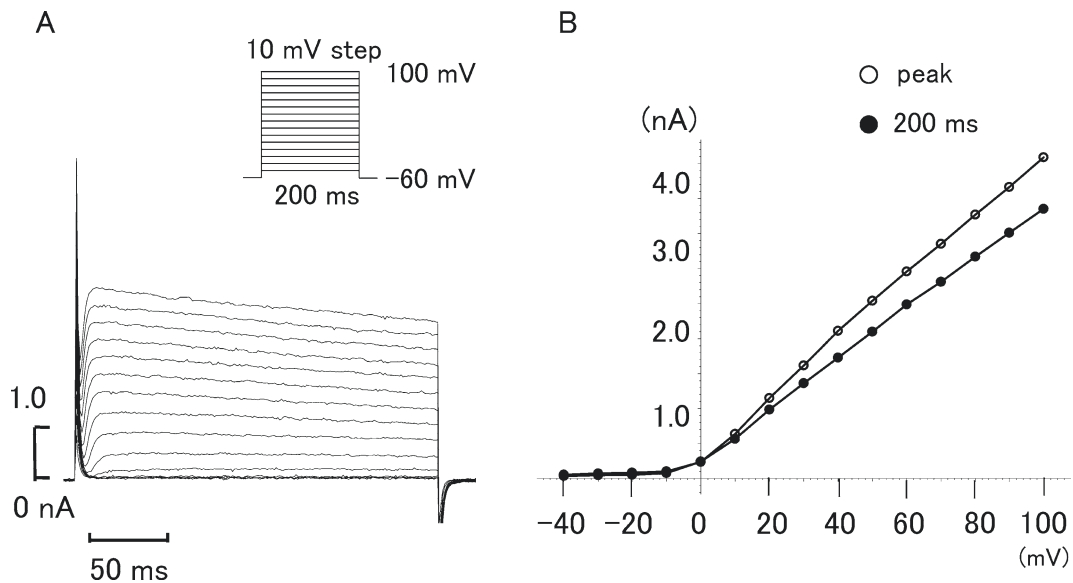


Fig. 1. Typical currents (A) and their I-V curves (B) obtained from an H9c2 cell.

A: A set of representative whole-cell currents from an H9c2 cell. A family of voltage pulses given every 10 sec is shown in the inset.

B: I-V curves constructed from the trace in A. Current amplitudes were measured at the peak (○) and at the end of 200 ms pulses (●).

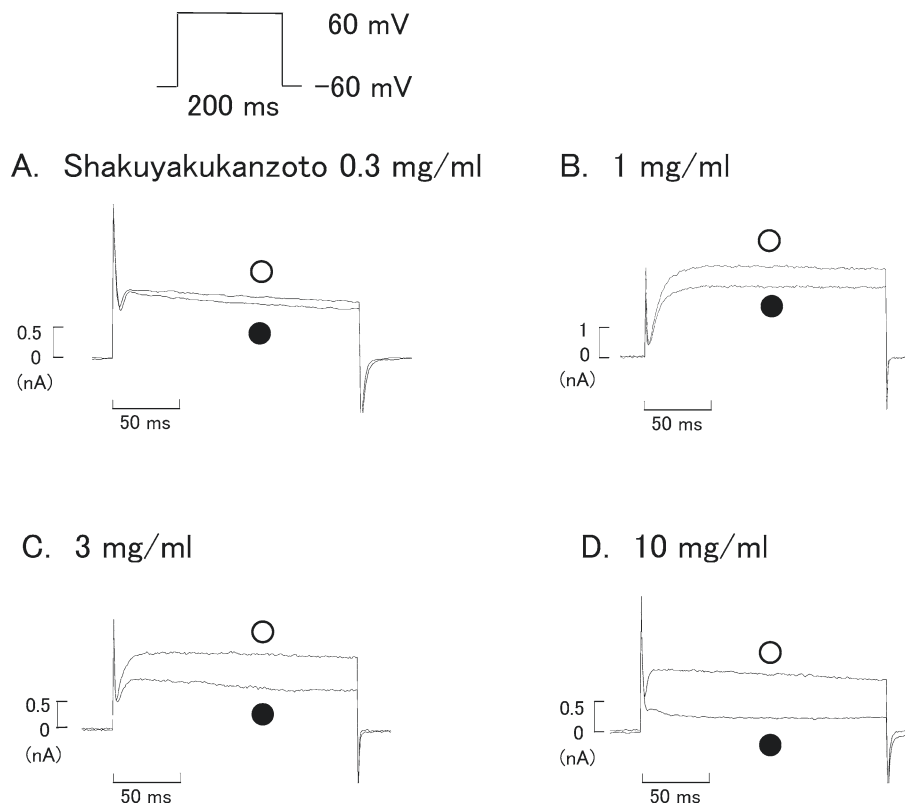


Fig. 2. Effect of Shakuyakukanzoto (SKT) on outward K⁺ currents in H9c2 cells. SKT concentrations are (A) 0.3 mg/ml, (B) 1 mg/ml, (C) 3 mg/ml, and (D) 10 mg/ml. The pulses are shown in the inset. A-D are obtained from different cells. (○) is control and (●) in shakuyakukanzoto.

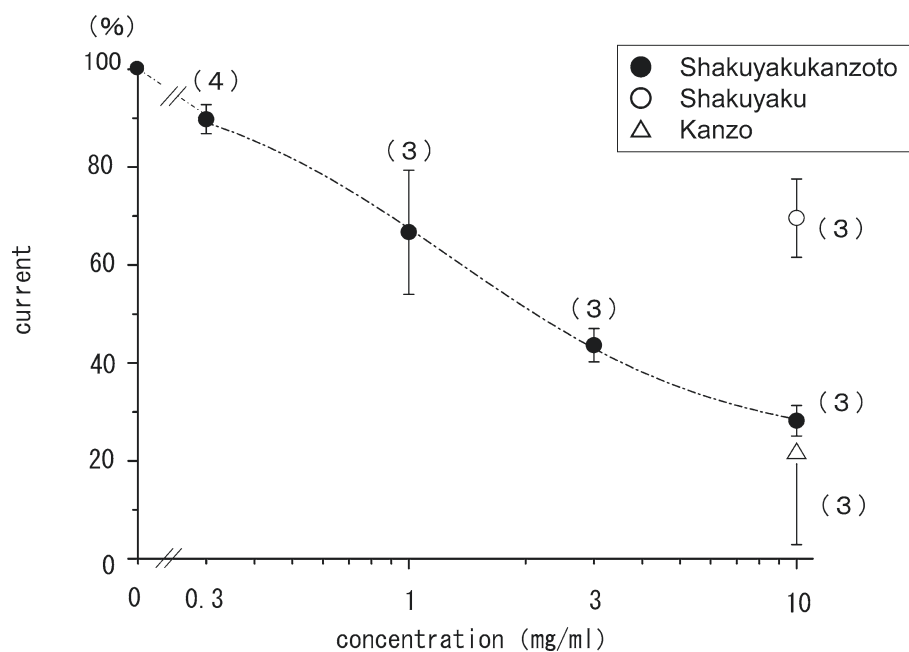


Fig. 3. Concentration-response curve of Shakuyakukanzoto (●) on I_{Kur}. The average values of current were fitted. IC₅₀ was 1.3 mg/ml and Hill coefficient was 1.2. The effects of Shakuyaku (10 mg/ml) and Kanzo (10 mg/ml) were also plotted. Data are mean ± S.D. of 3 to 4 cells.

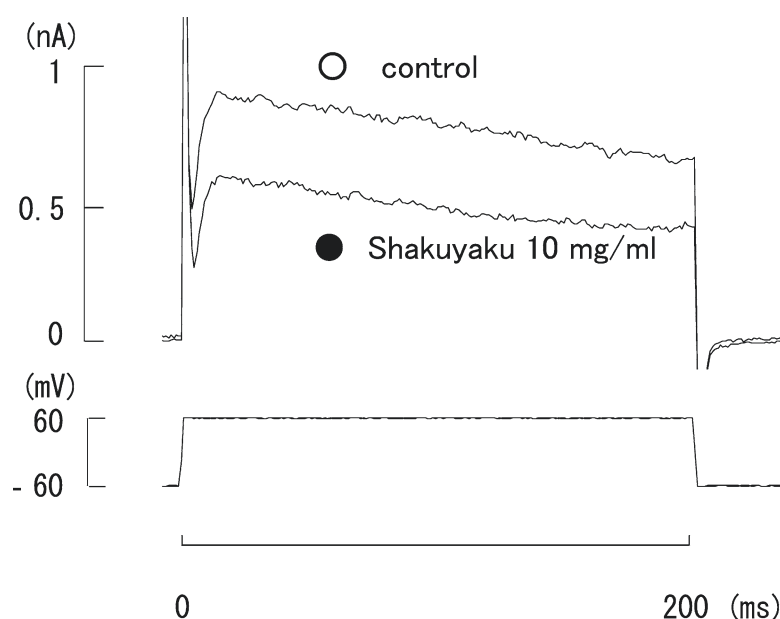


Fig. 4. Inhibitory effect of Shakuyaku on K^+ current in H9c2 cells. After recording the control current ($\circ$), Shakuyaku at 10 mg/ml was perfused for 5 min ($\bullet$).

Effect of Kanzo on K^+ current

Noguchi *et al.*¹⁸⁾ reported that Kanzo (licorice) inhibited I_{Kur} in H9c2 cells. To confirm their result, we perfused H9c2 cells with Kanzo solution under the whole cell clamp using the above protocol. Typical data are shown in Fig. 5. Kanzo at 10 mg/ml suppressed the peak I_{Kur} to $21.5 \pm 18.6\%$ of the control ($n=3$) (Fig. 3). These results are consistent with those previously reported by Noguchi *et al.*¹⁸⁾. The inhibitory effect of 10 mg/ml Kanzo was equivalent to that of 10 mg/ml Shakuyakukanzoto. We further tested the effect of glycyrrhizin, a major component of Kanzo. As shown in Fig. 6, glycyrrhizin at 100 μ M had no effect on K^+ current during 5 min of perfusion.

Effect of Kv2.1 siRNA on K^+ current in H9c2 cells

Suzuki *et al.*¹⁴⁾ and Wang *et al.*¹⁵⁾ suggested that I_{Kur} in H9c2 cells was Kv2.1. To confirm their results, we compared the level of Kv2.1 mRNA and the magnitude of I_{Kur} between H9c2 cells transfected with control siRNA or Kv2.1 siRNA. We used a Kv2.1 siRNA, which showed the best suppression effect among three siRNAs tested (Figure not shown). After 48 hours of transfection, the expression level of Kv2.1 mRNA was decreased significantly in Kv2.1 siRNA transfected cells compared to those with control siRNA ($37.5 \pm 7.6\%$ ($n=3$) $p < 0.001$, Fig. 7A). When we measured I_{Kur} , the

peak values were decreased significantly in the siRNA transfected cells (control siRNA 1.26 ± 0.79 nA ($n=8$) Kv2.1 siRNA 0.32 ± 0.24 nA ($n=10$) $p=0.006$) (Fig. 7B). From these results, we confirmed that I_{Kur} is most likely to be Kv2.1 in H9c2 cells.

Expression of Kv2.1 mRNA in rat skeletal muscle

We performed RT-PCR to determine Kv2.1 expression in rat skeletal muscle, and detected mRNAs of Kv1.1, Kv1.3, Kv2.1, Kv3.1, Kv3.4, and Kv4.3 (Fig. 8). Therefore, we confirmed the expression of Kv2.1 in rat skeletal muscles.

DISCUSSION

The present study demonstrated that Shakuyakukanzoto inhibited I_{Kur} type K^+ current in a concentration-dependent manner in H9c2 cells. The IC_{50} of Shakuyakukanzoto was approximately 1.3 mg/ml and the Hill coefficient was 1.2. Considering that the Hill coefficient (1.2) was close to one, it is reasonable to assume that one molecule of active ingredient in Shakuyakukanzoto components may bind to a single site in the K^+ channel.

The clinical oral dose of Shakuyakukanzoto is 7.5 g per day. If a person with 60 kg body weight takes 7.5 g Shakuyakukanzoto, and if her/his circulating blood volume is 4.2 L (7% of body weight), the blood concentration of Shakuyakukanzoto would be

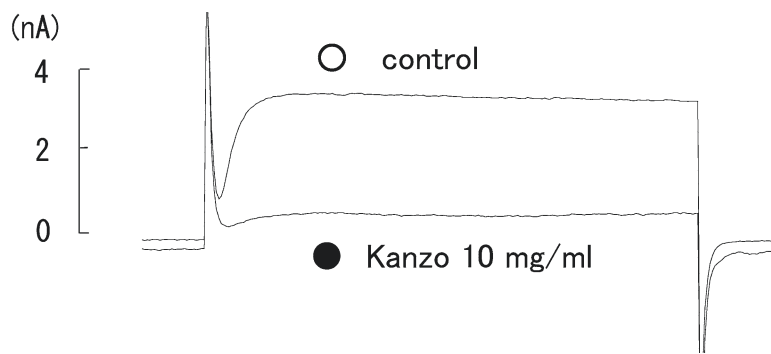


Fig. 5. Inhibitory effect of Kanzo on K⁺ current in H9c2 cells. After recording the control current (○), Kanzo at 10 mg/ml was perfused for 5 min (●). The voltage pulse was the same as in Fig. 4 and Fig. 6.

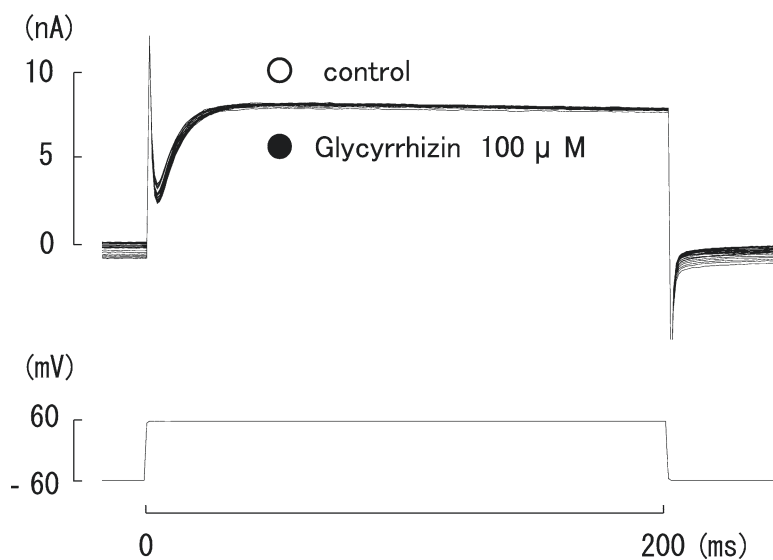


Fig. 6. Effect of Glycyrrhizin on K current in H9c2 cells. Control current (○) and currents during perfusion of 100 μM glycyrrhizin for 5 min (●) were superimposed.

1.8 mg/ml (7.5 g / 4.2 L). As this value is near the IC₅₀ of 1.3 mg/ml, Shakuyakukanzoto is possibly estimated to inhibit IK_{ur} in the clinical concentration range. However, this estimation is insufficient, because herbal medicine is often absorbed from the intestine not only directly but also after being metabolized by enteric bacteria.

Shakuyakukanzoto consists of 50% Shakuyaku and 50% Kanzo. We therefore compared the effects of those three components. Kanzo, Shakuyakukanzoto, and Shakuyaku at 10 mg/ml suppressed the peak IK_{ur} to $21.5 \pm 18.7\%$ ($n=3$), $28.2 \pm 3.1\%$ ($n=3$), and $69.6 \pm 8.9\%$ ($n=3$) of the control, respectively. Noguchi *et al.*¹⁸⁾ reported that 10 μM Kanzo already inhibited IK_{ur} to 50% in H9c2 cells. Thus the order of potency of IK_{ur} inhibition was Kanzo>Shakuyakukanzoto>Shakuyaku. This order matched with *in vivo* rat results reported by Lee *et al.*²⁰⁾. They induced tetanus in rat gastroc-

nemius muscle by electrical stimulation under anesthesia. Shakuyakukanzoto, Shakuyaku, and Kanzo (0.5, 1, or 2 g/kg) were administered into the duodenum 1 hour before the tests. Shakuyakukanzoto and Kanzo both released tetani, whereas Shakuyaku had no such effect. Lee *et al.* therefore concluded that the potency of releasing tetani was Kanzo>Shakuyakukanzoto>Shakuyaku.

In the present study, we confirmed Kv2.1 mRNA expression not only in H9c2 cells, but also in rat skeletal muscle (Fig. 8). Moreover, IK_{ur} was decreased significantly in H9c2 cells transfected with Kv2.1siRNA. Thus, Shakuyakukanzoto most likely inhibited Kv2.1, which may have a function in skeletal muscle.

We propose a possible implication of the inhibitory effects of Shakuyakukanzoto on Kv2.1 for resolving muscle cramp. Suppression of IK_{ur} would reduce K⁺ efflux from muscle fibers and

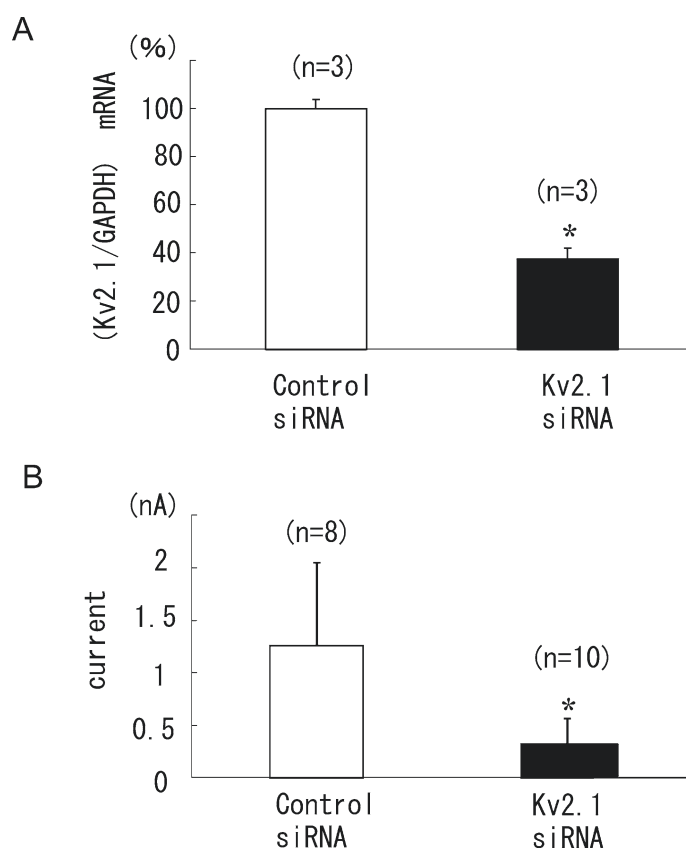


Fig. 7. Effect of siRNA on Kv2.1 mRNA expression and K⁺ current in H9c2 cells

A : The level of expression of Kv2.1 mRNA in H9c2 cells after 48 hours of control siRNA or Kv2.1 siRNA transfection. The ratio against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was calculated. B : Comparison of K⁺ currents between H9c2 cells transfected with control siRNA and Kv2.1 siRNA.

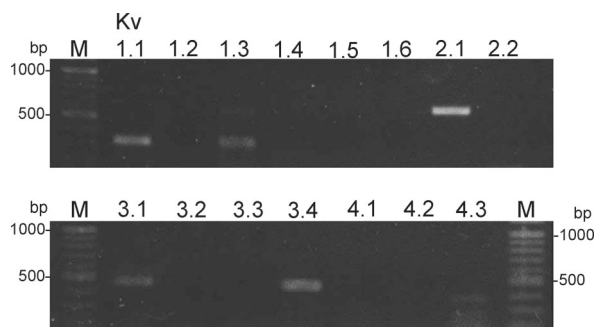


Fig. 8. RT-PCR analysis of various Kv (1.1-4.3) channels using mRNAs isolated from rat skeletal muscle. M indicates protein markers.

cramped myofibers are tetanized²¹⁾. If so, their membrane potentials may be depolarized, whereby Kv2.1 channels are open. Shakuyakukanzoto inhibits IK_{Kr}, and reduces K⁺ efflux from muscle fibers. Meanwhile, Na⁺-K⁺ ATPase induces K⁺ influx into the myofibers. Excessive K⁺ is consequently reduced from the extracellular space of myofibers, and intracellular and extracellular balance of K⁺ concentration may be restored. This could

be part of the reason why Shakuyakukanzoto relieves muscle cramps.

So far there have been various papers regarding Shakuyakukanzoto. Kimura²²⁾ reported that paeoniflorin (a major component of Shakuyaku) and glycyrrhizin depolarized and blocked the neuromuscular junctions. Jin *et al.*²³⁾ found that paeoniflorin dilated blood vessels and improved the peripheral circulation. Endo *et al.*²⁴⁾ investigated the different ratios of Shakuyaku and Kanzo on blocking the intestinal movement in rats and found the best effective ratio of Shakuyaku and Kanzo as 1 : 1.

In the present study, we found that Shakuyakukanzoto inhibited Kv2.1 K⁺ current in H9c2 cells. We propose that the mechanism of Shakuyakukanzoto for resolving muscle cramp may be at least partly due to the normalization of intra- and extracellular K⁺ balance. Further study is necessary to elucidate the mechanism of action of Shakuyakukanzoto.

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CONFLICT OF INTEREST

We have no conflict of interest to disclose.

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