

## Effects of Lizhong Tang on cultured mouse small intestine interstitial cells of Cajal

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### Abstract

**AIM:** To investigate the effects of Lizhong Tang, an herbal product used in traditional Chinese medicine, on mouse small intestine interstitial cells of Cajal (ICCs).

**METHODS:** Enzymatic digestions were used to dissociate ICCs from mouse small intestine tissues. The ICCs were morphologically distinct from other cell types in culture and were identified using phase contrast microscopy after verification with anti c-kit antibody. A whole-cell patch-clamp configuration was used to record potentials (current clamp) from cultured ICCs. All of the experiments were performed at 30-32 °C.

**RESULTS:** ICCs generated pacemaker potentials, and Lizhong Tang produced membrane depolarization in current-clamp mode. The application of flufenamic acid (a nonselective cation channel blocker) abolished the generation of pacemaker potentials by Lizhong Tang. Pretreatment with thapsigargin (a Ca<sup>2+</sup>-ATPase inhibi-

tor in the endoplasmic reticulum) also abolished the generation of pacemaker potentials by Lizhong Tang. However, pacemaker potentials were completely abolished in the presence of an external Ca<sup>2+</sup>-free solution, and under this condition, Lizhong Tang induced membrane depolarizations. Furthermore, When GDP-β-S (1 mmol/L) was in the pipette solution, Lizhong Tang still induced membrane depolarizations. In addition, membrane depolarizations were not inhibited by chelerythrine or calphostin C, which are protein kinase C inhibitors, but were inhibited by U-73122, an active phospholipase C inhibitors.

**CONCLUSION:** These results suggest that Lizhong Tang might affect gastrointestinal motility by modulating pacemaker activity in interstitial cells of Cajal.

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**Key words:** Interstitial cells of Cajal; Lizhong Tang; Motility; Gastrointestinal tract; Whole-cell patch clamp configuration

**Core tip:** The gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in interstitial cells of Cajal (ICCs). Taken together, our data suggest that the gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs. Considering the effects of this drug on ICCs, further research is required to identify the compounds responsible for the effects of Lizhong Tang and to determine their mechanisms of action.

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## INTRODUCTION

Lizhong Tang, was first reported 1800 years ago in “Shanghan Lun”, and it remains a classical herbal product in traditional Chinese medicine. Lizhong Tang is composed of Radix Ginseng (*Panax ginseng* C.A. Meyer), Rhizoma Zingiberis (*Zingiber officinale* Roscoe), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.) and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch)<sup>[1]</sup>, and it is widely used in traditional medicine to treat spleen deficiency patterns in many diseases with common symptoms, such as vomiting, diarrhea, stomach pain, poor appetite, cold limbs, and stomach bleeding which, are caused by cold and weak organs<sup>[1,2]</sup>. However, little is known of the molecular basis of the effects of Lizhong Tang on gastrointestinal (GI) motility.

Interstitial cells of Cajal (ICCs) are pacemaker cells in GI muscles that generate rhythmic oscillations in membrane potentials known as slow waves<sup>[3-5]</sup>. Slow waves propagate within ICC networks and are conducted into smooth muscle cells *via* gap junctions. Furthermore, they initiate phasic contractions by activating Ca<sup>2+</sup> entry through L-type Ca<sup>2+</sup> channels. Pacemaker activity in the murine small intestine is mainly due to periodic activations of nonselective cation channels<sup>[6,7]</sup> or Cl<sup>-</sup> channels<sup>[8,9]</sup>. ICCs also mediate or transduce inputs from the enteric nervous system. However, the effects of Lizhong Tang in mouse small intestine ICCs have not been investigated, and therefore, we undertook this study to investigate the characteristics of Lizhong Tang in mouse small intestine ICCs.

## MATERIALS AND METHODS

### Preparation of cells and cell cultures

Balb/c mice (3-7 d old) of either sex were anesthetized 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. The luminal contents were removed by washing with Krebs-Ringer bicarbonate solution. The tissues were then pinned to the base of a Sylgard dish, and the mucosae were removed by sharp dissection. Small tissue strips of intestinal muscle (consisting of both circular and longitudinal muscles) were equilibrated in Ca<sup>2+</sup>-free Hanks solution (containing the following in mmol/L: KCl 5.36, NaCl 125, NaOH 0.34, NaHCO<sub>3</sub> 0.44, glucose 10, sucrose 2.9, and HEPES 11) for 30 min, and then, the cells were dispersed using an enzyme solution containing collagenase (Worthington Biochemical Co., Lakewood, NJ, United States) 1.3 mg/mL, bovine serum albumin (Sigma Chemical Co., St. Louis, MO, United States) 2 mg/mL, trypsin inhibitor (Sigma) 2 mg/mL and ATP 0.27 mg/mL. The cells were plated onto sterile glass coverslips coated with murine collagen (2.5 µg/mL, Falcon/BD, Franklin Lakes, NJ, United States) in a 35-mm culture dish and then cultured at 37 °C in a 95% O<sub>2</sub>, 50 mL/L CO<sub>2</sub> incubator in smooth muscle growth medium (Clo-

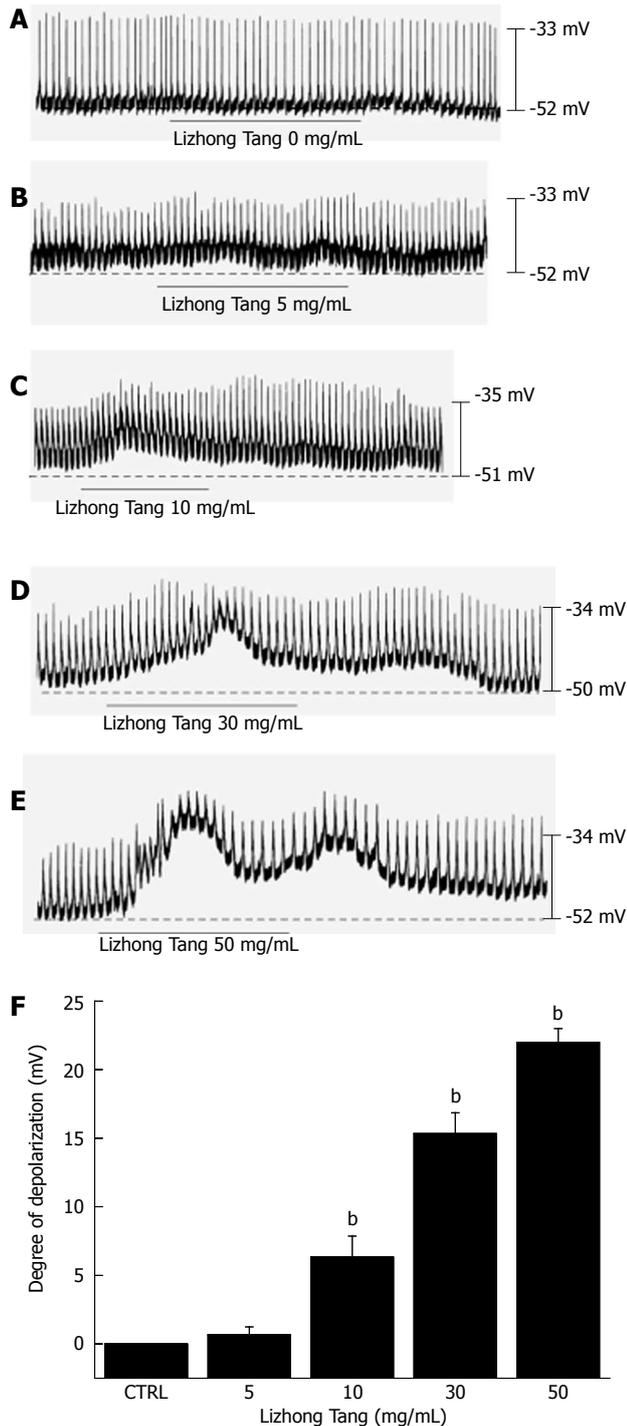
netics Corp., San Diego, CA, United States) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, United States) and murine stem cell factor (SCF 5 ng/mL, Sigma). ICCs were identified immunologically using an anti-c-kit antibody (phycoerythrin-conjugated rat anti-mouse c-kit monoclonal antibody; eBioscience, San Diego, CA, United States) at a dilution of 1:50 for 20 min<sup>[10]</sup>. The ICCs were morphologically distinct from other cell types in culture and were identified using phase contrast microscopy after verification with anti c-kit antibody.

### Patch-clamp experiments

A whole-cell patch-clamp setup was used to record the membrane potentials (current clamp) of cultured ICCs. An axopatch ID (Axon Instruments, Foster, CA, United States) was used to amplify membrane currents and potentials. The command pulse was applied using an IBM-compatible personal computer running pClamp software (version 6.1; Axon Instruments). The data obtained were filtered at 5 kHz displayed on an oscilloscope and a computer monitor, and printed using a pen recorder (Gould 2200, Gould, Valley View, OH, United States). The results were analyzed using pClamp and Origin (version 6.0) software. All of the experiments were performed at 30-32 °C.

### Solutions and drugs

The physiological salt solution used to bathe cells (Na<sup>+</sup>-Tyrode) contained the following (in mmol/L): KCl 5, NaCl 135, CaCl<sub>2</sub> 2, glucose 10, MgCl<sub>2</sub> 1.2 and HEPES 10, adjusted to pH 7.4 with NaOH. The pipette solution contained the following (in mmol/L): KCl 140, MgCl<sub>2</sub> 5, K<sub>2</sub>ATP 2.7, NaGTP 0.1, creatine phosphate disodium 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with KOH. Lizhong Tang was purchased from I-WORLD Pharmaceuticals (South Korea). Lizhong Tang is composed of Radix Ginseng (*Panax ginseng* C.A. Meyer), Rhizoma Zingiberis (*Zingiber officinale* Roscoe), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.) and Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.). The adult dosage is 10-15 g (crude material) per day. More information about Lizhong Tang can be obtained at the I-WORLD Pharmaceuticals Homepage (<http://i-pharm.koreasme.com>). The pills were dissolved with distilled water at a concentration of 0.5 g of crude drug/ml and stored in the refrigerator. All of the drugs were obtained from Sigma (Sigma Chemical Co., United States). The drugs were dissolved in distilled water, and added to the bathing solution to the desired concentrations immediately prior to use. The addition of these chemicals to the bathing solution did not alter its pH. Thapsigargin, U-73122, and U-73343 were dissolved in dimethyl sulfoxide (DMSO) to produce 50 and 100 mmol/L stock solutions and added at 1000 times dilutions to the bathing solution on the day of the experiment. The final concentration of DMSO in the bathing solution was always < 0.1%, and we confirmed that this



**Figure 1** Effects of Lizhong Tang on pacemaker potentials in cultured interstitial cells of Cajal from murine small intestines. A-E: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (0-50 mg/mL) in current-clamp mode ( $I = 0$ ); F: The responses to Lizhong Tang are summarized. Bars represent mean  $\pm$  SE.  $^bP < 0.01$  vs control group. CTRL: Control.

concentration did not affect the results.

### Statistical analysis

All of the data are expressed as the mean  $\pm$  SE. Student's *t*-test for unpaired data was used to compare the control and experimental groups. Statistical significance was accepted for *P* values  $< 0.05$ .

## RESULTS

### Effect of Lizhong Tang on the pacemaker potentials of cultured ICCs

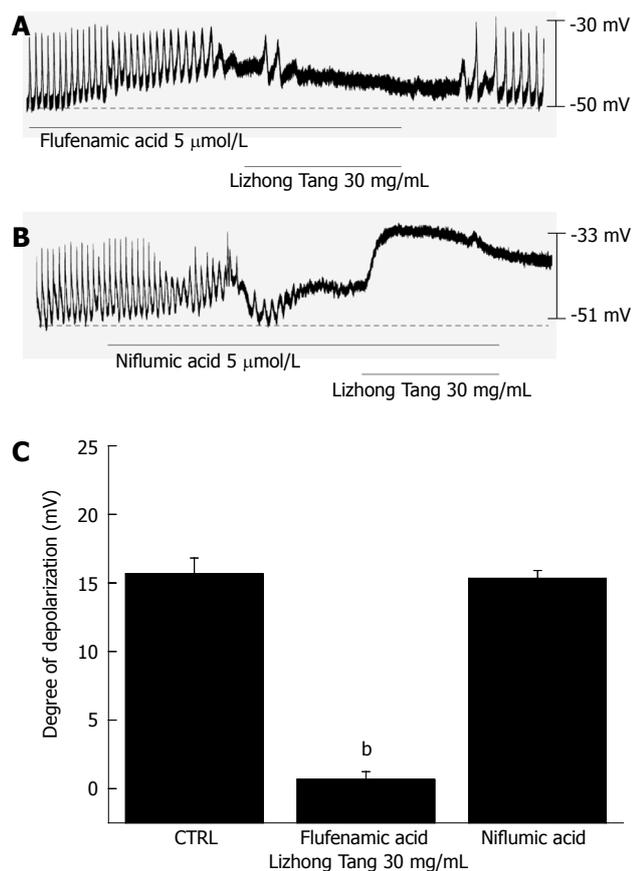
The patch-clamp technique was tested on ICCs, which had formed network-like structures in culture after 2-4 d. Spontaneous rhythms were routinely recorded from cultured ICCs under current- and voltage-clamp conditions; ICCs within networks displayed more robust electrical rhythms. Tissue-like spontaneous slow waves have been previously recorded from these cells<sup>[11]</sup>. To understand the relationship between Lizhong Tang and the modulation of pacemaker activity in ICCs, we examined the effects of Lizhong Tang on pacemaker potentials. Recordings from cultured ICCs under current-clamp mode ( $I = 0$ ) showed spontaneous pacemaker potentials. The mean resting membrane potential was  $-52 \pm 1.3$  mV, and the mean amplitude was  $23 \pm 2$  mV. In the presence of Lizhong Tang (0-50 mg/mL), the membrane potentials were depolarized to  $0.6 \pm 0.5$  mV at 5 mg/mL,  $6.3 \pm 1.5$  mV at 10 mg/mL,  $15.2 \pm 1.3$  mV at 30 mg/mL, and  $22.1 \pm 1.2$  mV at 50 mg/mL (Figure 1A-E). Summarized values and a bar graph of the effects of Lizhong Tang on pacemaker potentials are provided in Figure 1F ( $n = 4$ ).

### Effects of non-selective cation channel blocker or Cl channel blocker on Lizhong Tang-induced pacemaker potentials in cultured ICCs

To determine the characteristics of the membrane depolarizations induced by Lizhong Tang, flufenamic acid (a non-selective cation channel blocker)<sup>[12,13]</sup> and niflumic acid (a Cl channel blocker)<sup>[12,14]</sup> were used. In the presence of flufenamic acid (5  $\mu$ mol/L), pacemaker potentials were abolished and the subsequent application of Lizhong Tang (30 mg/mL) did not produce membrane depolarization (Figure 2A). In the presence of flufenamic acid, the membrane depolarizations produced by Lizhong Tang were  $0.6 \pm 0.4$  mV, which was significantly different from the control values obtained in the absence of flufenamic acid ( $n = 4$ , Figure 2C). Pacemaker potentials were also abolished in the presence of niflumic acid (5  $\mu$ mol/L), but Lizhong Tang still produced membrane depolarization (Figure 2B). In the presence of niflumic acid, the mean membrane depolarization produced by Lizhong Tang was  $15.3 \pm 0.4$  mV, which was not significantly different from the control condition ( $n = 4$ , Figure 2C).

### Effects of external $Ca^{2+}$ -free solution and $Ca^{2+}$ -ATPase inhibitor in the endoplasmic reticulum on Lizhong Tang-induced pacemaker potentials in cultured ICCs

External  $Ca^{2+}$  influx is necessary for GI smooth muscle contractions and is essential for the generation of pacemaker potentials by ICCs. The generation of pacemaker currents is known to be dependent on intracellular  $Ca^{2+}$  oscillations<sup>[15]</sup>. To investigate the roles of external and of internal  $Ca^{2+}$ , Lizhong Tang was tested under external  $Ca^{2+}$ -free conditions and in the presence of thapsigargin, an inhibitor of  $Ca^{2+}$ -ATPase in the endoplasmic reticu-

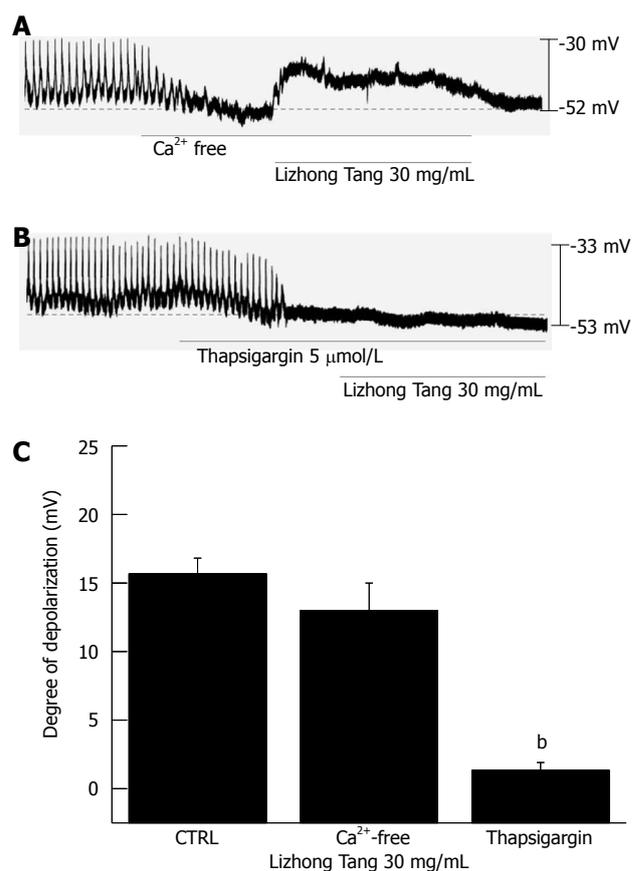


**Figure 2** Effects of flufenamic acid (a nonselective cation channel blocker) or niflumic acid (a Cl<sup>-</sup> channel blocker) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal from murine small intestines. A: The application of flufenamic acid (5 μmol/L) abolished the generation of pacemaker potentials, and in the presence of flufenamic acid, Lizhong Tang (30 mg/mL) did not cause membrane depolarization; B: In contrast, although niflumic acid (5 μmol/L) abolished the generation of pacemaker potentials, it did not block Lizhong Tang-induced (30 mg/mL) membrane depolarization; C: The responses to Lizhong Tang in the presence of flufenamic acid or niflumic acid are summarized. Bars represent mean ± SE. <sup>b</sup>*P* < 0.01 vs control group. CTRL: Control.

lum<sup>[6,12]</sup>. Pacemaker potentials were completely abolished in the presence of an external Ca<sup>2+</sup>-free solution, and under this condition, Lizhong Tang induced membrane depolarizations (*n* = 4, Figure 3A). However, under external Ca<sup>2+</sup>-free conditions, membrane depolarizations by Lizhong Tang (30 mg/mL) were not significantly different from the depolarizations induced by Lizhong Tang (30 mg/mL) under normal Ca<sup>2+</sup> conditions (*n* = 4, Figure 3C). In addition, Lizhong Tang-induced membrane depolarizations were inhibited by thapsigargin pretreatment (Figure 3B). Furthermore, the membrane depolarizations induced by Lizhong Tang were significantly affected by the presence of thapsigargin (*n* = 4, Figure 3C).

### The involvement of G protein on Lizhong Tang-induced pacemaker potentials in cultured ICCs

The effects of GDP-β-S (a non-hydrolysable guanosine 5'-diphosphate analogue that permanently inactivates G-protein binding proteins<sup>[16]</sup>) were examined to determine whether G-proteins are involved in the effects of

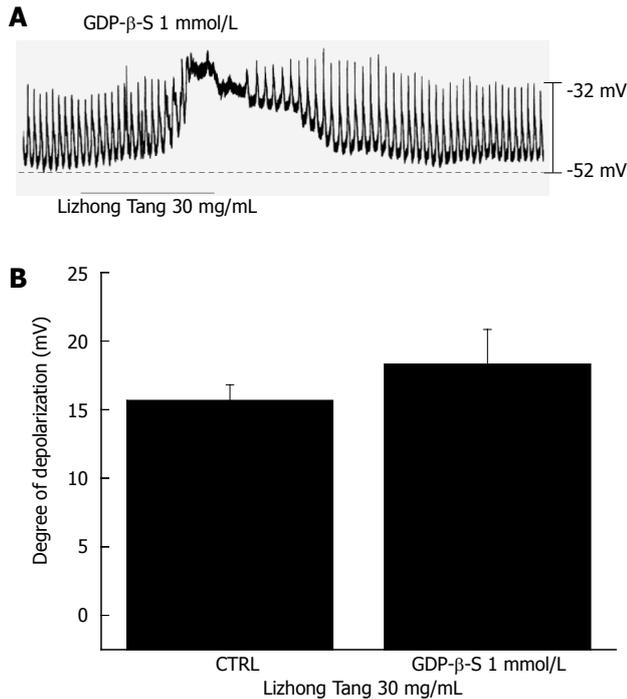


**Figure 3** Effects of an external Ca<sup>2+</sup>-free solution, thapsigargin (an inhibitor of Ca<sup>2+</sup>-ATPase in the endoplasmic reticulum), or U-73122 (an active phospholipase C inhibitor) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal. A: External Ca<sup>2+</sup>-free solution abolished the generation of pacemaker potentials, but failed to block Lizhong Tang-induced (30 mg/mL) membrane depolarization; B: Thapsigargin (5 μmol/L) abolished the generation of pacemaker potentials, and blocked Lizhong Tang-induced (30 mg/mL) membrane depolarization; C: The responses to Lizhong Tang in external Ca<sup>2+</sup>-free solution in the presence of thapsigargin are summarized. Bars represent mean ± SE. <sup>b</sup>*P* < 0.01 vs control group. CTRL: Control.

Lizhong Tang on ICCs. When GDP-β-S (1 mmol/L) was in the pipette solution, Lizhong Tang (30 mg/mL) still induced membrane depolarizations (Figure 4A). However, the membrane depolarizations induced by Lizhong Tang were not significantly affected by the presence of GDP-β-S (1 mmol/L) in the pipette solution (*n* = 4, Figure 4C).

### Effects of phospholipase C inhibitor on Lizhong Tang-induced pacemaker potentials in cultured ICCs

Because membrane depolarizations induced by Lizhong Tang are related to intracellular Ca<sup>2+</sup> mobilization, we examined whether the effects of Lizhong Tang on pacemaker potentials required phospholipase C (PLC) activation. To test this possibility, Lizhong Tang-induced membrane depolarizations were measured in the absence or presence of U-73122 (an active PLC inhibitor<sup>[17]</sup>). Pacemaker membrane depolarizations currents were completely abolished by U-73122 (5 μmol/L), and under these conditions, Lizhong Tang-induced (30 mg/mL)



**Figure 4** Effects of GDP-β-S in the pipette on Lizhong Tang-induced pacemaker potentials in cultured murine small intestine interstitial cells of Cajal. A: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (30 mg/mL) in the presence of GDP-β-S (1 mmol/L) in the pipette. Under these conditions, Lizhong Tang (30 mg/mL) caused membrane depolarization; B: The responses to Lizhong Tang in the presence of GDP-β-S in the pipette are summarized. Bars represent mean  $\pm$  SE. CTRL: Control.

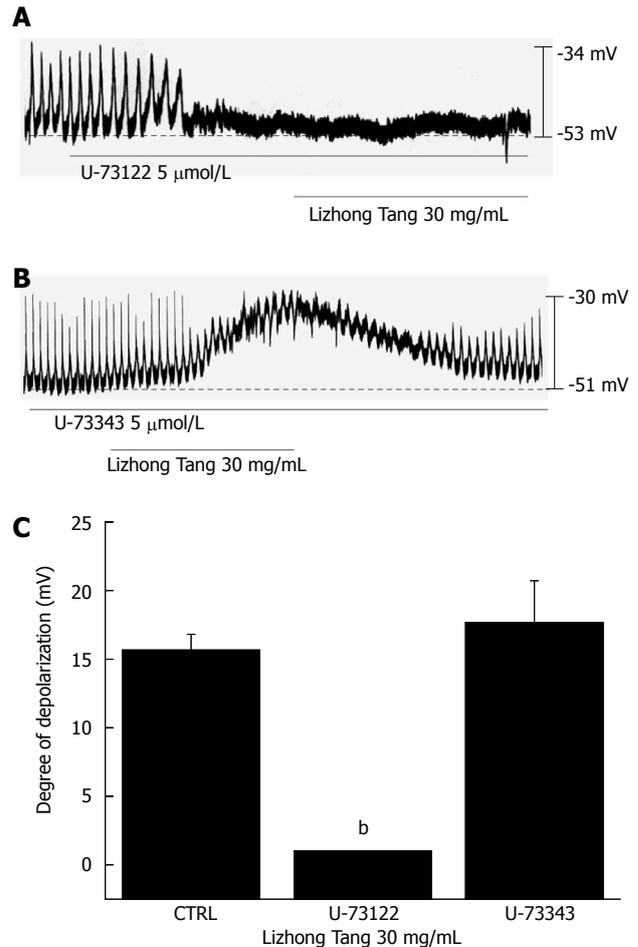
membrane depolarizations were suppressed ( $n = 4$ , Figure 5A). In the presence of U-73122, the mean membrane depolarization produced by Lizhong Tang was  $0.5 \pm 0.3$  mV, and this was significantly different than the depolarization observed in the absence of U-73122 ( $n = 4$ , Figure 5C). Treatment with U-73343 (5  $\mu$ mol/L; an inactive analog of U-73122) had no influence on Lizhong Tang-induced pacemaker potentials, and Lizhong Tang-induced (30 mg/mL) membrane depolarizations were not suppressed by U-73343 (Figure 5C).

#### Effects of protein kinase C inhibitor on Lizhong Tang-induced pacemaker potentials in cultured ICCs

We tested the effects of chelerythrine and of calphostin C (both inhibitors of protein kinase C (PKC)<sup>[12,18]</sup>) to investigate whether Lizhong Tang-induced pacemaker potential responses are mediated by the activation of PKC. Neither chelerythrine (1  $\mu$ mol/L) nor calphostin C (1  $\mu$ mol/L) had any effect on membrane depolarizations induced by Lizhong Tang (30 mg/mL; Figure 6) and the value was also not significantly different when compared with the membrane depolarizations induced by Lizhong Tang in the absence of chelerythrine or calphostin C ( $n = 5$ , Figure 6C).

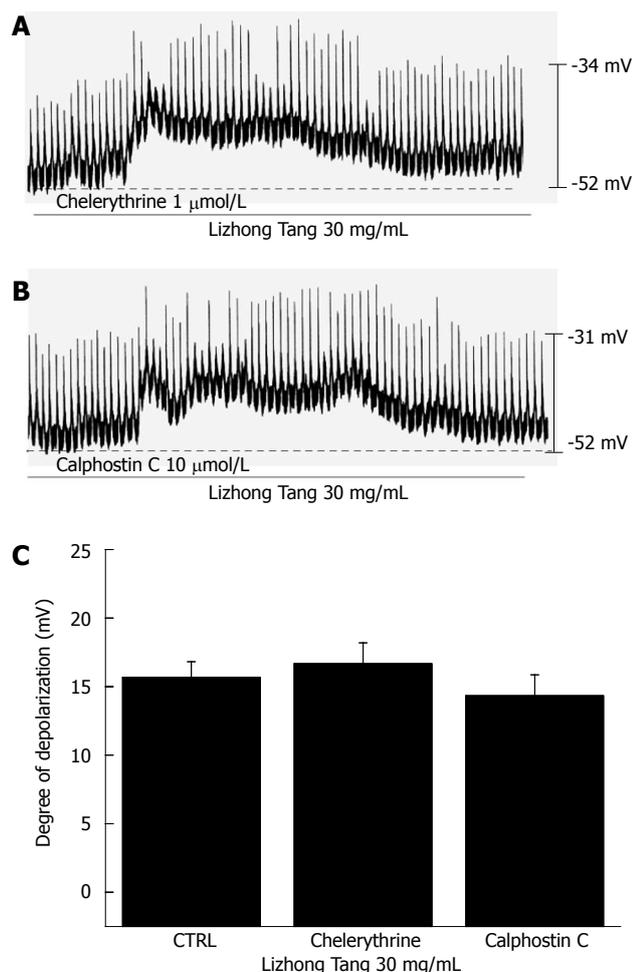
## DISCUSSION

The GI tract exhibits spontaneous mechanical con-



**Figure 5** Effects of phospholipase C inhibitors on Lizhong Tang-induced potentials in cultured interstitial cells of Cajal. A: U-73122 (5  $\mu$ mol/L; a phospholipase C inhibitor) abolished the generation of pacemaker potentials, and blocked Lizhong Tang-induced (30 mg/mL) membrane depolarization; B: The application of U-73343 (5  $\mu$ mol/L) did not influence the generation of pacemaker currents or block Lizhong Tang-induced (30 mg/mL) membrane depolarization; C: The responses to Lizhong Tang in the presence of phospholipase C inhibitors are summarized. Bars represent mean  $\pm$  SE. <sup>b</sup> $P < 0.01$  vs control group. CTRL: Control.

tractions that are mediated by the periodic generation of electrical pacemaker potentials, which are the basic determinant of GI smooth muscle activity<sup>[3]</sup>. Recent studies have shown that the ICCs act as the pacemakers and conductors of electrical slow waves in GI smooth muscles<sup>[3-5]</sup>. Moreover, evidence indicates that endogenous agents, such as, neurotransmitters, hormones, and paracrine substances modulate GI tract motility by influencing ICCs<sup>[5-7,19,20]</sup>. Therefore, one of the best ways to investigate the role of GI motility is to use ICCs. Many types of ICCs with different immunohistochemical and electrical properties, including myenteric ICCs (ICC-MY), intramuscular ICCs (ICC-IM), deep muscular plexus ICCs (ICC-DMP), and submucosal ICCs (ICC-SM), are distributed throughout the GI tract<sup>[16]</sup>. In animal models lacking ICC-MY, slow waves in the small intestine are strongly attenuated, which shows that these cells are indeed essential for pacemaker activity in the GI tract<sup>[21]</sup>. Furthermore, ICCs are involved in physiological GI mo-



**Figure 6** Effects of chelerythrine or calphostin C (inhibitors of protein kinase C) on Lizhong Tang-induced pacemaker potentials in cultured interstitial cells of Cajal. A, B: Pacemaker potentials in interstitial cells of Cajal exposed to Lizhong Tang (30 mg/mL) in the presence of chelerythrine (1  $\mu$ mol/L) or calphostin C (10  $\mu$ mol/L). Lizhong Tang caused membrane depolarization in the presence of both inhibitors; C: The responses to Lizhong Tang in the presence of chelerythrine or calphostin C are summarized. Bars represent mean  $\pm$  SE. CTRL: Control.

tility and are therefore clinically important in many bowel disorders, including inflammatory bowel disease, chronic idiopathic intestinal pseudo-obstruction, intestinal obstruction with hypertrophy, achalasia, Hirschsprung's disease, juvenile pyloric stenosis, juvenile intestinal obstruction, and anorectal malformation<sup>[16]</sup>.

Lizhong Tang warms the liver and spleen and strengthens the spleen and stomach. It has been widely used as treatment from deficiency, diarrhea with watery stool, nausea and vomiting. In addition, it also has ameliorative effects on loss of appetite, abdominal pain, acute or chronic gastritis gastric or duodenal ulcers, irritable bowel syndrome, chronic colitis, chronic bronchitis, oral herpes, and functional uterine bleeding<sup>[1,22,23]</sup>. However, the effects of Lizhong Tang on GI tract motility and ICCs have not been investigated.

In this study, Lizhong Tang produced membrane depolarization in current-clamp mode, and the application of flufenamic acid (a non-selective cation channel

blocker), but not niflumic acid (a  $\text{Cl}^-$  channel blocker), abolished the generation of the pacemaker potentials induced by Lizhong Tang, suggesting that the Lizhong Tang-induced membrane depolarizations may be mediated by non-selective cationic channels. In addition, pretreatment with a  $\text{Ca}^{2+}$ -free solution or with thapsigargin (a  $\text{Ca}^{2+}$ -ATPase inhibitor in the endoplasmic reticulum), abolished the generation of pacemaker potentials. Under  $\text{Ca}^{2+}$ -free conditions, Lizhong Tang also showed membrane depolarization; however, in the presence of thapsigargin, Lizhong Tang did not show membrane depolarization, suggesting that intracellular calcium release is necessary. Furthermore, pacemaker membrane depolarizations were inhibited by U-73122 (PLC inhibitor), but not by GDP- $\beta$ -S, which permanently binds G-binding proteins. In addition, the PKC inhibitors chelerythrine and calphostin C did not block Lizhong Tang-induced pacemaker potentials, suggesting that PLC is involved in the induction of the pacemaker potentials, but that PKC is not. In summary, Lizhong Tang affects GI motility by modulating pacemaker activity in ICCs, and this activation is associated with non-selective cationic channels via phospholipase C activation, and  $\text{Ca}^{2+}$  release from internal storage in an external  $\text{Ca}^{2+}$ -, G-protein-, and PKC-independent manner.

Taken together, our data suggest that the gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs. Considering the effects of this drug on ICCs, further research is required to identify the compounds responsible for the effects of Lizhong Tang and to determine their mechanisms of action.

## COMMENTS

### Background

Interstitial cells of Cajal (ICCs) are the pacemaker cells that generate slow waves in the gastrointestinal (GI) tract. Lizhong Tang is a classic herbal product in traditional Chinese medicine. However, the effects of Lizhong Tang in mouse small intestine ICCs have not been investigated.

### Research frontiers

The gastroprokinetic effects of Lizhong Tang are mediated by the induction of pacemaker potentials in ICCs.

### Innovations and breakthroughs

Lizhong Tang affects GI motility by modulating pacemaker activity in ICCs, and this activation is associated with non-selective cationic channels via phospholipase C activation, and  $\text{Ca}^{2+}$  release from internal storage in an external  $\text{Ca}^{2+}$ -, G-protein-, and protein kinase C-independent manner.

### Applications

Lizhong Tang may be a new target for pharmacological treatment of GI motility disorders.

### Peer review

In their manuscript, authors studies the effect of Lizhong Tang, an herbal product used in traditional Chinese medicine, on the pacemaking activity of mouse small ICCs. It was well written.

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