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ORIGINAL ARTICLE

Effects of *Atractylodes Japonica* Extract on Pacemaker Potentials Generated by Interstitial Cells of Cajal from Murine Small Intestine

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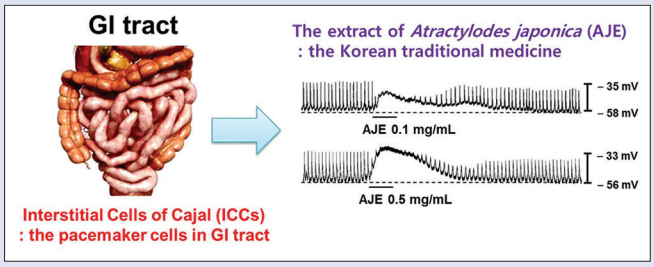
ABSTRACT

Background: *Atractylodes japonica* has been commonly used to treat gastrointestinal (GI) disorders in Korean traditional medicine. Interstitial cells of Cajal (ICCs) are pacemaker cells in the GI tract and can regulate GI motility. **Objective:** To investigate the effects of the extract of *Atractylodes japonica* (AJE) on pacemaker potentials generated by ICCs from murine small intestine. **Materials and Methods:** Enzymatic digestion was performed to dissociate ICCs. All experiments on ICCs were performed after 12 h of culture. The whole-cell patch-clamp configuration was used to record pacemaker potentials generated by ICC. **Results:** AJE (0.1–1 mg/mL) depolarized pacemaker potentials in a concentration-dependent manner and decreased the amplitudes of pacemaker potentials at all concentrations in the current-clamp mode. Pretreatment with Y25130 (a 5-HT_3 receptor antagonist), RS39604 (a 5-HT_4 receptor antagonist), or SB269970 (a 5-HT_7 receptor antagonist) had no effects on depolarization of pacemaker potentials induced by AJE. In addition, pretreatment with 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (a muscarinic M_3 receptor antagonist) inhibited AJE-induced depolarization of pacemaker potential; however, pretreatment with methoctramine (a muscarinic M_2 receptor antagonist) did not affect depolarization of pacemaker potentials induced by AJE. In the presence of an external Na^+-free solution, the pacemaker potentials decreased, and under this condition, AJE did not depolarize the pacemaker potentials. Flufenamic acid, a nonselective cation channel (NSCC) blocker, decreased the pacemaker potential, which in turn inhibited AJE-induced depolarization of pacemaker potential. **Conclusion:** The results of this study suggest that AJE depolarized the pacemaker potentials generated by ICC by stimulating muscarinic M_3 receptors, but not 5-HT receptors, through NSCCs. Therefore, AJE can be a novel prokinetic agent.

Key words: *Atractylodes japonica*, gastrointestinal tract, interstitial cells of Cajal, pacemaker potential, prokinetic agent

SUMMARY

- AJE can depolarize pacemaker potentials and decrease the amplitudes of pacemaker potentials in a concentration-dependent manner in ICCs
- 5-HT receptors are not involved in AJE-induced depolarization of pacemaker potential
- Muscarinic M3 receptors are involved in AJE-induced depolarization of pacemaker potential
- Flufenamic acid (NSCC blocker) inhibited the depolarization of pacemaker potentials induced by AJE.



Abbreviations used: ICCs:Interstitial cells of Cajal, GI:Gastrointestinal, NSCC:Non-selective cation channel, TRP:Transient receptor potential.

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INTRODUCTION

Atractylodes japonica has commonly been used in Korean traditional medicine as a remedy for digestive disorders such as disorders of gastrointestinal (GI) motility and gastric secretion.[1,2] Despite the considerable use of *A. japonica* for modulating GI functions, its pharmacological roles in the regulation of GI motility have not been understood yet.

Interstitial cells of Cajal (ICCs) are pacemaker cells in the GI tract that generate rhythmic oscillations in membrane potentials known as slow waves.[3,4] In addition, it has been known that endogenous or exogenous agents, such as neurotransmitters, hormones, or traditional medicines, can regulate GI motility by influencing ICCs.[5-7] Therefore, ICCs have important roles in the regulation of GI motility.[8] However, little is known about the effects of the extract of *A. japonica* (AJE) on the pacemaker potentials generated by ICCs in the GI tract. In the present study, we investigated whether AJE can modulate the pacemaker potentials generated by ICCs from murine small intestine.

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MATERIALS AND METHODS

Preparation of extract of *Atractylodes japonica*

The dried root of *A. japonica* was purchased from Boncho Co. (Daejoen, Korea). The plant sample was identified by Dr. Yun Tai Kim according to the "Illustrated Guide to Clinical Medical Herbs,"[9] and a voucher specimen (#NP-3009) was deposited with the Research group of innovative special food, Korea Food Research Institute. The dried root of *A. japonica* (600 g) was extracted with 70% ethanol (6000 mL) for 3 h at 80°C. The extracts were filtered through a filter paper (No. 4, 185 mm, Whatman™, Buckinghamshire, UK), and the solvents were removed using rotary evaporator (N-21NS, EYELA, Tokyo, Japan). The remaining extracts were freeze dried to yield around 32.86% of the dried root weight (w/w).

Preparation of cells and cell cultures

Animal care and experiments were conducted in accordance with the guidelines issued by the Institutional Animal Care and Use Committee at Pusan National University (Busan, Republic of Korea; Approval no. PNU-2016-1370). The small intestines of ICR mice (3 to 5 day old) were excised (from 1 cm below the pyloric ring to the cecum) and opened along the mesenteric border. The luminal contents were removed using Krebs–Ringer bicarbonate solution, and the tissues were pinned to the base of Sylgard dishes. Mucosae were removed by sharp dissection. Small tissue strips of intestinal muscle (comprising circular and longitudinal muscles) were equilibrated for 30 min in Ca^{2+}-free Hank's solution. The cells were then dispersed in an enzyme solution containing collagenase (Worthington Biochemical, Lakewood, NJ, USA; 1.3 mg/mL), bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA; 2 mg/mL), trypsin inhibitor (Sigma-Aldrich; 2 mg/mL), and ATP (0.27 mg/mL) and plated on sterile glass coverslips coated with murine collagen (2.5 mg/mL; Falcon/BD, Franklin Lakes, NJ, USA) in 35 mm culture dishes. The cells were cultured in smooth muscle growth medium (Clonetics, San Diego, CA, USA) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, USA) and murine stem cell factor (5 ng/mL; Sigma-Aldrich) in a 95% oxygen/5% carbon dioxide incubator at 37°C. Because the ICC morphology differed from other cell types in the culture, they could be identified under a phase contrast microscope after incubation with anti-c-Kit antibody.

Patch clamp experiments

Physiological salt solution was used to bathe cultured ICC clusters (Na^+-Tyrode), and it contained the following: KCl, 5 mM; NaCl, 135 mM; calcium chloride ($CaCl_2$), 2 mM; glucose, 10 mM; magnesium chloride ($MgCl_2$), 1.2 mM; and HEPES, 10 mM (adjusted to pH 7.4 with NaOH). The pipette solution used to examine pacemaker activity contained the following: KCl, 140 mM; $MgCl_2$, 5 mM; dipotassium ATP (K_2ATP), 2.7 mM; sodium GTP (NaGTP), 0.1 mM; creatine phosphate disodium, 2.5 mM; HEPES, 5 mM; and ethylene glycol tetraacetic acid, 0.1 mM (adjusted to pH 7.2 with potassium hydroxide). The patch clamp techniques were conducted in whole-cell configuration to record potentials (i.e., current clamp mode) from cultured ICCs using Axopatch I-D and Axopatch 200B amplifiers (Axon Instruments, Foster, CA, USA). The results were analyzed using pClamp and Origin software (version 6.0, Microcal, Northampton, MA, USA). All experiments were performed at 30°C–33°C.

Drugs

The drugs used in the experiments including 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), methoctramine, Y25130, RS39604, SB269970, and flufenamic acid were purchased from Sigma-Aldrich. Stock solutions were prepared and stored according to the manufacturer's instructions.

Statistical analysis

The results are expressed as means ± standard error of the means. N values refer to the number of cells used in the experiments. For multiple comparison analysis, one-way ANOVA with Bonferroni's *post hoc* comparison was used. For statistical analyses, Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and Origin version 8.0 (OriginLab Corporation, Northampton, MA, USA) were used. $P < 0.05$ was considered as statistically significant.

RESULTS

Depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine by extract of *Atractylodes japonica*

Under current-clamp mode (I = 0), ICCs generated pacemaker potentials [Figure 1] with a mean resting membrane potential of −56.7 ± 2.1 mV and a mean amplitude of 24.5 ± 0.9 mV. AJE (0.1–1 mg/mL) depolarized pacemaker potentials in a concentration-dependent manner and decreased the amplitudes of pacemaker potentials at all concentrations [Figure 1b and c]. The mean degrees of depolarization at 0.1, 0.5, and 1 mg/mL were 14.1 ± 0.8, 23.7 ± 1.2, and 24.8 ± 0.9 mV, respectively [Figure 1d, n = 18], and the mean amplitudes at 0.1, 0.5, and 1 mg/mL were 1.5 ± 0.4, 2.3 ± 0.4, and 3.1 ± 0.6 mV, respectively [Figure 1e, n = 18). These results suggest that AJE dose dependently depolarized the pacemaker potentials generated by ICC.

Serotonergic receptor subtypes were not involved in extract of *Atractylodes japonica*-induced depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine

To investigate the relationship between AJE and its receptors, we first examined the involvement of 5-HT receptors because they can mediate GI motility and are strongly associated with prokinetic activity.[8,10,11] Only three receptors (5-HT_3R, 5-HT_4R, and 5-HT_7R) are present in ICCs,[8,12,13] and to check the 5-HT receptor subtypes involved in the effects of AJE, we pretreated ICCs with various 5-HT receptor antagonists, followed by treatment with AJE. ICCs were pretreated with Y25130 (a 5-HT_3 receptor antagonist), RS39604 (a 5-HT_4 receptor antagonist), and SB269970 (a 5-HT7 receptor antagonist) at a concentration of 10 μM for 5 min, respectively, followed by addition of AJE. However, pretreatment with Y25130, RS39604, or SB269970 showed no effects on AJE-induced depolarization of pacemaker potentials [Figure 2a-c]. In the presence of Y25130, RS39604, or SB269970, the mean depolarization of pacemaker potentials induced by AJE was 25.1 ± 1.4, 22.3 ± 0.9, or 23.0 ± 1.2 mV, and the mean amplitudes were 2.5 ± 0.5, 3.5 ± 0.9, or 2.9 ± 0.7 mV, respectively [n = 5, respectively; Figure 2d and e]. These results suggest that 5-HT receptors are not involved in AJE-induced depolarization of pacemaker potential.

Involvement of muscarinic receptor subtypes in extract of *Atractylodes japonica*-induced depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine

Next, we investigated the involvement of muscarinic receptor subtypes. Muscarinic receptors mediate membrane depolarization in GI smooth muscle cells.[14,15] Isolated ICCs express muscarinic M_2 and M_3 receptor subtypes in murine small intestine.[16] To investigate the involvement of muscarinic receptor subtypes, ICCs were pretreated with muscarinic

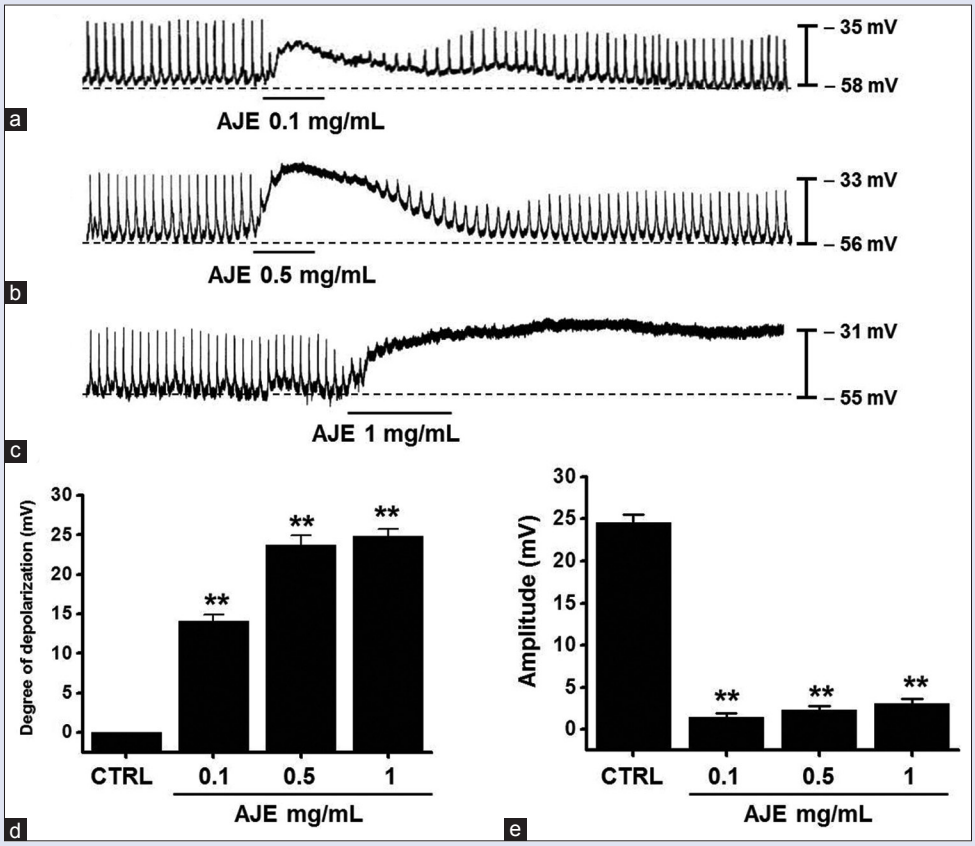

Figure 1: Effects of AJE on pacemaker potentials generated by interstitial cells of Cajals from murine small intestine. (a-c) AJE (0.1–1 mg/mL) depolarized the pacemaker potentials generated by interstitial cells of Cajals in the current-clamp mode ($I = 0$). (d and e) Summary of responses to AJE. Bars represent mean ± standard error of the means. $^{**}P < 0.01$: significantly different from nontreated controls. AJE: Extract of *Atractylodes japonica*; CTRL: Control

receptor antagonists, followed by treatment with AJE. Pretreatment with 4-DAMP (a muscarinic M_3 receptor antagonist) inhibited the depolarization of pacemaker potentials induced by AJE [Figure 3a]; however, pretreatment with methoctramine (a muscarinic M_2 receptor antagonist) did not block AJE-induced depolarization of pacemaker potentials [Figure 3b]. In the presence of 4-DAMP, the mean AJE-induced depolarization of pacemaker potentials and the mean amplitudes were 0.5 ± 0.6 and 24.4 ± 0.8 mV, respectively [$n = 5$; Figure 3c and 3d]. In the presence of methoctramine, the mean AJE-induced depolarization of pacemaker potentials and the mean amplitudes were 24.6 ± 1.0 and 4.5 ± 1.3 mV, respectively [$n = 5$; Figure 3c and 3d]. These results suggest that muscarinic M3 receptors are involved in AJE-induced depolarization of pacemaker potential.

Involvement of nonselective cation channels in extract of *Atractylodes japonica*-induced depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine

To investigate the characteristics of AJE-induced depolarization of pacemaker potential, we studied the effects of AJE in the presence of an external Na^+-free solution or flufenamic acid, a nonselective cation channel (NSCC) blocker. The pacemaker potentials decreased in the external Na^+-free solution, and under this condition, AJE did not depolarize the pacemaker potentials [Figure 4a]. In addition, flufenamic acid (30 μM) decreased the pacemaker potentials and subsequently inhibited AJE-induced depolarization of pacemaker potentials [Figure 4b]. In the presence of external Na^+-free solution, the mean AJE-induced depolarization of pacemaker potentials and the mean amplitudes were 0.9 ± 0.7 and 1.6 ± 0.4 mV, respectively [$n = 4$; Figure 4c and d]. In the presence of flufenamic acid, the mean AJE-induced depolarization of pacemaker potentials and the mean amplitudes were 0.8 ± 0.6 and 1.4 ± 0.3 mV, respectively [$n = 4$; Figure 4c and d]. These results suggest that the AJE-induced depolarization of pacemaker potentials is mediated by NSCCs.

DISCUSSION

In the present study, we investigated the effect of AJE on pacemaker potentials generated by ICCs from murine small intestine. AJE depolarized pacemaker potentials by stimulating muscarinic M_3 receptors, but not 5-HT receptors, through NSCCs, indicating that AJE can be a novel prokinetic agent that can modulate GI motility.

The rhizome of *A. japonica* Koidz (Compositae) has been known to exhibit various pharmacological properties, including anti-oxidant,[17] anti-inflammatory,[18] and GI modulatory effects.[2] *A. japonica* is commonly used to treat disorders of GI motility and gastric secretion in Korean traditional medicine.[19] Choi *et al.*[19] reported that *A. japonica* may act on the longitudinal muscles of distal colon, and the contraction of longitudinal muscles of distal colon induced by *A. japonica* is mediated by the activation of choline acetyltransferase and muscarinic receptors. In addition, Park *et al.*[1] suggested that AJE increased the colonic transit time, and among animals pretreated with thyrotropin-releasing hormone, the weight and number of fecal pellets were significantly decreased in those treated with *A. japonica*. Although

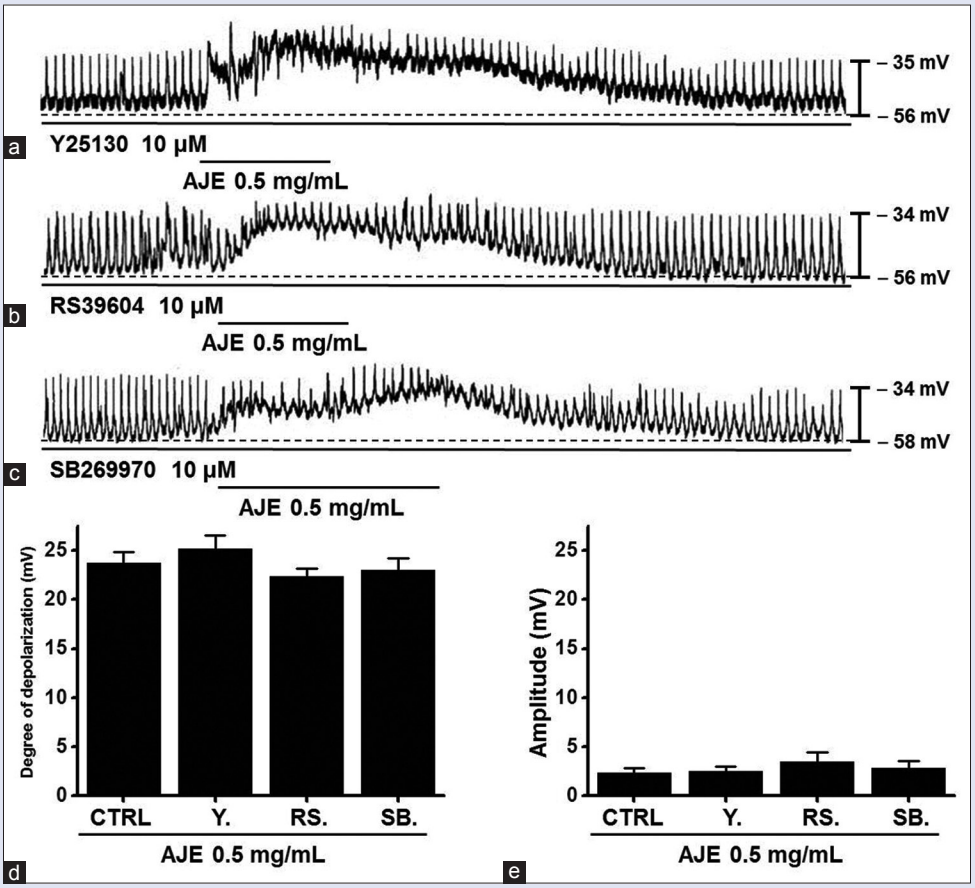

Figure 2: Effects of 5-HT receptor subtype antagonists on AJE-induced depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine. (a-c) AJE-induced depolarization of pacemaker potentials was not inhibited by treatment with 5-HT3 receptor antagonist (Y25130), 5-HT4 receptor antagonist (RS39604), and 5-HT7 receptor antagonist (SB269970). (d and e) Summary of effects of pretreatment with 5-HT receptor subtype antagonists. Bars represent mean ± standard error of the means. AJE: Extract of *Atractylodes japonica*; CTRL: Control; Y.: Y25130; RS.: RS39604; SB.: SB269970.

A. japonica is commonly used as a herbal remedy to improve GI motility disorders,[2] its pharmacological role in the regulation of GI motility has not been clarified yet. In this study, we observed that AJE modulates the pacemaker potentials generated by ICCs. Therefore, it can be suggested that AJE may regulate GI motility through ICC pacemaker potential. In future, we will investigate the effects of AJE on GI motility, including intestinal transit time or gastric emptying *in vivo*.

Muscarinic receptors are commonly expressed in the digestive tract.[20-23] The muscarinic receptors comprise five subtypes: muscarinic M_1, M_2, M_3, M_4, and M_5 receptors.[24,25] The major muscarinic receptors expressed in GI smooth muscle and ICCs are M_2 and M_3.[16,26] The muscarinic receptor subtype involved in the contractile response of GI smooth muscle or ICCs has been investigated both at mRNA and protein levels, and the studies revealed that both M_2 and M_3 receptors are present.[27,28] Furthermore, the activation of muscarinic M_1 receptors evokes the relaxation of gastric smooth muscles in M_3 knockout mice through an NO-mediated mechanism.[29,30] Muscarinic M_2 and M_4 receptors preferentially are coupled to Gai/o and muscarinic M_1, M_3, and M_5 receptors are coupled to Gaq/11. Muscarinic M_2 and M_4 receptors may inhibit adenylate cyclase activity and prolong the opening of K+, NSCCs, and transient receptor potential (TRP) channels.[31] In contrast, muscarinic M_1, M_3, and M_5 receptors increase intracellular calcium by mobilizing phosphoinositides that generate inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG).[32,33] In this study, AJE depolarized the pacemaker potentials from ICCs through muscarinic M_3 receptors [Figure 3]. Carbachol (CCh) induced the pacemaker potential depolarizations of ICCs,[34] and we think that AJE have similar effects with CCh on ICCs. ICCs regulated by CCh were modulated by G-protein through Ca^{2+} modulation[34] like AJE-induced depolarizations. Therefore, we think that TRPC4 or TRPC6 channels may be involved in MOE-induced muscarinic receptor activation through G protein in ICCs. Therefore, we hypothesize that AJE may exert its effects through G-protein-, IP_{3-}, and DAG-dependent pathways. In future, we will investigate the association of the effects of AJE with these pathways.

ICCs are pacemaker cells in the GI tract, and they generate and propagate slow waves that regulate GI motility.[8] A loss or deficiency of ICCs has been shown to result in GI dysfunction and is believed to contribute to the development of motility disorders.[35,36] Therefore, ICCs play a critical physiological role in the coordination of GI motility and therefore are good tools for studying GI motility.

CONCLUSION

The present study shows that AJE can depolarize pacemaker potentials and decrease the amplitudes of pacemaker potentials in a concentration-dependent manner. 5-HT receptors are not involved in AJE-induced depolarization of pacemaker potential. However, 4-DAMP (a muscarinic M_3 receptor antagonist) inhibited AJE-induced depolarization of pacemaker potential. In addition, flufenamic acid (NSCC blocker) inhibited the depolarization of pacemaker potentials induced by AJE. These findings suggest that AJE may be a novel prokinetic agent.

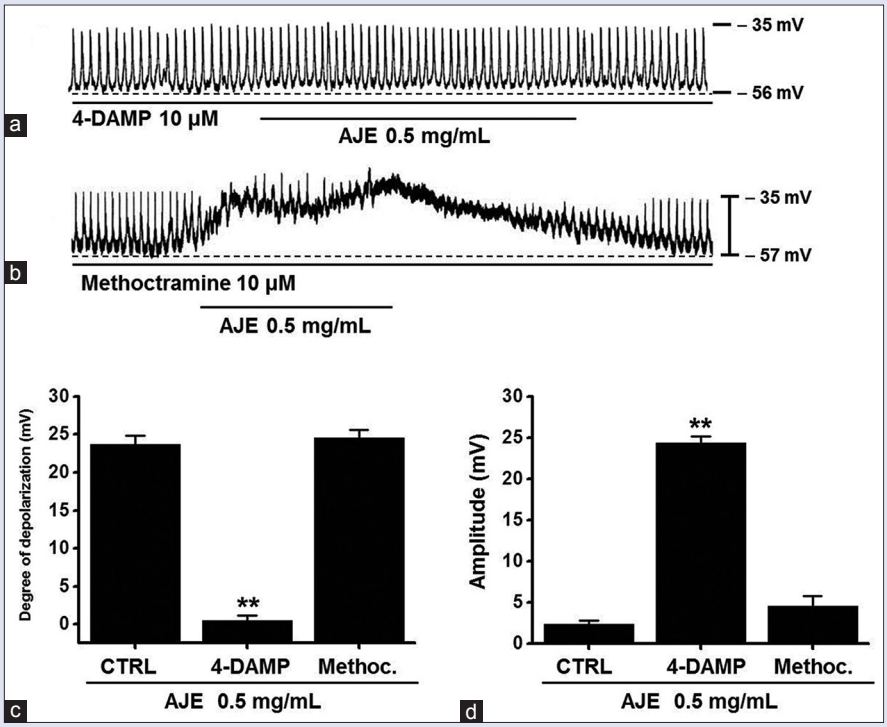

Figure 3: Effects of muscarinic receptor subtype antagonists on AJE-induced depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine. (a) In the presence of 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (a M3 receptor antagonist), AJE-induced depolarization was inhibited. (b) However, AJE-induced depolarization was not inhibited in the presence of methoctramine (a M2 receptor antagonist). (c and d) Summary of effects of pretreatment with muscarinic receptor subtype antagonists. Bars represent mean ± standard error of the means. ***P* < 0.01. AJE: Extract of *Atractylodes japonica*; CTRL: Control; Methoc: Methoctramine

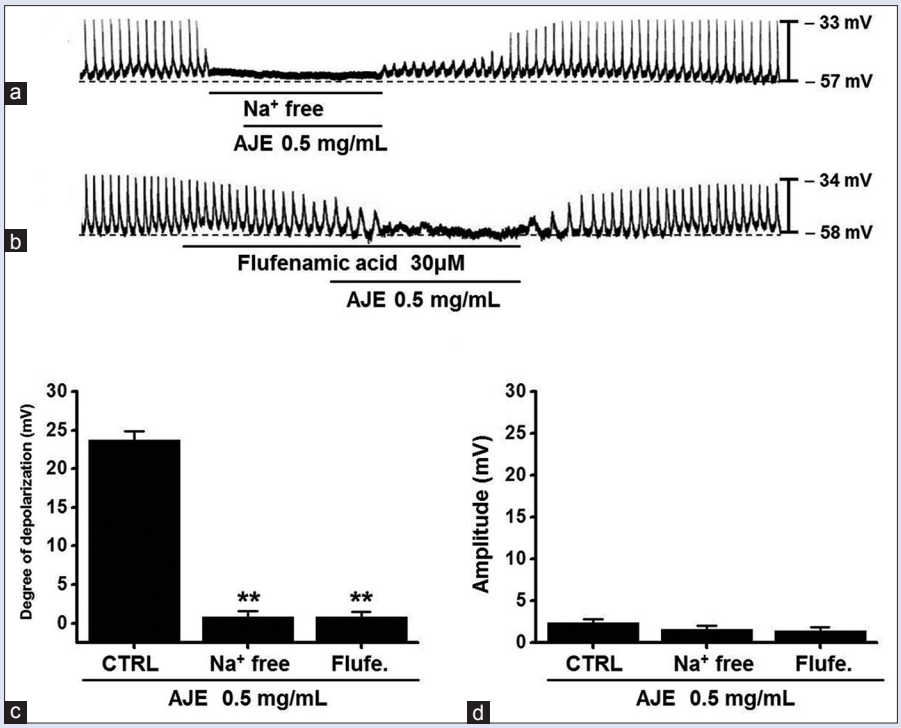

Figure 4: Effects of an external Na^+-free solution or flufenamic acid (an nonselective cation channel channel blocker) on AJE-induced depolarization of pacemaker potentials generated by interstitial cells of Cajals from murine small intestine. (a) In the presence of an external Na^+-free solution, AJE did not depolarize the pacemaker potentials. (b) Also, flufenamic acid (30 μM) inhibited AJE-induced depolarization of pacemaker potential. (c and d) Summary of effects of an external Na^+-free solution or flufenamic acid. Bars represent mean ± standard error of the means. ***P* < 0.01. AJE: Extract of *Atractylodes japonica*; CTRL: Control; Flufe: Flufenamic acid

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Nil.

Conflicts of interest

There are no conflicts of interest.

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