Natural Product Sciences
19(4) : 290-296 (2013)

Effects of Pine Needle Extract on Spontaneous Pacemaker Potentials in Interstitial Cells of Cajal from the Mouse Colon

Pawan Kumar Shahi[1], Dong Chaun Zuo[1], Seok Choi[1], Mi Jung Lee[1], Hyeon Sook Cheong[2], Dong Yoon Lim[3], and Jae Yeoul Jun[1,*]

[1]Department of Physiology, College of Medicine, Chosun University, Gwangju 501-759, Korea
[2]Department of Life Science, College of Natural Science, Chosun University, Gwangju 501-759, Korea
[3]Department of Pharmacology, College of Medicine, Chosun University, Gwangju 501-759, Korea

Abstracts – In preliminary tests, we examined the effect of several fractions isolated from fermented pine needle extract on pacemaker potentials in cultured interstitial cells of Cajal (ICCs) from the mouse colon using a whole cell patch clamp technique. Among these fractions, Fraction 3 (F3) elicited the most powerful depolarization of membrane. Therefore, the aim of the present study was to investigate the effect of F3 obtained from fermented extract of *Pinus densiflora* needle on pacemaker potentials in ICCs and to establish its mechanism of action. Colonic ICCs generated spontaneous periodic pacemaker potentials in the current-clamp mode. F3 depolarized the membrane and decreased the frequency and amplitude of pacemaker potentials in a dose-dependent fashion. The F3-induced effects on pacemaker potentials were blocked by methoctramine, a muscarinic M_2 receptor antagonist, and by glycopyrrolate, a muscarinic M_3 receptor antagonist. The F3-induced effects on pacemaker potentials were blocked by external Na^+-free solution and by flufenamic acid, a non-selective cation channel blocker, as well as by the removal of external Ca^{2+} and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum. Taken together, these results suggest that F3 of pine needle extract modulates the pacemaker activity of colonic ICCs by the activation of non-selective cation channels via muscarinic M_2 and M_3 receptors. And external Ca^{2+} influx and intracellular Ca^{2+} release are involved in F3 actions on ICCs.
Keywords – Fraction of pine needle extract, Interstitial cells of Cajal, Colon, Muscarinic receptor

Introduction

The gastrointestinal (GI) motility is regulated by interactions between smooth muscle cells, neurons, and interstitial cells of Cajal (ICCs) (Sanders *et al.,* 2012). ICCs are pacemaker cells driven to generate slow waves in smooth muscles, and mediate neural signals to smooth muscles, thus regulate gastrointestinal motility (Thomsen *et al.,* 1998; Sanders *et al.,* 2006). ICCs formed networks within GI tracts. The disruption of networks or reduction of cell number related with several motility disorders (Jain *et al.,* 2003; Streutker *et al.,* 2007). Therefore, ICCs are pivotal in regulation of GI motility and considered as therapeutic targets of motility disorders. Pine needle extracts as folk medicine or as food have been used to treat diseases of liver, GI tract, circulatory system, and skin problems (Yoon, 1997). The actions of pine needle extracts include the lowering blood lipid level, antioxidants, antitumor, and antibiotics (Kim *et al.,* 1998; Kong *et al.,* 1995; Lee, 2003; Moon *et al.,* 1993; Park *et al.,* 2002). Also, pine needle extracts caused vascular relaxation in vitro study and vasodepressor responses in animal studies (Cheong & Lim, 2010). Recently, Cheong's colleagues isolated several fractions from pine needle extracts, and called fraction 1 - 12. They reported that fraction 4 and 5 inhibited catecholamine release from adrenal medulla, suggesting its potential roles in reducing blood pressure as an antihypertensive component (Choi *et al.,* 2013). We also reported that pine needle extracts inhibited pacemaker activity of ICCs by activating ATP-sensitive K^+ channels via the production of prostaglandins, suggesting that pine needle extracts may suppress small intestinal motility (Cheong *et al.,* 2005). However, in this study, we found that fraction 3 isolated from pine needle extracts has an excitatory action on electrical activities of colonic ICCs. Thus, we report the possible role of motility-improving effects of fraction 3 obtained from pine extracts.

*Author for correspondence
Jae Yeoul Jun, Department of Physiology, College of Medicine, Chosun University, Sesuk-dong, Dongku 501-759, Gwanju, Korea.
Tel: +82-62-230-6412; E-mail: jyjun@chosun.ac.kr

Experimental

Preparation of pine needle fractions – The leaves of Korean red pine trees (*Pinus densiflora*) cultivated in Gokseong, Korea were harvested to prepare for extraction. They were washed with distilled water, chopped with a knife into approximately 2 cm pieces, ground for 1 min with water and homogenized. The preparation was allowed to settle for 3 h at 4 °C and the supernatant (extract) was recovered. This procedure was repeated three times and the three extracts were combined. Finally the combined extract was freeze-dried. This dried sample is referred to as the pine needle extract. Next, pine needle extract was fractionated to six fractions; Fr 1-2, Fr 3, Fr 4-5, Fr 6, Fr 7, and Fr 8-11 (Choi *et al.,* 2013).

Preparation of cells and tissues – Balb/C mice (8 - 13 days old) of both sexes were anesthetized with ether and killed by cervical dislocation. The large intestine below the cecum to the rectum were cut out and opened along the mesenteric border. The luminal contents were removed by washing with Krebs-Ringer bicarbonate solution, the tissues were pinned to the base of a Sylgard dish, and the mucosa was removed by sharp dissection. Small strips of colonic muscle (containing both circular and longitudinal muscles) were equilibrated in Ca^{2+}-free Hanks solution (containing in mM: KCl 5.36, NaCl 125, NaOH 0.34, Na_2HCO_3 0.44, glucose 10, sucrose 2.9 and HEPES 11) for 30 min. The cells were then dispersed with an enzyme solution containing collagenase (Worthington Biochemical Co., USA) 1.3 mg/ml, bovine serum albumin (Sigma) 2 mg/ml, trypsin inhibitor (Sigma, USA) 2 mg/ml and ATP 0.27 mg/ml. Thereafter they were plated onto sterile glass coverslips coated with poly-l-lysine (0.01%, Sigma) in 35 mm culture dishes, and cultured at 37 °C in a 95% O_2-5% CO_2 incubator in SMGM (smooth muscle growth medium, Lonza, Walkersville, USA) supplemented with 1% antibiotics/antimycotics (Gibco, USA) and murine stem cell factor (SCF, 5 ng/ml, Sigma).

Patch clamp experiments – The whole-cell configuration patch clamp technique was used to record membrane potentials of the cultured ICCs (current clamp). Membrane potentials were amplified by Axopatch 200B (Axon Instruments, USA). Command pulses were applied using an IBM-compatible personal computer and pClamp software (version 9.2; Axon Instruments). Data were filtered at 5 kHz and displayed on an oscilloscope and a computer monitor. The cells were bathed in a solution containing (in mM): KCl 5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1.2 and HEPES 10, adjusted to pH 7.4 with tris. The pipette solution contained (in mM) KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, disodium creatine phosphate 2.5, HEPES 5 and EGTA 0.1, adjusted to pH 7.2 with tris. Results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments were performed at 30 °C.

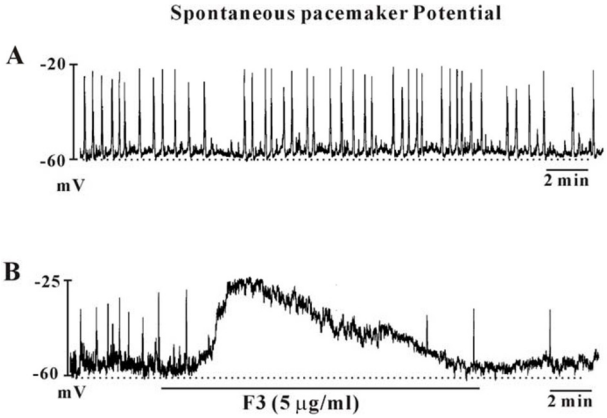

Fig. 1. Typical trace of pacemaker potentials and effects of fraction 3 (F3) isolated from pine needle extract in current clamping mode ($I = 0$) in cultured ICCs from the mouse colon. (A) shows the generation of spontaneous pacemaker potentials. (B) shows the effects of F3 (5 μg/ml) on pacemaker potentials. Dotted lines indicate resting level of membrane potentials.

Statistical analysis – Data are expressed as means ± standard errors. Differences were evaluated using Student's t-test. P values of < 0.05 were taken to be statistically significant. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

Results

Effects of fraction 3 isolated from pine needle extract on pacemaker potentials in ICCs – Cultured cells showed networks that had gross morphological properties similar to ICCs in situ. Under current clamp mode ($I = 0$), the colonic ICCs generated spontaneous pacemaker potentials (Fig. 1A). We tested 3 types of fractions isolated from pine extracts, fraction 2 (F2), fraction 3 (F3), and fraction 4 (F4). 5 μg/ml Fraction 3 (F3) of pine needle extracts when administered to bathing solution depolarized the membrane and decreased the frequency and amplitude of pacemaker potentials (Fig. 1B). However, F2 and F4 had hyperpolarized the membrane and showed the inhibitory action (data not shown). Thus, we chose F3 to find the excitatory mechanism in this study. F3 (1~5 μg/ml) dose-dependently depolarized the pacemaker potentials of colonic ICCs (Fig. 2A, B, C). Under control conditions, the resting membrane potential, frequency, and amplitude of

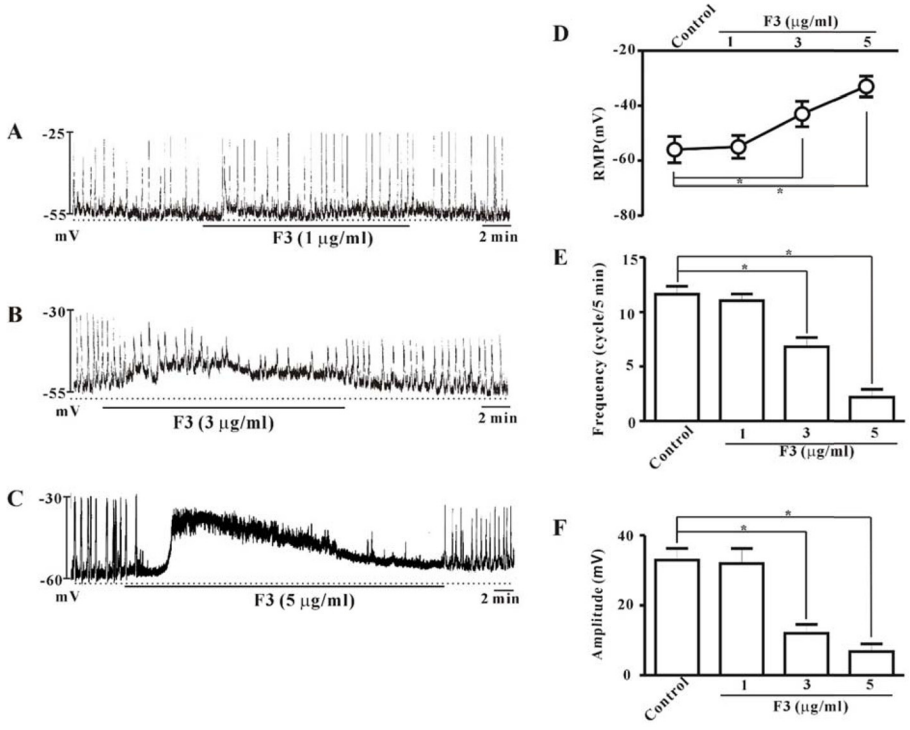

Fig. 2. Dose-dependent effects of F3 of pine needle extract on spontaneous pacemaker potentials in cultured colonic ICCs. (A), (B), and (C), show pacemaker potentials of ICCs exposed to 1, 3, 5 μg/ml F3 of pine needle extract. (D), (E), and (F) summarize the effect of F3 of pine needle extract on pacemaker potentials. Bars are means ± SE (n = 12). * $P < 0.05$, significant different from the untreated controls. Dotted lines indicate resting level of membrane potentials.

pacemaker potentials were – 56.1 ± 4.8 mV, 11.6 ± 0.7 cycles/5 min, and 33.64 ± 3.39 mV, respectively (n = 12). The effects of F3-induced actions on pacemaker potentials were summarized in Fig. 2 D, E, F. In the presence of F3 at 3 and 5 μg/ml, the resting membrane potential, frequency, and amplitude of pacemaker potentials were significantly different from control values. These results suggest that F3 isolated from pine needle extract has an excitatory action on colonic ICCs.

Involvement of muscarinic receptors in the effect of F3 on pacemaker potentials in ICCs – Muscarinic receptor stimulation depolarized the membrane with the decrease of amplitude of pacemaker activities in cultured ICCs from mouse small intestine (So *et al.,* 2009). Therefore, in the present study, we tested with muscarinic receptor antagonists to know whether F3-induced depolarization of membrane is mediated by stimulation of muscarinic receptors. Atropine (a non-selective muscarinic receptor antagonist), methoctramine (a selective muscarinic M_2 receptor antagonist), and glycopyrrolate (a selective muscarinic M_3 receptor antagonist) were used. Pretreatment of atropine (10 μM) (n = 5, Fig. 3A), methoctramine (10 μM) (n = 5, Fig. 3B), and glycopyrrolate (10 μM) (n = 5, Fig. 3C) for 10 min blocked the F3-induced depolarization of the membrane. However, atropine, methoctramine, and glycopyrrolate itself did not have any effects on pacemaker potentials. The effects of F3-induced actions in the presence of muscarinic antagonists on pacemaker potentials were summarized in Fig. 3D, E. These results suggest that the membrane depolarization by F3 is mediated through activation of muscarinic receptors.

Effects of extracellular Na^+-free or inhibitor of non-selective cation channels in the effect of F3 on pacemaker potentials in ICCs – To determine the characteristic of the depolarized pacemaker potentials induced by F3, we tested the effects of F3 in the presence of an external Na^+-free solution or flufenamic acid, a non-selective cation channel (NSCC) blocker. Exposure to the external Na^+-free solution abolished the generation of pacemaker potentials. Under this condition, the depolarized pacemaker potentials induced by F3 (5 μg/ml) were blocked (Fig. 4A). Under normal condition, the resting membrane potential and frequency induced by F3 were – 35.1 ± 5.4 mV and 1.5 ± 0.64 cycles/5 min. In the presence of external Na^+-free solution, the resting membrane potential and frequency induced by F3 were – 54.32 ± 2.46 mV and 0.25 ± 0.2 cycles/5 min (Fig. 4C, D). In addition, pretreatment with flufenamic acid (10 μM)

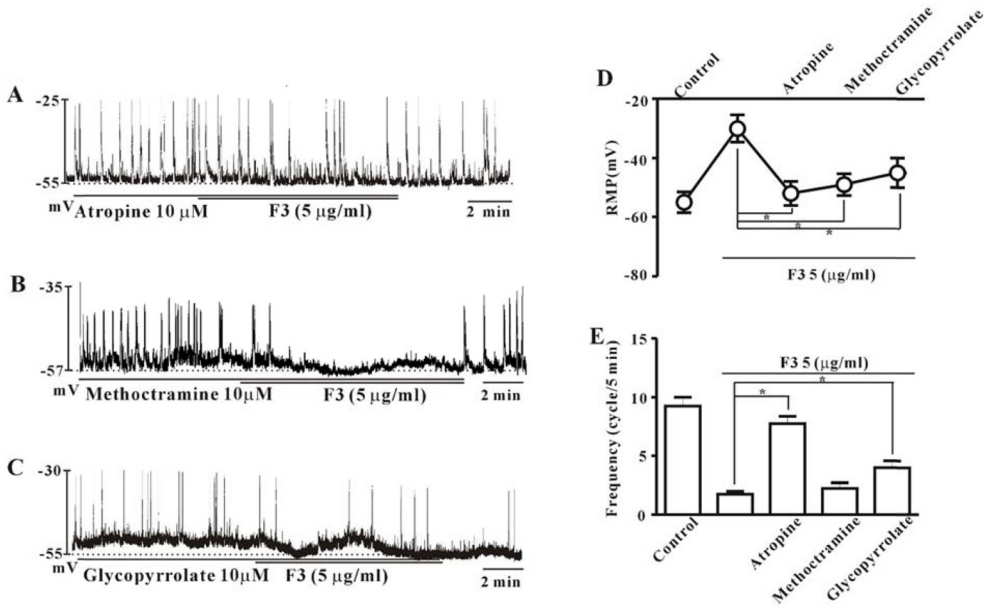

Fig. 3. Effects of muscarinic receptor blockers on F3-induced actions on pacemaker potentials in cultured colonic ICCs. (A), (B), and (C), show pacemaker potentials of ICCs exposed to F3 in the pretreatment with atropine (10 μM), a general muscarinic receptor antagonist, methoctramine (10 μM), a specific muscarinic M_2 receptor antagonist, and glycopyrrolate (10 μM), a specific muscarinic M_3 receptor antagonist. (D) and (E) summarize the effect of F3 of pine needle extract on pacemaker potentials in the presence of atropine, methoctramine, and glycopyrrolate. Bars are means ± SE (n = 5). **P* < *0.05,* significant different from F3 treatment alone. Dotted lines indicate resting level of membrane potentials.

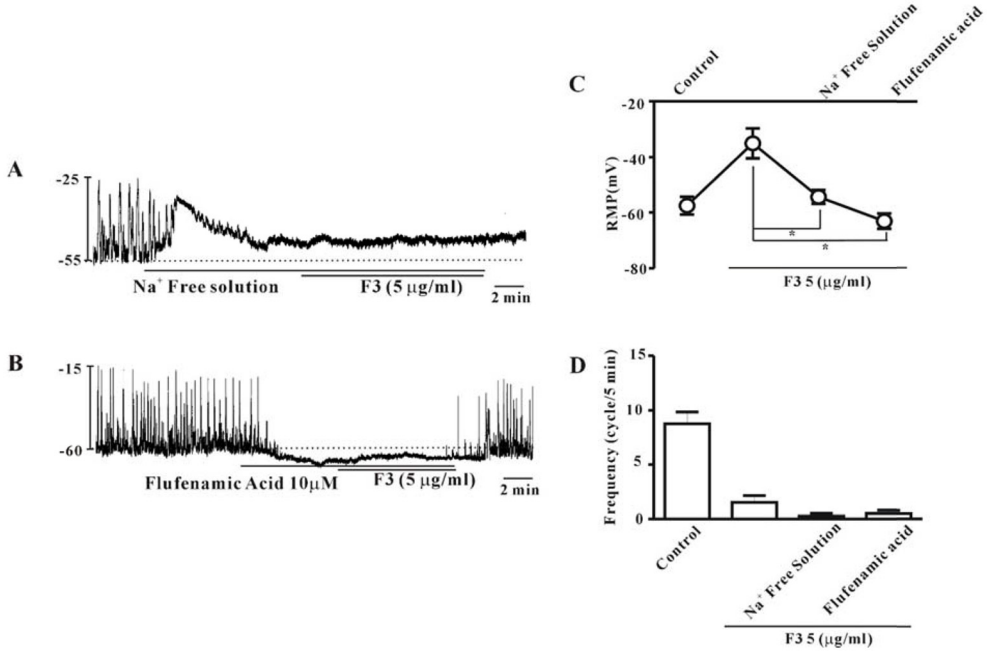

Fig. 4. Effects of external Na^+-free solution and flufenamic acid, a non-selective cation channel blocker, on F3-induced actions on pacemaker potentials in cultured colonic ICCs. (A) The use of an external Na^+-free solution abolished the generation of pacemaker potentials. Under these conditions, F3 (5 μg/ml) did not depolarize the membrane. (B) Flufenamic acid (10 μM) abolished the generation of pacemaker potentials and blocked the F3-induced depolarization of the membrane of pacemaker potentials. Responses to F3 in the external Na^+-free solution and in the presence of flufenamic acid are summarized in (C) and (D). Bars are means ± SE (n = 5). $^*P < 0.05$: significantly different from F3 treatment alone. Dotted lines indicate resting level of membrane potentials.

(n = 5, Fig. 4B) abolished the generation of pacemaker potentials and blocked the F3-induced depolarization of the membrane. In the presence of flufenamic acid, the resting membrane potential and frequency induced by F3

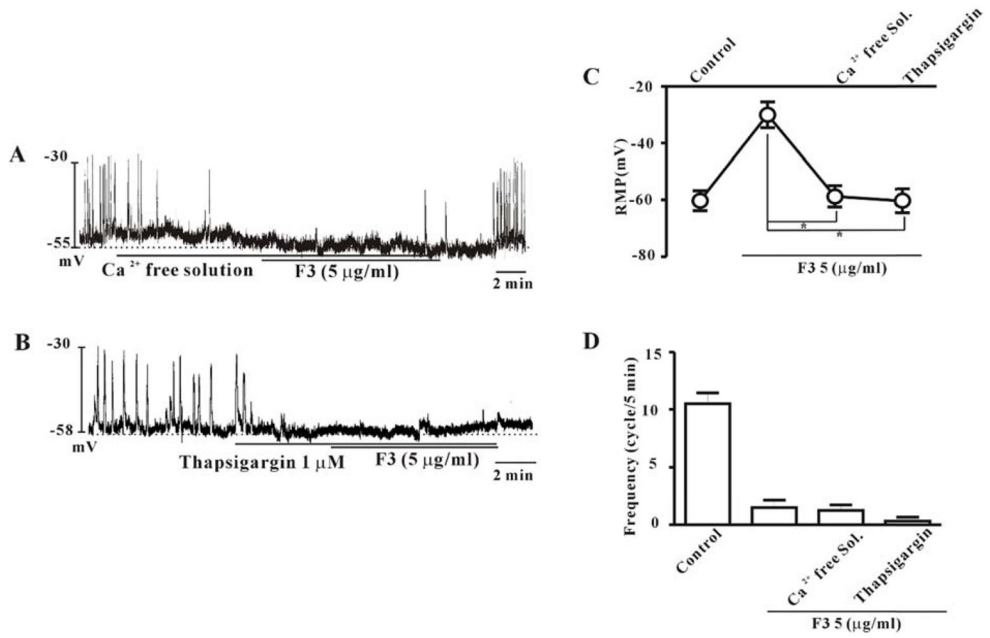

Fig. 5. Effect of an external Ca^{2+}-free solution or thapsigargin, a Ca^{2+} ATPase inhibitor of the endoplasmic reticulum, on the F3-induced actions on pacemaker potentials in cultured colonic ICCs. (A) The use of an external Ca^{2+}-free solution abolished the generation of pacemaker potentials. Under these conditions, F3 (5 μg/ml) did not depolarize the membrane. (B) Thapsigargin (1 μM) abolished the generation of pacemaker potentials and blocked the F3-induced depolarization of the membrane of pacemaker potentials. Responses to F3 in the external Ca^{2+}- free solution and in the presence of thapsigargin are summarized in (C) and D. Bars are means ± SE (n = 5). $^{*}P < 0.05$: significantly different from F3 treatment alone. Dotted lines indicate resting level of membrane potentials.

were − 63.1 ± 2.76 mV and 0.5 ± 0.28 cycles/5 min (Fig. 4C, D). These results suggest that the F3-induced effects on pacemaker potentials are mediated at least partly by the activation of NSCC.

Role of extracellular or intracellular Ca^{2+} in the effect of F3 on pacemaker potentials in ICCs – To investigate the role of external Ca^{2+} or internal Ca^{2+}, the effect of F3 on pacemaker potentials was tested under external Ca^{2+}-free conditions and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum. In the condition of an external Ca^{2+}-free solution, the generation of pacemaker potentials was completely abolished. In this condition, F3 did not induce the depolarization of membrane (Fig. 5A). The value of the F3-induced resting membrane potential in the Ca^{2+}-free solution was not significantly different with a control value obtained in normal solution (Fig. 5C, D). Also, thapsigargin (1 μM) completely abolished the generation of pacemaker potentials. Depolarization induced by F3 was not observed in the presence of thapsigargin (Fig. 5B). The value of the the F3-induced resting membrane potential in the thapsigargin solution was not significantly different with a control value obtained from in normal solution (Fig. 5C, D).

Discussion

Spontaneous contractions of GI tracts are initiated by periodic membrane depolarization, called as slow waves, which determine the frequency and membrane potential of smooth muscles (Szurszewski, 1987). Diverse enteric neurotransmitters, hormones and drugs regulate GI motility by modulating the frequency and membrane potential of slow waves (Olsson & Holmgren, 2001). Interstitial cells of Cajal (ICCs) are pacemaker cells that transmit the pacemaker potentials to smooth muscles via gap junctions. Therefore, the regulatory effects of pacemaker potential in ICCs are important to modulate GI motility (Sanders, 2010). We previously found that pine needle extracts inhibits pacemaker activities of intestinal ICCs, suggesting a possible role of suppressive agents in GI motility (Cheong *et al.,* 2005). However, in the present study, we found that fractions isolated from fermented pine extracts have excitatory role in regulating pacemaker activities of colonic ICCs.

It has been shown that Fraction 3 (F3) depolarized the membrane of pacemaker potentials dose-dependently, suggesting its alteration in the colonic motility. GI tracts are innervated by enteric motor neurons. It was well-known that acetylcholine (ACh) and substance P are

major excitatory neurotransmitters, while nitric oxide (NO) and VIP are major inhibitory neurotransmitters. ACh and substance P depolarized the membrane and induce mechanical contractions in intact tissues of GI tracts. NO and VIP hyperpolarized the membrane and induce mechanical relaxations (Olsson & Holmgren, 2001; Sanders, 1998). Carbachol, a muscarinic agonist also depolarized the membrane in isolated intestinal ICCs (So *et al.,* 2009). Therefore, we examined the effect of muscarinic receptor antagonists to know whether F3-induced actions are mediated by activation of muscarinic receptor or not. In the present study, atropine greatly inhibited the F3-induced effects on pacemaker potentials, suggesting that F3 actions are mediated through muscarinic receptor activations. It has been known that muscarinic M_2 and M_3 receptors involved in mechanical contractions in GI tract (Lecci *et al.,* 2002; Sanders, 1998; Unno *et al.,* 2006). In this study, both muscarinic M_2 and M_3 receptor antagonists also blocked the F3-induced deporaization of pacemaker potentials, indicating that both muscarinic M_2 and M_3 receptors contribute to the F3-induced actions in colonic ICCs.

Although the pacemaking mechanisms of ICCs were uncertain, non-selective cation channels (NSCC) or Ca^{2+}-activated Cl^- channels was candidate as pacemaker channels in intestinal ICCs (Kim *et al.,* 2009; Zhu *et al.,* 2009). It has been known that ACh induced inward currents in isolated GI smooth muscles and intestinal ICCs which were mediated through NSCC activation (Inoue & Chen, 1993; So *et al.,* 2009). Activation of NSCC induces net inward currents predominantly by Na^+ under physiological conditions, which leads to depolarization of the membrane in GI smooth muscles cells (Kuriyama *et al.,* 1998). In the present study, F3 did not produce depolarization of the membrane in the external Na^+-free condition. In addition, flufenamic acid, a blocker of NSCC, also inhibited the F3-induced effects on pacemaker potentials, indicating that depolarization of the membrane by F3 may be due to activation of NSCC in colonic ICCs.

Extracellular Ca^{2+} influx and intracellular Ca^{2+} release are essential to generate pacemaker potentials (Torihashi, *et al.,* 2002; Nakayama *et al.* 2007). Particularly, periodic release of IP_3 dependent intracellular Ca^{2+} release from endoplasmic reticulum is essentially required for pacemaker potential generations in ICCs (Liu *et al.,* 1995; Ward *et al.,* 2002). In this study, F3-induced depolarization of pacemaker potentials was blocked in an external Ca^{2+} free solution and also by thapsigargin, a Ca^{2+} ATPase inhibitor in the endoplasmic reticulum, suggesting that extracellular Ca^{2+} influx and intracellular Ca^{2+} release are associated with depolarization of membrane by F3. In support of this idea, it has been reported that ACh-induced depolarization through activation NSCC was dependent intracellular Ca^{2+} release in gastrointestinal smooth muscle cells (Inoue & Chen, 1993).

In conclusion, these experimental results have shown that F3 isolated from pine needle extract has an excitatory action on colonic ICCs and modulate the motility of colonic smooth muscles through activation of muscarinic receptors. Therefore, it seems that F3 isolated from pine needle extracts can be helpful to improve the altered colonic motility, especially in decreased motility disorders. However, we need further study to find the major compounds responsible for these effects.

Acknowledgements

This research was supported by Technology Commercialization Support Program, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (2012-2013).

References

Cheong, H.S. and Lim, D.Y., Pine needle extracts inhibit contractile responses of the isolated rat aortic strips. *Natural Product Sciences*. **16**, 123-132 (2010).

Cheong, H.S., Paudyal, D.P., Jun, J.Y., Yeum, C.H., Yoon, P.J., Park, C.G., Kim, M.Y., So, I., Kim, K.W., and Choi, S., Effects of pine needle extract on pacemaker currents in interstitial cells of Cajal from the murine small intestine. *Mol. Cells.* **20**, 235-240 (2005).

Choi, M.S., Seo, Y.H., Cheong, H.S., and Lim, D.Y., Inhibitory effects of self-fermented pine needle extract on catecholamine release in the rat adrenal medulla. *Natural Product Sciences.* **19**, 36-48 (2013).

Inoue, R. and Chen, S., Physiology of muscarinic receptor-operated nonselective cation channels in guinea-pig ileal smooth muscle. *EXS.* **66**, 261-268 (1993).

Jain, D., Khalid, M., Manish, T., Joan, C. M., and Deborah, D.P., Role of interstitial cells of Cajal in motility disorders of the bowel. *Am. J. Gastroenterol.* **98**, 618-624 (2003).

Kim, E.J., Choi, K.P., Ham, S.S., and Kang, H.Y. Inhibitory effect of pine needle extracts on the chemically induced mutagenicity. *Korean J. Food Sci. Technol.* **30**, 450455 (1998).

Kim, B.J., Park, K.J., Kim, H.W., Choi, S., Jun, J.Y., Chang, I.Y., Jeon, J.H., So, I., and Kim, S.J., Identification of TRPM7 channels in human intestinal interstitial cells of Cajal. *World J. Gastroenterol.* **15**, 5799-5804 (2009)

Kong, Z., Liu, Z., and Ding, B., Study on antimutagenic effect of pine needle extract. *Mutat. Res.* **347**, 101104 (1995).

Kuriyama, H., Kitamura, K., Itoh, T., and Inoue, R., Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. *Physiol. Rev.* **78**, 811-920 (1998).

Lecci, A., Santicioli, P., and Maggi, C.A., Pharmacology of transmission to gastrointestinal muscle. *Curr. Opin. Pharmacol.* **2**, 630-641 (2002).

Lee, E., Effects of powdered pine needle (*Pinus densiflora* seib et Zucc.) on serum and liver lipid composition and antioxidative capacity in rats fed high oxidized fat. *J. Korean Soc. Food Sci. Nutr.* **32**, 926930 (2003).

Liu, L.W., Thuneberg, L., and Huizinga, J.D., Cyclopiazonic acid, inhibiting the endoplasmic reticulum calcium pump, reduces the canine colonic pacemaker frequency. *J. Pharmacol. Exp. Ther.* **275**, 1058-1068 (1995).

Moon, J.J., Han, Y.B., and Kim, J.S., Studies on antitumor effects of pine needles, *Pinus densiflora* Sieb et Zucc. *Korean Vet. Res.* **33**, 701710 (1993).

Nakayama, S., Kajioka, S., Goto, K., Takaki, M., and Liu, H.N., Calcium-associated mechanisms in gut pacemaker activity. *J. Cell. Mol. Med.* **11**, 958-968 (2007).

Olsson, C. and Holmgren, S., The control of gut motility. *Comp. Biochem. Physiol.* **128**, 481-503 (2001).

Park, C.S., Kwon, C.J., Choi, M.A., Park, G.S., and Choi, K.H., Antioxidative and nitrite scavenging activities of mugwort and pine needle extracts. *Korean J. Food Pres.* **9**, 248252 (2002) .

Sanders, K.M., Interstitial cells in smooth muscles. Review series. *J. Cell. Mol. Med.* **14**, 1197-1198 (2010).

Sanders, K.M., G protein-coupled receptors in gastrointestinal physiology. IV. Neural regulation of gastrointestinal smooth muscle. *Am. J. Physiol.* **275**, G1-G7 (1998).

Sanders, K.M., Koh, S.D., Ro, S., and Ward, S.M., Regulation of gastrointestinal motility--insights from smooth muscle biology. *Nat. Rev. Gastroenterol. Hepatol.* **9**, 633-645 (2012).

Sanders, K.M., Koh, S.D., and Ward, S.M., Interstitial cells of Cajal as pacemakers in the gastrointestinal tract. *Annu. Rev. Physiol.* **68**, 307-343 (2006).

So, K.Y., Kim, S.H., Sohn, H.M., Choi, S.J., Parajuli, S.P., Choi, S., Yeum, C.H., Yoon, P.J., and Jun, J.Y., Carbachol regulates pacemaker activities in cultured interstitial cells of Cajal from the mouse small intestine. *Mol. Cells.* **27**, 525-531 (2009).

Streutker, C.J., Huizinga, J.D., Driman, D.K., and Riddell, R.H., Interstitial cells of Cajal in health and disease. Part I: normal ICC structure and function with associated motility disorders. *Histopathology.* **50**, 176-189 (2007).

Szurszewski, J.H., Electrical basis for gastrointestinal motility. In: Physiology of the Gastrointestinal Tract, 2nd edn, ed. Johnson LR, pp. 383-422. *Raven Press, New York* (1987).

Thomsen, L., Robinson, T.L., Lee, J.C., Farraway, L.A., Hughes, M.J., Andrews, D.W., and Huizinga, J.D., Interstitial cells of Cajal generate a rhythmic pacemaker current. *Nat. Med.* **4**, 848-851 (1988).

Torihashi, S., Fujimoto, T., Trost, C., and Nakayama, S., Calcium oscillation linked to pacemaking of interstitial cells of Cajal: requirement of calcium influx and localization of TRP4 in caveolae. *J. Biol. Chem.* **277**, 19191-19197 (2002).

Unno, T., Matsuyama, H., Okamoto, H., Sakamoto, T., Yamamoto, M., Tanahashi, Y., Yan, H. D., and Komori, S., Muscarinic cationic current in gastrointestinal smooth muscles: signal transduction and role in contraction. *Auton. Autacoid. Pharmacol.* **26**, 203-217 (2006).

Ward, S.M., Ordog, T., Koh, S.D., Baker, S.A., Jun, J.Y., Amberg, G., Monaghan, K., and Sanders, K.M., Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. *J. Physiol.* **525**, 355-361 (2002).

Yoon, S.U., Pine trees and natural remedy. *Academy Press Inc.,* pp. 4043, Seoul (1997).

Zhu, M.H., Kim, T.W., Ro, S., Yan, W., Ward, S.M., Koh, S.D., and Sanders, K.M., A Ca^{2+}-activated Cl^- conductance in interstitial cells of Cajal linked to slow wave currents and pacemaker activity. *J. Physiol.* **587**, 4905-4918 (2009).

Received October 16, 2013
Revised December 7, 2013
Accepted December 10, 2013