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Molecules and Cells

Induction of Pacemaker Currents by DA-9701, a Prokinetic Agent, in Interstitial Cells of Cajal from Murine Small Intestine

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The interstitial cells of Cajal (ICC) are pacemaking cells required for gastrointestinal motility. The possibility of whether DA-9701, a novel prokinetic agent formulated with Pharbitis Semen and Corydalis Tuber, modulates pacemaker activities in the ICC was tested using the whole cell patch clamp technique. DA-9701 produced membrane depolarization and increased tonic inward pacemaker currents in the voltage-clamp mode. The application of flufenamic acid, a non-selective cation channel blocker, but not niflumic acid, abolished the generation of pacemaker currents induced by DA-9701. Pretreatment with a Ca^{2+}-free solution and thapsigargin, a Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum, abolished the generation of pacemaker currents. In addition, the tonic inward currents were inhibited by U-73122, an active phospholipase C inhibitor, but not by GDP-β-S, which permanently binds G-binding proteins. Furthermore, the protein kinase C inhibitors, chelerythrine and calphostin C, did not block the DA-9701-induced pacemaker currents. These results suggest that DA-9701 might affect gastrointestinal motility by the modulation of pacemaker activity in the ICC, and the activation is associated with the non-selective cationic channels via external Ca^{2+} influx, phospholipase C activation, and Ca^{2+} release from internal storage in a G protein-independent and protein kinase C-independent manner.

INTRODUCTION

Gastrointestinal (GI) smooth muscles spontaneously produce contractions controlling peristaltic activity and the movement of intestinal contents. The mechanical contractions are initiated by periodic membrane depolarization, called slow waves (Szurszewski, 1987). The interstitial cells of Cajal (ICC) are the pacemaker cells that generate slow waves by producing spontaneous inward currents (pacemaker currents; Thomsen et al., 1998). These cells connect with each other to form a network, which closely attach to neuron cells and electrically couple to the adjacent muscle layers. Diverse signals entering the ICC are converted to slow waves, which propagate into smooth muscle cells via a gap junction, leading to GI movement (Koh et al., 1998). Recently, the ICC has been suggested as a therapeutic target based on the observations that ablation of the ICC network results in the elimination of slow waves (Huizinga et al., 1995). Further, it has been proposed that there is loss or damage to the ICC networks leading to impaired slow wave propagation in a variety of GI motility disorders in animals (Sanders et al., 2002) and humans (Pardi et al., 2002). For example, disturbed GI motility has been associated with reduced areas of the ICC in rodents with diabetic gastropathy (Long et al., 2004; Ordog et al., 2000; Yamamoto et al., 2008). It has also been reported that failure in gastric myoelectrical activity is responsible for delayed gastric emptying in patients with functional dyspepsia (FD; Lin et al., 1999), and slow-wave propagation and coupling are impaired in approximately two-thirds of FD patients (Zhang et al., 2006). Furthermore, reduction in the number of the ICC and an abnormal distribution of the cells is associated with many human gut motility diseases (Geraldino et al., 2006; Nakahara et al., 2002; Vanderwinden and Rumessen, 1999).

We previously found that DA-9701 has strong gastroprokinetic effects and a safety profile superior to conventional prokinetics, including cisapride and mosapride (Lee et al., 2008). DA-9701 not only accelerates gastric emptying and the GI transit of meals in normal and abnormally delayed conditions, but also enhances gastric accommodation in conscious dogs (Lee et al., 2008). DA-9701 is currently in a Phase III clinical trial to be developed as a therapeutic agent for FD patients in Korea. In this study, we determined whether DA-9701 modulates the electrical properties in the ICC of murine small intestine to investigate the underlying therapeutic target for DA-9701. Our data indicated that DA-9701 provokes pacemaker currents in the ICC by means of intracellular mobilization of Ca^{2+} through phospholipase C (PLC), which might be one of the cellular and molecular targets for the gastroprokinetic effects of DA-9701.

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MATERIALS AND METHODS

Animals

Balb/C mice were purchased from OrientBio, Inc. (Korea). All experiments were carried out according to the guiding principles for the care and use of animals approved by the Ethics Committee in Chosun University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Preparation of DA-9701

DA-9701 is the standardized extract of Pharbitis Semen and Corydalis Tuber, which was prepared as previously reported (Lee et al., 2008). Briefly, dried herbs (water content < 10%) were mixed in a specific ratio of the two herbs, and extracted with a mixed ethanol solution (50% v/v) at room temperature for 48 hours. After filtration, the extract was evaporated in a vacuum, and lyophilized for a complete removal of the residual solvent to yield brown powder. The standard method evaluating the quality of DA-9701 has been established using quantitative HPLC, as previously reported (Lee et al., 2008). The content of at least three compounds present in two herbs of DA-9701 was determined.

Preparation of cells

Balb/C mice (8-13 days 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 content was washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of Sylgard dishes and the mucosa removed by sharp dissection. Small strips of intestinal muscle were equilibrated in Ca^{2+}-free Hank's solution (KCl, 5.36; NaCl, 125; NaOH, 0.336; Na_2HCO_3, 0.44; glucose, 10; sucrose, 2.9; and HEPES, 11 [all in mM]) adjusted to pH 7.4 with Tris for 30 min and after incubation for 15 min at 37°C with an enzyme solution containing collagenase (1.3 mg/ml; Worthington Biochemical Co., USA), bovine serum albumin (2 mg/ml; Sigma Chemical Co., USA), trypsin inhibitor (2 mg/ml; Sigma Chemical Co.), and ATP (0.27 mg/ml), and the cells were dispersed. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD) in 35 mm culture dishes. The cells were then cultured at 37°C in a 95% O_2-5% CO_2 incubator in smooth muscle growth medium (SMGM; Clonetics Corp., USA) supplemented with 2% antibiotics/antimycotics (Gibco, USA) and murine stem cell factor (SCF, 5 ng/ml, Sigma Chemical Co.). The ICC were identified immunologically with a monoclonal antibody for Kit protein (ACK_2) labeled with Alexa Fluor 488 (Molecular Probes, USA).

Patch clamp experiments

The whole-cell configuration of the patch-clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC. Currents or potentials were amplified by Axopatch 1-D (Axon Instruments, USA). Command pulse was applied using an IBM-compatible personal computer and pClamp software (version 6.1; Axon Instruments). The data were filtered at 5 kHz and displayed on an oscilloscope, a computer monitor, and a pen recorder (Gould 2200; Gould, USA). The results were analyzed using pClamp and Graph Pad Prism (version 2.01) software. All experiments were performed at 30°C.

Solutions and drugs

The cells were bathed in a solution (KCl, 5; NaCl, 135; $CaCl_2$, 2; glucose, 10; $MgCl_2$, 1.2; and HEPES, 10 [all in mM]) adjusted to pH 7.2 with Tris. The pipette solution (K-aspartate, 120; KCl, 20; $MgCl_2$, 5; K_2ATP, 2.7; Na_2GTP, 0.1; creatine phosphate disodium, 2.5; HEPES, 5; and EGTA, 0.1 [all in mM]) was adjusted to pH 7.2 with Tris. The drugs used included guanosine 5′-[β-thio]diphosphate trilithium salt (GDP-β-S), U-73122, U-73343, calphostin C, chelethrine, and thapsigargin. All drugs were purchased from Sigma Chemical Co.

Statistical analysis

Data are expressed as the means ± standard errors. Differences in the data were evaluated by Student' s *t*-test. A P values < 0.05 were taken as a statistically significant difference. The n values reported in the text refer to the number of cells used in the patch-clamp experiments.

RESULTS

Effect of DA-9701 on slow waves and pacemaker currents in cultured ICC

Isolated ICC, which express c-kit receptor tyrosine kinase (KIT; Torihashi et al., 1997), were identified with the Kit immunofluroescence. Kit-positive cells had a distinctive morphology that was easily recognized in cultures. Recording from cultured ICC under current clamp mode (I = 0) showed spontaneous pacemaker potentials. The resting membrane potential was -51 ± 2 mV and the amplitude was 22 ± 4 mV. In the presence of DA-9701 (10 μg/ml), the membrane potentials were depolarized to -32 ± 5.4 mV and the amplitude of the pacemaker potentials was decreased to 2.4 ± 1.1 mV (n = 5 ; Fig. 1A, bar graph not shown). Converting the amplifier to voltage clamp mode at a holding potential of -70 mV, the ICC generated spontaneous inward currents called 'pacemaker currents' and DA-9701 produced tonic inward currents and decreased the frequency and amplitude of pacemaker currents in a dose-dependent manner (Figs. 1B, 1C, and 1D). The summarized values and bar graph of the DA-9701 effects on pacemaker currents are indicated in Figs. 1E, 1F, and 1G (n = 4).

Effects of non-selective cation channel blocker or Cl^- channel blocker in DA-9701-induced pacemaker currents in cultured ICC

To determine the characteristics of the tonic inward currents produced by DA-9701, flufenamic acid (a non-selective cation channel blocker; Sanders et al., 2004) or niflumic acid (a Cl^- channel blocker; Kuriyama et al., 1998) were tested. In the presence of flufenamic acid (10 μM), the pacemaker current was abolished and then the application of DA-9701 (10 μg/ml) did not produce tonic inward currents (Fig. 2A). The resting currents produced by DA-9701 were -24 ± 7 pA in the presence of flufenamic acid and this value was significantly different when compared to the control condition (n = 4; Fig. 2D). In the presence of the application of niflumic acid (10 μM), the pacemaker currents also were abolished. In this condition, DA-9701 still produced tonic inward currents (Fig. 2B). In the presence of niflumic acid, the resting currents produced by DA-9701 were -126 ± 12 pA; this value was not significantly different when compared with control values obtained in the absence of niflumic acid (n = 5; Fig. 2E).

No involvement of G proteins on DA-9701 pacemaker currents in cultured ICC

The effects of GDP-β-S, a non-hydrolysable guanosine 5′-diphosphate analogue which permanently inactivates G protein binding proteins (Sanders, 1998), were examined to determine whether the G protein is involved in the effects of DA-9701 in

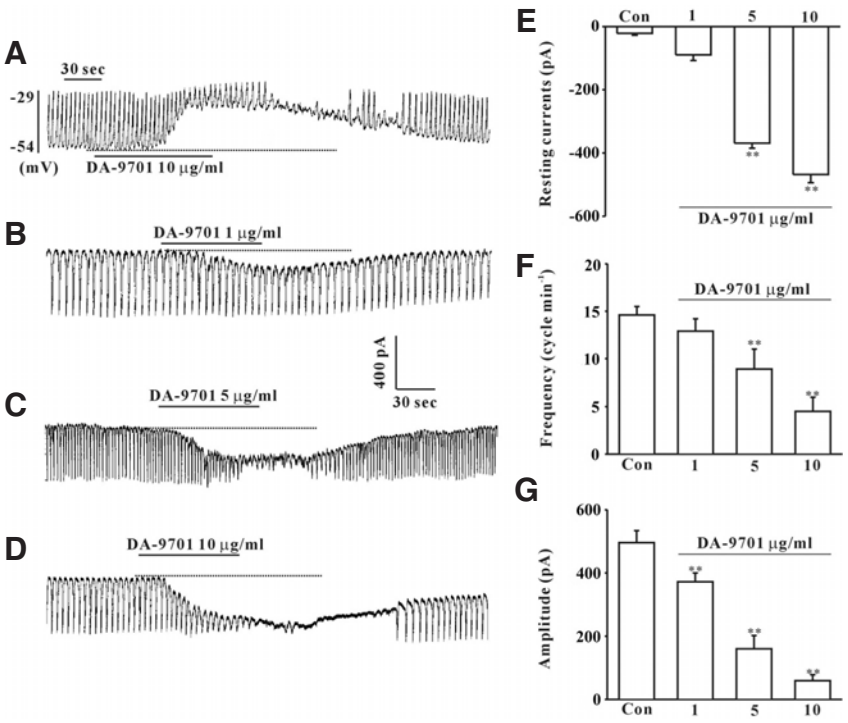

Fig. 1. Effects of DA-9701 on pacemaker potentials and pacemaker currents recorded in cultured ICC from murine small intestine. (A) shows the pacemaker potentials of the ICC exposed to DA-9701 (10 μg/ml) in the current clamping mode (I = 0). (B, C, and D) show the pacemaker currents of the ICC recorded at a holding potential of -70 mV exposed to various concentrations of DA-9701 (1, 5, and 10 μg/ml). Dotted lines indicate zero current levels. Responses to DA-9701 are summarized in (E), (F), and (G). Bars represent mean values ± SE. **($P < 0.01$) Significantly different from the untreated control. Con, Control.

the ICC. When GDP-β-S (10 mM) was in the pipette, DA-9701 (10 μg/ml) still showed tonic inward currents (Fig. 2C). In the presence of GDP-β-S in the pipette, the resting currents were -23 ± 9 pA. Upon application of DA-9701 (10 μg/ml), the resting currents produced by DA-9701 were -526.2 ± 36 pA (n = 4; Fig. 2F).

Effects of external Ca^{2+}-free solution and Ca^{2+}-ATPase inhibitor of endoplasmic reticulum in DA-9701-induced pacemaker currents in cultured ICC

The external Ca^{2+} influx is necessary for GI smooth muscle contractions and is essential for generating pacemaker currents in the ICC. The generation of pacemaker currents was dependent upon intracellular Ca^{2+} oscillation (Ward et al., 2000a). To investigate the role of external Ca^{2+} or internal Ca^{2+}, DA-9701 was tested under external Ca^{2+}-free conditions and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum (Koh et al., 2002). The pacemaker currents recorded at a holding potential of -70 mV were completely abolished by an external Ca^{2+}-free solution. In this condition, DA-9701-induced tonic currents were blocked (n = 5; Fig. 3A). Under external Ca^{2+}-free conditions, the value of resting currents with DA-9701 (10 μg/ml) was significantly different when compared with DA-9701 (10 μg/ml) in normal Ca^{2+} solution (Fig. 3C). In addition, DA-9701-induced tonic inward currents were inhibited by pretreatment of thapsigargin (Fig. 3B). In the presence of thapsigargin (5 μM), the value of resting currents with DA-9701 was significantly different when compared to DA-9701 in the absence of thapsigargin (n = 4, Fig. 3D).

Effects of phospholipase C inhibitor on DA-9701-induced pacemaker currents in cultured ICC

Since the tonic inward currents by DA-9701 was related to intracellular Ca^{2+} mobilization, we examined whether the effects on pacemaker currents require PLC activation. To test this possibility, DA-9701-induced tonic inward currents were measured in the absence and presence of U-73122, an active PLC inhibitor (Sakamoto et al., 2006). The pacemaker currents recorded at a holding potential of -70 mV were completely abolished by application of U-73122 (5 μM) and under these conditions, DA-9701-induced (10 μg/ml) tonic currents were suppressed (n = 4; Fig. 4A). In the presence of U-73122, the tonic inward currents produced by DA-9701 were -21 ± 11 pA. The value of the resting currents by DA-9701 was significantly different when compared with DA-9701 in the absence of U-73122 (n = 5, Fig. 4B). The treatment of U-73343 (5 μM), an inactive analog of U-73122, had no influence on the DA-9701-induced pacemaker currents and under these conditions, DA-9701-induced (10 μg/ml) tonic currents were not suppressed by U-73343 (Fig. 4C).

No involvement of protein kinase C inhibitor on DA-9701-induced pacemaker currents in cultured ICC

We tested the effects of chelerythrine or calphostin C, an inhibitor of protein kinase C (Aiello et al., 1996), to investigate whether the DA-9701-induced responses to the pacemaker currents are mediated by the activation of protein kinase C. Chelerythrine (1 μM) or calphostin C (1 μM), did not have an effect on tonic inward currents by DA-9701 (10 μg/ml; Figs. 5A and 5B) and the value was also not significantly different when compared with the tonic inward currents by DA-9701 obtained in the absence of chelerythrine or calphostin C (n = 5; Figs. 5C and 5D).

DISCUSSION

Since the ICC are considered the pacemaker cells for GI motility, and loss of the ICC network has been associated with the pathogenesis of a variety of gut motility disorders, specific pharmacologic interventions with the ICC seem to be a promising target for restoring the impaired motor functions of the GI tract (Farrugia, 2008; Huizinga et al., 1997).

In this study, we showed that the ICC is one of the cellular

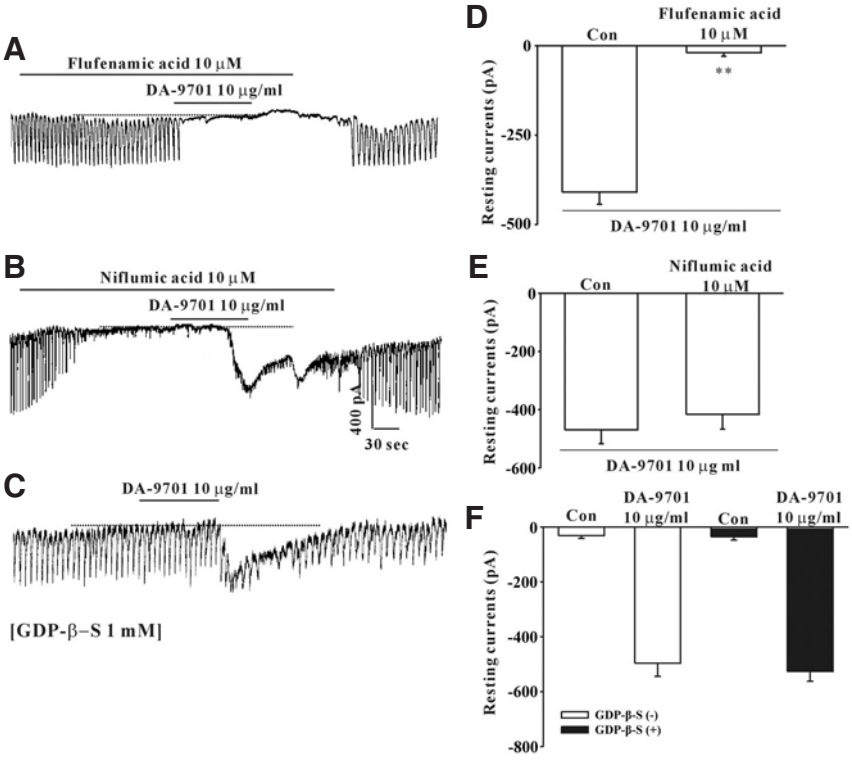

Fig. 2. Effects of flufenamic acid, a non-selective cation channel blocker, or niflumic acid, a Cl^- channel blocker, and GDP-β-S on DA-9701-induced pacemaker currents in cultured ICC of murine small intestine. (A) The application of flufenamic acid (10 μM) abolished the generation of the pacemaker currents. Under these conditions, DA-9701 (10 μg/ml) did not produce tonic inward currents. (B) Also, niflumic acid (10 μM) abolished the generation of pacemaker currents. However, niflumic acid did not block the DA-9701-induced (10 μg/ml) tonic inward currents. The dotted lines indicate the zero current levels. (C) The pacemaker currents of ICC exposed to DA-9701 (10 μg/ml) in the presence of GDP-β-S (1 mM) in the pipette. The dotted lines indicate the zero current levels. The responses to DA-9701 in the presence of flufenamic acid or niflumic acid and GDP-β-S are summarized in (D), (E), and (F). Bars represent mean values ± SE. **($P < 0.01$) Significantly different from the untreated control. Con, Control.

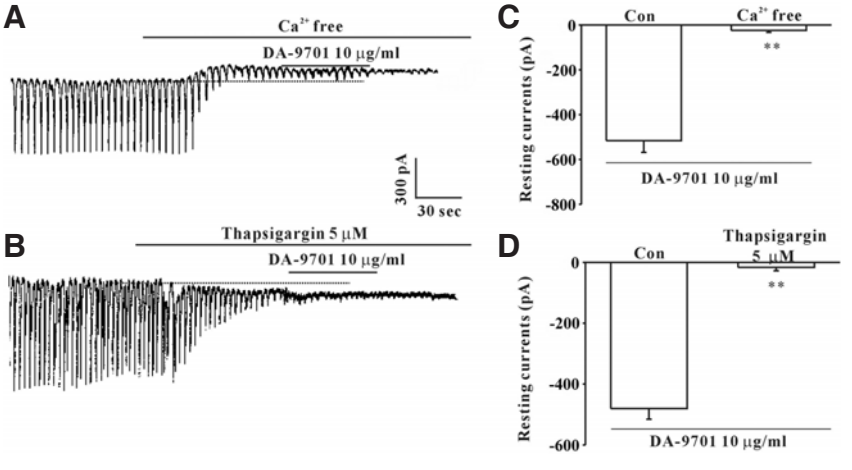

Fig. 3. Effects of an external Ca^{2+}-free solution or thapsigargin, a Ca^{2+}-ATPase inhibitor of endoplasmic reticulum, and U-73122, an active phospholipase C inhibitor, on DA-9701-induced pacemaker currents in cultured ICC (A) The external Ca^{2+}-free solution abolished the generation of pacemaker currents. Under these conditions, DA-9701-induced (10 μg/ml) tonic inward currents were blocked. (B) Thapsigargin (5 μM) abolished the generation of pacemaker currents. Also, thapsigargin blocked the DA-9701-induced (10 μg/ml) tonic inward currents. The responses to DA-9701 in the external Ca^{2+}-free solution, in the presence of thapsigargin are summarized in (C) and (D), respectively. Bars represent the mean values ± SE. **($P < 0.01$) Significantly different from the untreated control. Con, Control.

targets for the gastroprokinetic effects of DA-9701, a new prokinetic agent, and examined the various characteristics of the pacemaker currents induced by DA-9701. This drug efficiently depolarized the membrane of the ICC by increasing tonic inward currents. The role of ion channels was first examined as it has been suggested that the pacemaker currents in the ICC are mediated by the activation of non-selective cationic channels (Koh et al., 2002) or inwardly rectifying Cl^- channels (Park et al., 2005). The generation of the pacemaker currents by DA-9701 was abolished by flufenamic acid, a non-cationic ion blocker, but not by niflumic acid, a Cl^- channel blocker (Fig. 2), suggesting that the DA-9701-induced pacemaker currents may be mediated by non-selective cationic channels. Next, we examined the role of Ca^{2+} mobilization and PLC, as it is known that the generation of the pacemaker current is initiated by the release of Ca^{2+} from the endoplasmic reticulum, as well as external calcium influx (Torihashi et al., 2002), and some stimuli require the activation of PLC, which leads to the formation of inositol triphosphate (IP_3) and diacylglycerol in the ICC for the activation of pacemaker currents (Kim et al., 2002). The tonic inward currents induced by DA-9701 were blocked by thapsigarin, a Ca^{2+} ATPase inhibitor in the endoplasmic reticulum (Figs. 3B and 3D), suggesting that intracellular calcium release is necessary. Additionally, we reasoned that calcium entry from the external compartment might be required for the induction of pacemaker currents by DA-9701, as the removal of external calcium abolished DA-9701 induced inward currents (Figs. 3A and 3C). Furthermore, the inward currents were blocked by U-73122, an active PLC inhibitor, but not U-73343, an inactive analogue for U-73122 (Fig. 4), suggesting that PLC is involved

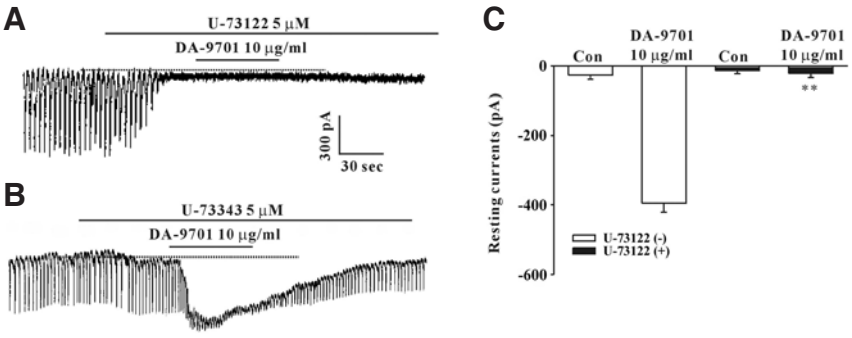

Fig. 4. Effects of DA-9701 on phospholipase C in cultured ICC (A) U-73122 (5 μM), a phospholipase C inhibitor, abolished the generation of pacemaker currents by DA-9701. (B) U-73122 blocked the DA9701-induced (10 μg/ml) tonic inward currents. (C) The application of U-73343 (5 μM) did not show any influence on the generation of pacemaker currents. Also, U-73343 did not block the DA9701-induced (10 μg/ml) tonic inward currents. The dotted lines indicate the zero current levels. Bars represent the mean values ± SE. **(P < 0.01) Significantly different from the untreated control. Con, Control.

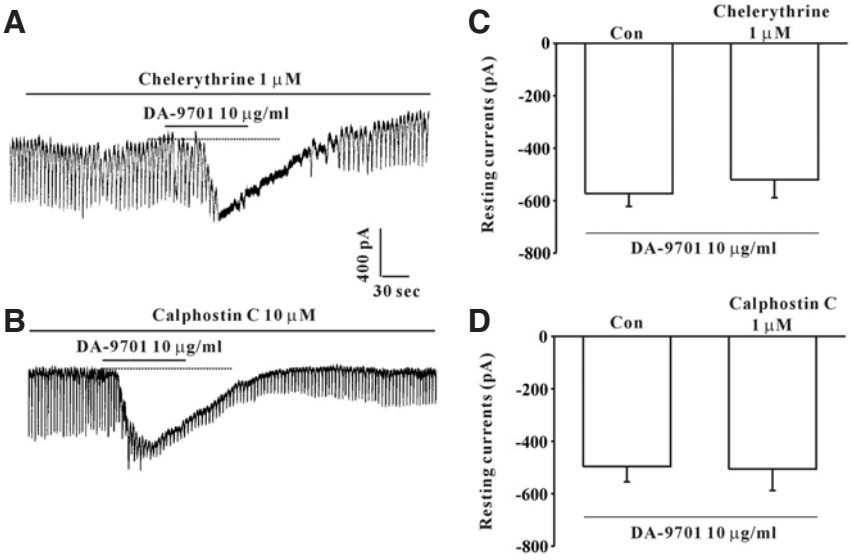

Fig. 5. Effects of chelerythrine or calphostin C, an inhibitor of protein kinase C, upon DA-9701-induced pacemaker currents in cultured ICC of murine small intestine. (A, B) Pacemaker currents of ICC exposed to DA-9701 (10 μg/ml) in the presence of chelerythrine (1 μM) or calphostin C (10 μM). Under these conditions, DA-9701 caused tonic inward currents. The dotted lines indicate the zero current levels. Responses to DA-9701 in the presence of chelerythrine or calphostin C are summarized in (C). Bars represent the mean values ± SE. Con, Control.

in the induction of the pacemaker current. We speculated that the pacemaker currents by DA-9701 are initiated by release of Ca^{2+} from the endoplasmic reticulum and followed by reuptake of mitochondria. One of the targets for the pacemaker mechanism by DA-9701 might be the spontaneous oscillation of intracellular Ca^{2+} in the ICC. In contrast, inactivation of G protein by GDP-β-S did not suppress the generation of the pacemaker current by DA-9701, as revealed in Figs. 2C and 2F, indicating no involvement of G proteins. Although the activation of G protein is generally associated with PLC, we found that DA-9701 stimulated PLC, but not G protein. We assume that because DA-9701 is a natural product formulated with two herbs, the effects of DA-9701 might be mediated by not a single compound, but by multiple compounds through several pathways using multiple receptors; it is feasible that DA-9701 may stimulate the PLC pathway without affecting the G protein and/or by another unknown mechanism through multiple pathways. In addition, neither chelerythrine nor calphostin C, (protein kinase C inhibitors) abolished the DA-9701-induced tonic inward currents (Fig. 4), suggesting that PKC is not involved in the effects induced by DA-9701. In summary, DA-9701 depolarized the membrane potential and induced tonic inward currents in the ICC, and may be mediated by the activation of non-selective cationic channels via external Ca^{2+} influx and intracellular Ca^{2+} release from internal storage by the action of inositol triphosphate via PLC activation in a G protein-independent and protein kinase C-independent manner. Based on the results obtained from this study, it appears that the induction processes for the pacemaker currents by DA-9701 are similar to those observed in the M_3 muscarinic receptor agonist (McKay and Huizinga, 2006). Acetylcholine (ACh) and carbachol increase GI motility and generates tonic inward currents in gastric ICC (Kim et al., 2003; 2006; Ward and Sanders, 2006; Ward et al., 2000b), which are very similar to data generated with DA-9701, assuming DA-9701 may increase intestinal motility by regulating the ICC using similar cellular machinery as Ach or carbachol. In like manner, we recently showed the action of carbachol on pacemaker currents in small intestinal ICC, which is very similar to those shown in DA-9701, and additionally the DA-9701-induced pacemaker currents were blocked by atropine treatment (data not shown).

Taken together, our data suggest that the gastroprokinetic effects of DA-9701 might be mediated by the induction of pacemaker currents in the ICC. Considering the superior effects of DA-9701 to conventional prokinetics and the effects of this drug on the ICC, further research, including finding active compound(s) and examining their action mechanisms, are clearly needed.

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