

# Regulation of the pacemaker activities in cultured interstitial cells of Cajal by *Citrus unshiu* peel extracts

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**Abstract.** The *Citrus unshiu* peel has been widely used for the treatment of gastrointestinal (GI) disorders in Eastern traditional medicine. The present study aimed to investigate the effects of *Citrus unshiu* peel extract (CPE) on the pacemaker activity of the GI tract in cultured interstitial cells of Cajal (ICCs) derived from the mouse small intestine. The whole-cell patch-clamp configuration was used to record pacemaker potentials. In current clamp mode, exposure to CPE caused membrane pacemaker depolarization in a concentration-dependent manner. In the presence of the muscarinic M<sub>2</sub> receptor antagonist, methoctramine, CPE induced membrane pacemaker depolarization, whereas treatment with the muscarinic M<sub>3</sub> receptor antagonist, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide, inhibited CPE-induced responses. When the pipette solution contained guanosine 5'-(β-thio) diphosphate trilithium salt (1 mM), CPE marginally induced membrane pacemaker depolarization. In addition, CPE-induced membrane pacemaker depolarization was inhibited following exposure to the active phospholipase C (PLC) inhibitor U-73122, but not the inactive PLC inhibitor U-73343. In the presence of a p42/p44 mitogen-activated protein kinase (MAPK) inhibitor (PD98059), a p38 MAPK inhibitor (SB203580) or a c-jun NH2-terminal kinase (JNK) II inhibitor, CPE failed to induce membrane pacemaker depolarization. These results suggest that CPE may affect GI motility through modulating ICC pacemaker activity by activating the muscarinic M<sub>3</sub> receptor and inducing the G-protein dependent PLC and MAPK signaling pathways.

## Introduction

Traditional plant-based medicines have served an important role in health care, and numerous drugs are known to originate from these medicines (1). *Citrus unshiu* (*C. unshiu*) Marcov, which belongs to the Rutaceae family is a seedless and easy-to-peel Korean citrus fruit, and constitutes 30% of the total volume of fruit produced in Korea (2). Its dried peel has been used to improve bronchial and asthmatic conditions, and blood circulation in Korea, China, and Japan for thousands of years (3,4).

The *C. unshiu* peel (also known as Jin-pee) is the primary waste product of citrus fruits and has been used as a source of molasses, pectin, cold-pressed oils and limonene (5). The peel has been studied extensively, as it contains numerous biologically active compounds, such as natural antioxidants (phenolic acids and flavonoids) (6,7). In addition, the *C. unshiu* peel is reported to possess anti-allergy (8,9), antibacterial, anti-fungal (10,11), anticancer (12), antidiabetic (13,14), anti-inflammatory (15,16), antioxidant (17-19), antiviral (20) and lipid-lowering activities (2,4). *C. unshiu* peel has been used in Korea to treat a variety of digestive disorders, including tympanites, nausea, vomiting and dyspepsia (16,21). Despite reports that the peel functions as a prokinetic agent to prevent or alleviate gastrointestinal (GI) motility dysfunctions (22), little is known about its effects on GI motility or its mechanisms of action.

Interstitial cells of Cajal (ICCs) are the pacemaker cells of the GI muscles that generate rhythmic oscillations in membrane potentials (termed 'slow waves') (23,24), and mediate or transduce inputs from the enteric nervous system (25). Research into the biology of ICCs has provided exciting and novel opportunities to understand the etiology of GI diseases (26). Therefore, the aim of the present study was to investigate the effect of *C. unshiu* peel extracts (CPE) on the pacemaker potentials of cultured ICCs from the murine small intestine.

## Materials and methods

*Preparation of samples and high-performance liquid chromatography (HPLC) analysis.* The dried peel of *C. unshiu* was

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purchased from Kapdang Co. (Seoul, Korea). The sample was identified by Dr Yun Tai Kim (Korea Food Research Institute, Seongnam, Korea) according to the 'Illustrated Guide to Clinical Medical Herbs' (27) and a voucher specimen (reference no. NP-1505) was deposited with the Research Group of Innovative Special Food (Korea Food Research Institute). *C. unshiu* dried peel (600 g) was incubated with 70% ethanol (6,000 ml) for 2 h at 20°C. This process was repeated with fresh 70% ethanol, and the extract solution was combined and filtered through a 0.45- $\mu$ m membrane filter (EMD Millipore, Billerica, MA, USA). The solvents were removed by rotary evaporation and the remaining extracts were freeze-dried, which yielded ~21.1% of the dried peel weight (w/w).

The freeze-dried extract powder (100 mg) was dissolved in 5 ml methanol/dimethyl sulfoxide (DMSO; 1:1, v/v), before it was filtered through a 0.45- $\mu$ m regenerated cellulose-membrane filter (Sartorius AG, Goettingen, Germany), and diluted in methanol/DMSO (1:1, v/v) to a final concentration of 10 mg/ml prior to injection of 10  $\mu$ l of the solution into the HPLC. Analytical HPLC was performed using a Jasco HPLC system (Jasco, Inc., Tokyo, Japan), which comprised a PU-980 pump, an AS-950-10 autosampler and an MD-2010 Plus multi-wavelength detector.

The chromatographic separation was conducted at 30°C using a Symmetry<sup>®</sup> C18 column (4.6x250 mm, particle size 5  $\mu$ m; Waters Corporation, Milford, MA, USA) with gradient elution using a mobile phase composed of 40% methanol (mobile phase A) and 100% methanol (mobile phase B). Alterations in the mobile phase was achieved using a linear gradient system from 100% mobile phase A to 100% mobile phase B over 30 min and with a 0.5 ml/min flow rate, before the samples were detected at 284 nm. Quantitative analysis was performed in triplicate. The regression equation and correlation coefficient ( $r^2$ ) of each standard curve were automatically calculated by the Jasco HPLC system. The regression equations for narirutin and hesperidin were  $y=35,103.0278x-55,481.6311$  ( $r^2$ , 0.99994) and  $y=39,824.0428x-72,092.8906$  ( $r^2$ , 0.99973), respectively, indicating that a high linear correlation was achieved for all standard curves. The concentration of narirutin and hesperidin were determined to be  $21.72\pm 0.716$  and  $8.51\pm 0.296$  mg/g, respectively using the peak area in the chromatogram and the regression equation (Fig. 1).

**Ethical approval.** Animal care and experiments were conducted in accordance with the guidelines issued by the ethics committee of Pusan National University (Busan, Korea; approval no. PNU-2014-0725) and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 2011).

**Preparation of cells and culture conditions.** A total of 82 male (52%) and female (48%) BALB/c mice (age, 3-7 days; weight, 1.9-2.2 g; Samtako Bio Korea Inc., Osan-si, Korea) were anesthetized with ether and euthanized by cervical dislocation. They were maintained under controlled conditions (temperature,  $21\pm 3^\circ\text{C}$ ; humidity  $50\pm 6\%$ ; 12 h light/dark cycles) and were allowed free access to food and water. Mice were fed a diet comprised of crude protein ( $\geq 18\%$ ), crude fat ( $\geq 5\%$ ), crude fiber ( $\leq 4.5\%$ ), crude ash ( $\leq 8\%$ ), calcium ( $\geq 0.7\%$ ) and phosphorus ( $\leq 1.2\%$ ) (Samtako Bio Korea Inc.). The small

intestines from 1 cm below the pyloric ring to the cecum were removed, opened along the mesenteric border, and the luminal contents were removed by washing with a Krebs-Ringer bicarbonate solution. Tissues were pinned to the base of a Sylgard dish and the mucosae were removed by sharp dissection. Small tissue strips of intestinal muscle, consisting of circular and longitudinal muscles, were equilibrated in a  $\text{Ca}^{2+}$ -free Hank's Balanced Salt Solution (containing 5.36 mmol/l KCl, 125 mmol/l NaCl, 0.34 mmol/l NaOH, 0.44 mmol/l  $\text{Na}_2\text{HCO}_3$ , 10 mmol/l glucose, 2.9 mmol/l sucrose and 11 mmol/l HEPES) for 30 min. Cells were then dispersed using an enzyme solution containing 1.3 mg/ml collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA), 2 mg/ml bovine serum albumin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany), 2 mg/ml trypsin inhibitor (Sigma-Aldrich; Merck Millipore) and 0.27 mg/ml adenosine triphosphate (ATP; Sigma-Aldrich; Merck Millipore). Cells were subsequently plated onto Falcon sterile glass coverslips coated with murine collagen (2.5  $\mu\text{g}/\text{ml}$ ; BD Biosciences, Franklin Lakes, NJ, USA) in a 35-mm culture dish, and maintained in smooth muscle growth medium (Clonetics Corporation, San Diego, CA, USA) supplemented with 2% Penicillin-Streptomycin solution (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) and 5 ng/ml murine stem cell factor (Sigma-Aldrich; Merck Millipore) at 37°C in an  $\text{O}_2$  (95%)/ $\text{CO}_2$  (5%) incubator. ICCs were identified immunocytochemically by incubating cells with a phycoerythrin-conjugated rat anti-mouse monoclonal anti-c-Kit antibody (cat. no. 12-1172; dilution, 1:50; eBioscience, Inc., San Diego, CA, USA) for 20 min as described previously (28). ICCs were morphologically distinct from other cell types in culture, and it was therefore possible to identify these cells by phase contrast microscopy after they had been stained with the anti-c-Kit antibody.

**Patch-clamp experiments.** The whole-cell patch-clamp configuration was used to record membrane potentials (in current clamp mode) in cultured ICCs. An Axopatch 1D (Molecular Devices, LLC, Sunnyvale, CA, USA) was used to amplify membrane currents and potentials. The command pulse was applied using pCLAMP software (version 6.1; Molecular Devices, LLC). Data were obtained by filtering at 5 kHz and were displayed on an oscilloscope, a computer monitor, and detected using a Gould 2200 Series Analog Recorder (Gould Instrument Systems, Inc., Valley View, OH, USA). Results were analyzed using pCLAMP and Origin software (version 6.0; MicroCal, Northampton, MA, USA). All experiments were performed at 30-32°C.

**Solutions and drugs.** The physiological salt solution used to bathe cells ( $\text{Na}^+$ -containing Tyrode's Solution) consisted of 5 mmol/l KCl, 135 mmol/l NaCl, 2 mmol/l  $\text{CaCl}_2$ , 10 mmol/l glucose, 1.2 mmol/l  $\text{MgCl}_2$  and 10 mmol/l HEPES, adjusted to pH 7.4 with NaOH. CPE (1-10 mg/ml) was added to ICC bath solutions for 2 min. The pipette solution consisted of 140 mmol/l KCl, 5 mmol/l  $\text{MgCl}_2$ , 2.7 mmol/l  $\text{K}_2\text{ATP}$ , 0.1 mmol/l NaGTP, 2.5 mmol/l creatine phosphate disodium, 5 mmol/l HEPES and 0.1 mmol/l EGTA adjusted to pH 7.2 with KOH. All drugs including, methocramine, diphenylacetoxypiperidinium iodide (4-DAMP), guanosine 5'-( $\beta$ -thio) diphosphate trilithium salt (GDP- $\beta$ -S), U-73312, U-73343,

PD98059, SB203580 and the JNK II inhibitor SP600125, were obtained from Sigma-Aldrich (Merck Millipore). Drugs were dissolved in distilled water and added to the physiological salt solution at the desired concentrations immediately prior to use. The addition of these drugs to the solution for 5 min did not alter the pH. 4-DAMP was dissolved in DMSO to produce a 50 mmol/l stock solution, which was subsequently added to the bathing solution at a final concentration of 10  $\mu$ M on the day of the experiment for 5 min. The final concentration of DMSO in the culture solution was <0.1% and preliminary experiments confirmed that this concentration of DMSO did not affect results. In addition, 25  $\mu$ l methocitramine was dissolved in distilled water to produce a 50 mmol/l stock solution, which was added to the culture solution at a final concentration of 10  $\mu$ M on the day of the experiment for 5 min. GDP- $\beta$ -S was dissolved in DMSO to produce a 1 mol/l stock solution, which was added to the pipette solution at a final concentration of 1 mM on the day of the experiment. Both U-73312 and U-73343 were dissolved in DMSO to produce a 5 mmol/l stock solution, which was added to the culture solution at a final concentration of 5  $\mu$ M on the day of the experiment for 5 min. PD98059, SB203580 and the JNK II inhibitor were dissolved in DMSO to produce 10 mmol/l stock solutions, which were added to the culture solution at a final concentration of 10  $\mu$ M on the day of the experiment for 5 min.

**Statistical analysis.** Results are expressed as the mean  $\pm$  standard error. The Student's *t*-test and one-way analysis of variance with Bonferroni's post-hoc tests were used to test for significance among groups.  $P < 0.05$  was considered to indicate a statistically significant difference. The *n* values refer to the number of cells used in patch-clamp experiments.

## Results

**Effect of CPE on pacemaker potentials in cultured ICCs.** The initial aim of the current study was to investigate the effects of CPE on ICC pacemaker potentials. Recordings from cultured ICCs under current clamp mode ( $I = 0$ ) demonstrated the occurrence of spontaneous pacemaker potentials, with a resting membrane potential of  $-58.2 \pm 1.2$  mV and an amplitude of  $25.3 \pm 1.7$  mV. In the presence of CPE (1-10 mg/ml), membrane potentials were significantly depolarized compared with the control group to  $6.8 \pm 1.0$  mV at 1 mg/ml ( $P = 0.0012$ ),  $24.8 \pm 1.3$  mV at 5 mg/ml ( $P < 0.0001$ ) and  $28.8 \pm 0.9$  mV at 10 mg/ml ( $P < 0.0001$ ) CPE, with corresponding significantly reduced amplitudes of  $20.2 \pm 1.5$  mV ( $P = 0.012$ ),  $2.3 \pm 0.5$  mV ( $P < 0.0001$ ) and  $2.2 \pm 0.6$  mV ( $P < 0.0001$ ), respectively (Fig. 2A-D). A summary of values, together with a bar graph demonstrating the effects of CPE on pacemaker potentials are provided in Fig. 2E and F ( $n = 7$ ).

**Identification of CPE-target receptor subtypes in cultured ICCs.** Muscarinic receptors are known to mediate membrane depolarization and excitatory junction potentials in the GI tract (29,30). In addition, it has been reported that isolated ICCs express  $M_2$  and  $M_3$  muscarinic receptor subtypes in the GI tract (31). Therefore, in order to determine whether CPE-induced membrane depolarization involves muscarinic receptors, the effect of CPE on  $M_2$  and  $M_3$  muscarinic receptors

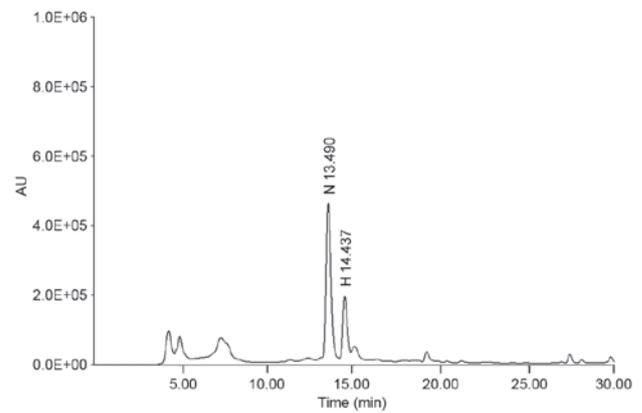


Figure 1. Chromatogram demonstrating the composition of *Citrus unshiu* peel extracts as determined by high-performance liquid chromatography. N, naringin; H, hesperidin; AU, absorption unit.

was investigated. ICCs were pretreated with muscarinic receptor antagonists prior to treatment with CPE. To achieve this, ICCs were first exposed to the muscarinic  $M_2$  receptor antagonist, methocitramine, and the muscarinic  $M_3$  receptor antagonist, 4-DAMP, at a concentration of 10  $\mu$ M for 5 min, before 5 mg/ml CPE was added. Treatment with methocitramine or 4-DAMP alone did not affect pacemaker potentials (data not shown), and pretreatment with methocitramine did not significantly inhibit the effect of CPE on the pacemaker potential compared with CPE treatment alone (Fig. 3A). Membrane depolarization in the presence of methocitramine by CPE was  $23.7 \pm 1.1$  mV ( $n = 6$ ), however, following the pretreatment of ICCs with 4-DAMP, membrane depolarization was inhibited compared with CPE treatment alone ( $P < 0.0001$ ; Fig. 3B and C). The membrane depolarization signal produced in the presence of 4-DAMP was  $0.6 \pm 0.6$  mV ( $n = 6$ ; Fig. 3C). These results suggest that CPE may affect ICC membrane potential through the  $M_3$  receptor.

**Involvement of G-proteins in CPE-induced depolarization of pacemaker potentials in cultured ICCs.** The effect of CPE-induced pacemaker potential depolarization in ICCs following treatment with GDP- $\beta$ -S, a non-hydrolysable guanosine 5'-diphosphate analogue that permanently inactivates G-protein binding proteins (32,33), was examined in order to determine the role of G proteins in mediating this effect. As demonstrated in Fig. 2C, CPE (5 mg/ml) induced ICC membrane depolarization. However, upon exposure to 1 mM GDP- $\beta$ -S, CPE membrane depolarization was only partially induced compared with CPE alone (Fig. 4A). As a result, the membrane depolarization induced by CPE was significantly reduced in the presence of GDP- $\beta$ -S ( $P = 0.0009$ ;  $n = 6$ ; Fig. 4B). These results suggest that G proteins may be involved in mediating CPE-induced pacemaker depolarization in ICCs.

**Effect of phospholipase C (PLC) inhibition on CPE-induced pacemaker potential depolarization.** A previous study demonstrated that membrane depolarization in ICCs may be associated with intracellular  $Ca^{2+}$  mobilization (28). Therefore, the current study aimed to determine whether the CPE-induced effects on the pacemaker potential of ICCs

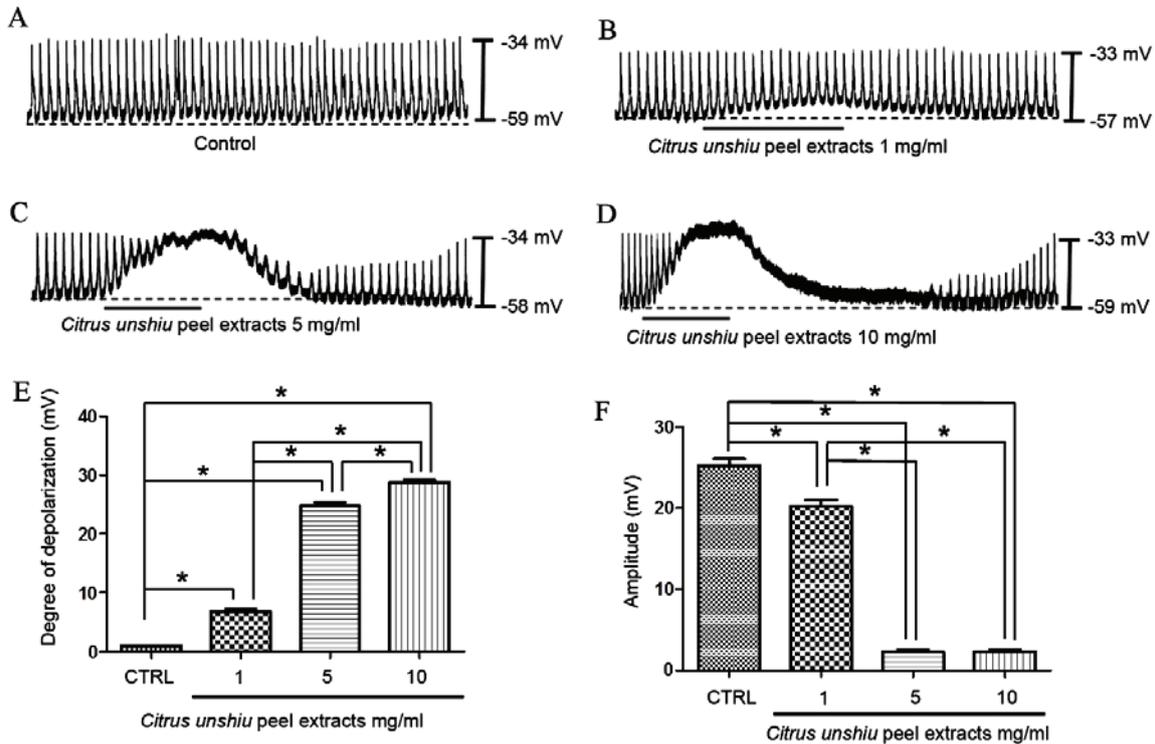


Figure 2. Effects of CPE on pacemaker potentials in cultured ICCs from the murine small intestine. The pacemaker potentials in current clamp mode ( $I=0$ ) of (A) untreated control ICCs, and those treated with (B) 1 mg/ml (C) 5 mg/ml and (D) 10 mg/ml CPE. Responses to CPE are summarized as (E) the degree of depolarization and (F) the amplitude of the pacemaker potential. Data are presented as the mean  $\pm$  standard error of 7 experiments. \* $P < 0.05$  vs. indicated treatment groups. CPE, *Citrus unshiu* peel extracts, ICCs, interstitial cells of Cajal; CTRL, control.

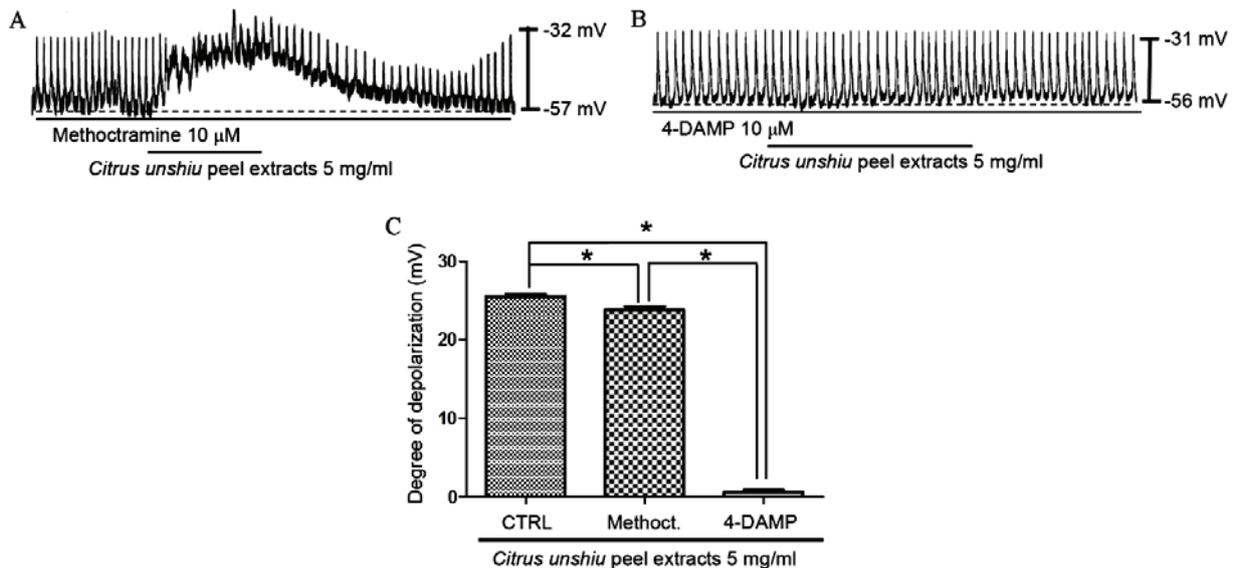


Figure 3. Effect of muscarinic receptor subtype antagonists on CPE-induced pacemaker potential depolarization in cultured ICCs. The pacemaker potentials of ICCs exposed to (A) 10  $\mu$ M methoctramine (a muscarinic  $M_2$  receptor antagonist) or (B) 10  $\mu$ M 4-DAMP (a muscarinic  $M_3$  receptor antagonist) prior to treatment with 5 mg/ml CPE. Pacemaker potentials were depolarized in the presence of methoctramine, but were unchanged following exposure to 4-DAMP. (C) Summary of the degree of membrane depolarization following exposure to CPE in the presence of methoctramine and 4-DAMP receptor antagonists. Results are presented as the mean  $\pm$  standard error of 6 experiments. \* $P < 0.05$  vs. indicated treatment groups. CPE, *Citrus unshiu* peel extracts, ICCs, interstitial cells of Cajal; 4-DAMP, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide; CTRL, control.

require PLC. To investigate this, CPE (5 mg/ml)-induced membrane depolarization in the absence and presence of the active PLC inhibitor U-73122 (5  $\mu$ M) was examined (34). As demonstrated in Fig. 5A, CPE-induced pacemaker

membrane depolarization was eliminated upon exposure of cells to U-73122. Under these conditions, CPE induced minor membrane depolarization ( $n=5$ ; Fig. 5A). In the presence of U-73122, the membrane depolarization produced by CPE was

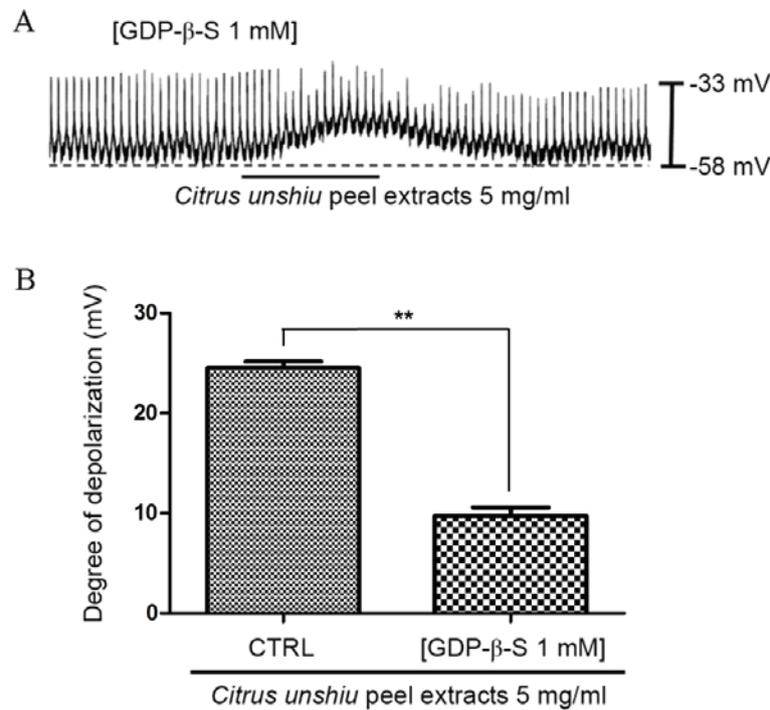


Figure 4. Effect of GDP-β-S on CPE-induced pacemaker potential depolarization in cultured ICCs. (A) Pacemaker potentials of ICCs exposed to CPE (5 mg/ml) in the presence of GDP-β-S (1 mM) in the pipette. Under these conditions, CPE induced minor pacemaker potential depolarization. (B) The degree of membrane depolarization following exposure to CPE in the presence of GDP-β-S. Data are presented as the mean ± standard error of 6 experiments. \*\*P<0.01 vs. CPE-only treated CTRL samples. GDP-β-S, guanosine 5'-(β-thio) diphosphate trilithium salt; CPE, *Citrus unshiu* peel extracts, ICCs, interstitial cells of Cajal; CTRL, control.

3.2±0.5 mV, and the membrane depolarization signal generated by exposure to CPE in the presence of U-73122 was significantly lower compared with CPE-only treated controls (P<0.0001). By contrast, pretreatment of ICCs with an inactive analog of U-73122 (U-73343; 5 μM) did not significantly alter the pacemaker potential, thus, CPE-induced membrane depolarization was not suppressed by U-73343 (n=5; Fig. 5B). These results suggest that the PLC pathway may be involved in CPE-induced pacemaker depolarization in ICCs.

*Involvement of mitogen-activated protein kinases (MAPKs) on CPE-induced ICC pacemaker potential depolarization.* Stimulation of muscarinic receptors has been demonstrated to activate MAPKs in a variety of cellular systems (35). Therefore, the role of MAPKs in the effects of CPE on membrane depolarization in cultured ICCs was investigated using a p42/44 MAPK inhibitor, PD98059, a p38 MAPK inhibitor, SB203580, and a c-jun NH2-terminal kinase (JNK) II inhibitor, SP600125. In the presence of PD98059 (10 μM), CPE generated partial membrane depolarization signal (n=5; Fig. 6A), which indicates that p42/44 may affect CPE-induced membrane depolarization. In addition, exposure to SB203580 (Fig. 6B) or SP600125 (Fig. 6C) partially inhibited the pacemaker potential depolarization induced by CPE (n=5). Depolarization was significantly reduced in the presence of the MAPK inhibitors compared with CPE treatment alone (P<0.0001, PD98059; P<0.0001, SB203580; P<0.0001, SP600125). Membrane depolarization was inhibited to the greatest degree upon exposure to PD98059, SB203580 and SP600125 (n=5; Fig. 6D and E). These results suggest that

MAPKs are important in modulating CPE-induced ICC pacemaker potential depolarization.

## Discussion

Citrus fruits contain sugar, organic acids and a number of physiologically functional components, including citric acid, ascorbic acid, minerals, coumarins and flavonoids (naringin, hesperidin, neohesperidin, rutin, naringenin, hesperetin, nairutin, and tangeretin) (36,37). *C. unshiu* is commonly known as the tangerine or mandarin orange. Traditionally, the *C. unshiu* peel has been used as a folk remedy to treat the common cold, dyspepsia, coughs and phlegm production (38). *C. unshiu* peel contains an abundance of flavonoids, which are known to possess a number of different beneficial effects (39-41). Hesperidin, naringin, and nobiletin (42,43) levels are high in citrus fruits (including *C. unshiu* peel), and are used as chemical quality control markers for *C. unshiu* peel products. Hesperidin is the most abundant flavonoid in citrus peel (42,43). In Korea, extracts of dried *C. unshiu* peel are sold as commercially available medicines for the treatment of a variety of GI disorders, and single extract doses of 0.5-15 g are generally recommended (21). However, despite the abundance of these biomolecules in citrus fruits, to the best of our knowledge, there is currently no data regarding the prokinetic activity of CPE. In addition, the molecular and physiological mechanisms underlying the therapeutic effects of *C. unshiu* peel on GI disorders has not yet been elucidated.

ICCs are known to be the pacemaker cells that modulate GI motility by generating pacemaker currents that produce

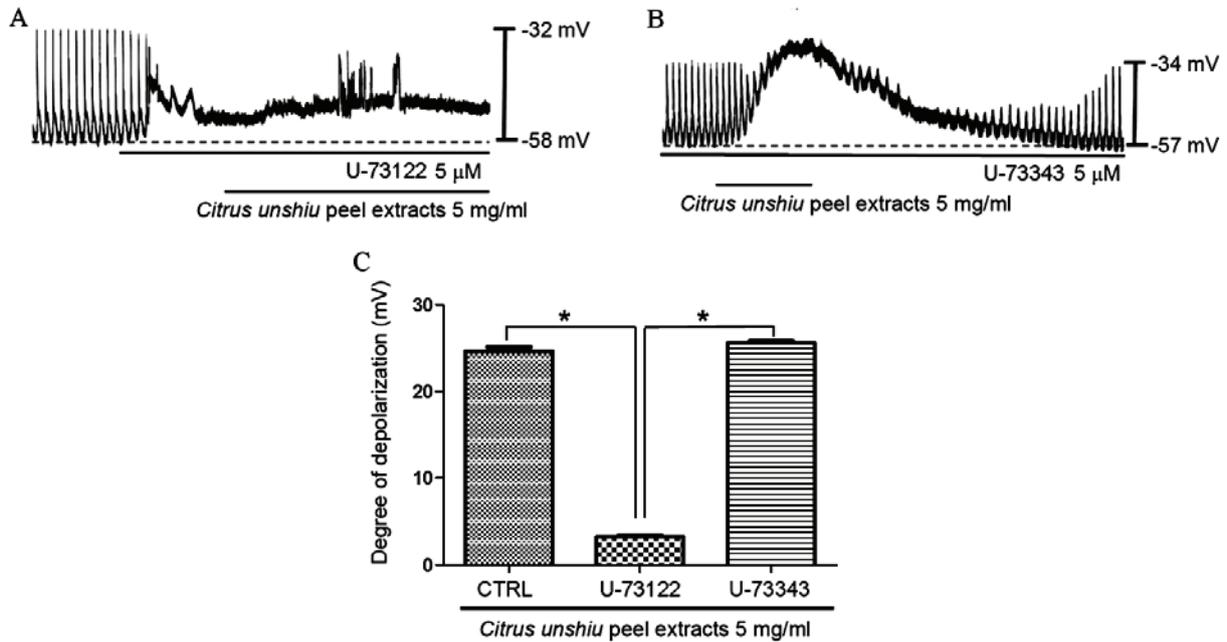


Figure 5. Effect of PLC inhibition on CPE-induced pacemaker potential depolarization in cultured ICCs. Pacemaker potentials of ICCs exposed to CPE (5 mg/ml) following treatment with (A) U-73122 (5  $\mu$ M; an active PLC inhibitor), which abolished the generation of pacemaker potentials, or (B) U-73343 (5  $\mu$ M; an inactive U-73122 analog), which demonstrated no significant effect on pacemaker potentials and failed to inhibit CPE-induced membrane depolarization. (C) Summary of the degree of membrane depolarization following exposure to CPE in combination with U-73122 or U-73343. Data are presented as the mean  $\pm$  standard error of 5 experiments. \* $P$ <0.05 vs. the U-73122-treated cells. PLC, phospholipase C; CPE, *Citrus unshiu* peel extracts, ICCs, interstitial cells of Cajal; CTRL, control.

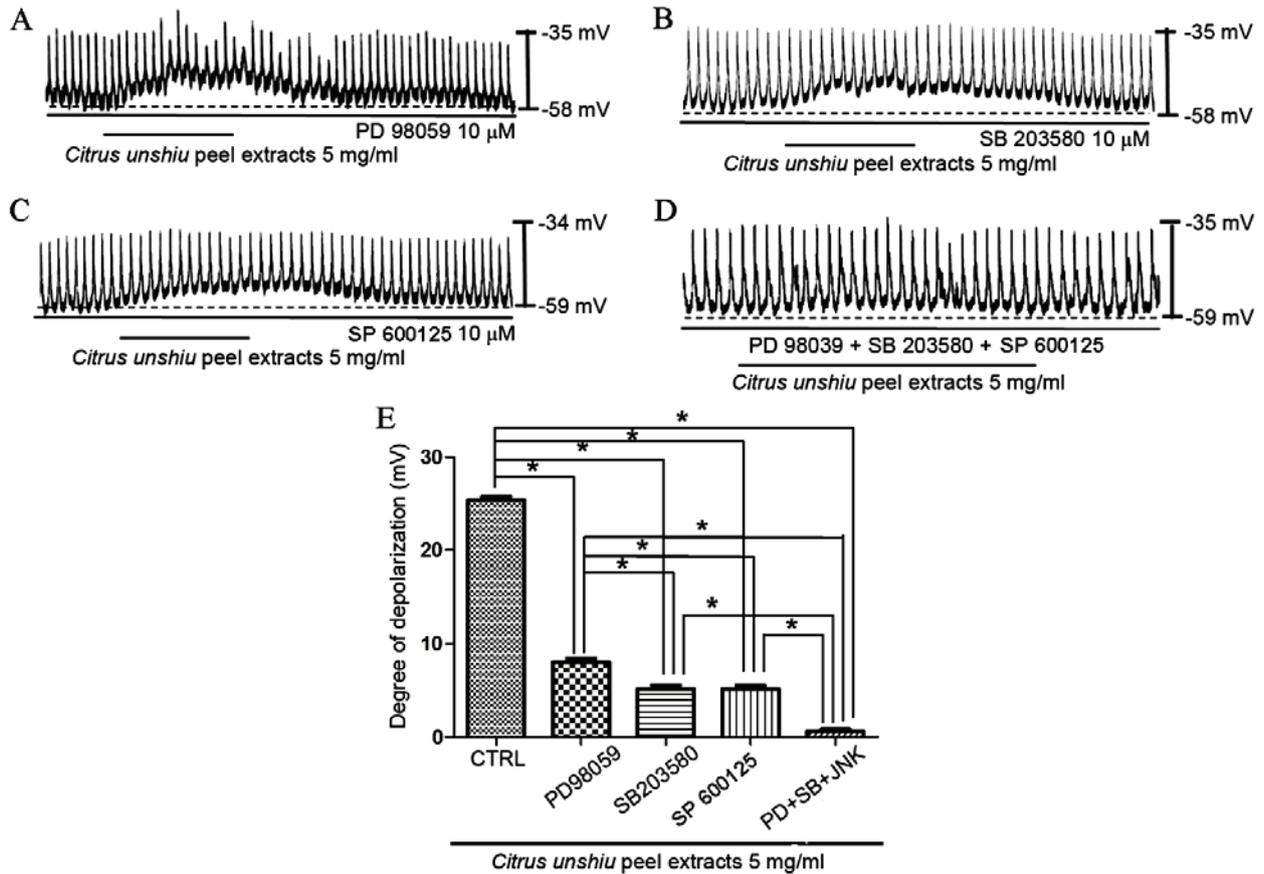


Figure 6. Effect of MAPK inhibitors on CPE-induced pacemaker potential depolarization in cultured ICCs. Pacemaker potentials of cultured ICCs exposed to CPE (5 mg/ml) in combination with (A) PD98059 (10  $\mu$ M; a p42/44 MAPK inhibitor), (B) SB203580 (10  $\mu$ M; a p38 MAPK inhibitor), (C) SP600125 (10  $\mu$ M; a c-jun NH2-terminal kinase II inhibitor) or (D) PD98039, SB203580 plus SP600125. (E) Summary of the degree of membrane depolarization following exposure to CPE and the MAPK inhibitors. Data are presented as the mean  $\pm$  standard error of 5 experiments. \* $P$ <0.05 vs. indicated treatment groups. MAPK, mitogen-activated protein kinase; CPE, *Citrus unshiu* peel extracts, ICCs, interstitial cells of Cajal; CTRL, control.

slow wave potentials. ICCs are connected to each other and to neighboring smooth muscle cells via gap junctions (23,24). Numerous neurotransmitters, including acetylcholine and 5-hydroxytryptamine, and diverse drugs or traditional herbal medicines (e.g. Ge-Gen-Tang) have been demonstrated to elicit excitatory or inhibitory effects on the pacemaker activity of ICCs (44,45), which supports the notion that ICCs are a critical in the control of smooth muscle motility in the GI tract.

In the present study, CPE was observed to modulate the pacemaker potential of ICCs. CPE produced pacemaker depolarization in current clamp mode. In addition, exposure of ICCs to the M<sub>3</sub> muscarinic receptor antagonist, 4-DAMP, inhibited CPE-induced pacemaker depolarization, whereas exposure to the M<sub>2</sub> receptor antagonist, methoctramine, did not. When GDP-β-S was present in the pipette solution, CPE induced minor pacemaker depolarization. In addition, membrane depolarization by CPE was inhibited following treatment of ICCs with the active PLC inhibitor U-73122. Furthermore, in the presence of MAPK inhibitors PD98059, SB203580 and SP600125, CPE produced slight membrane depolarization. These results suggest that CPE affects GI motility by modulating ICC pacemaker activity through G protein-dependent PLC and MAPK pathway-mediated activation of muscarinic M<sub>3</sub> receptors.

In the GI tract, M<sub>2</sub> and M<sub>3</sub> muscarinic receptors are involved in GI motility (46). However, no effect on CPE-induced pacemaker membrane depolarization was observed following exposure of ICCs to methoctramine in the present study. The GI tract is composed of smooth muscle, the enteric nervous system and ICCs. Therefore, we hypothesize that CPE may function to activate the M<sub>3</sub> receptor in ICCs, and the M<sub>2</sub> receptor may be involved in modulating smooth muscle or enteric nervous system functions. In support of this notion, So *et al* (47) suggested that the modulation of pacemaker currents by the muscarinic agonist carbachol is mediated by only muscarinic M<sub>3</sub> receptors and not M<sub>2</sub> receptors in ICCs. In addition, during the recording of intracellular Ca<sup>2+</sup> concentrations using fluo-3-AM dye, carbachol increased intracellular Ca<sup>2+</sup> concentrations and Ca<sup>2+</sup> oscillations. Therefore, it is possible that CPE may modulate ICC pacemaker potentials through muscarinic M<sub>3</sub> receptors only, through an intracellular Ca<sup>2+</sup> release-dependent mechanism. Future studies will aim to investigate the effects of CPE in Ca<sup>2+</sup> regulation. Acetylcholine muscarinic receptors are a family of G protein-couples receptors, and are composed of five subtypes (M<sub>1</sub>-M<sub>5</sub>). Of these, three (M<sub>1</sub>, M<sub>3</sub>, and M<sub>5</sub>) are coupled with PLC through a G<sub>q</sub> protein, whereas the M<sub>2</sub> and M<sub>4</sub> subtypes inhibit adenylate cyclase through G<sub>i</sub> or G<sub>o</sub> proteins (35). Stimulation of muscarinic receptors in a variety of cellular systems has been demonstrated to activate MAPKs (35), which are a family of protein kinases that with central roles in signal transduction (48). MAPKs regulate a variety of cellular responses, including inflammation, cell cycle progression, proliferation, differentiation and protein synthesis (49). However, the mechanisms underlying MAPK activation in response to muscarinic receptor stimulation remain to be elucidated. M<sub>2</sub> and/or M<sub>3</sub> receptors have been shown to mediate activation of the MAPK pathway (50,51) and muscarinic receptors and the MAPK signaling pathway are known to mediate proliferative responses in various

cell types (52-61). Matthiesen *et al* (52) suggested that these proliferative effects are due to M<sub>2</sub> receptor and G<sub>i</sub> protein-mediated MAPK activation, however, several G protein-coupled-MAPK activation pathways have been identified (53,54). Acetylcholine stimulates the proliferation of colon carcinoma cell lines through M<sub>3</sub> receptor-dependent phosphorylation of MAPK (55-57). In addition, cholinergic neurotransmitters stimulate the growth of astrocytoma and breast cancer cells through the AKT serine/threonine kinase or MAPK signaling pathways (58,59). Furthermore, acetylcholine stimulates ovarian or lung cancer growth through muscarinic receptor-mediated phosphorylation of MAPK (60,61). In a previous study, the effect of *C. unshiu* peel on the production of proinflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells was investigated (16). The results demonstrated that *C. unshiu* peel significantly reduced the phosphorylation of all LPS-stimulated MAPKs in a dose-dependent manner (16). Therefore, we hypothesize that MAPKs are important for the effect of *C. unshiu* peel on ICC membrane depolarization.

In conclusion, the results of the present study suggest that *C. unshiu* peel may be a suitable candidate for the development of prokinetic agents that prevent or alleviate GI disorders.

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