Mol. Cells *27*, 525-531, May 31, 2009
DOI/10.1007/s10059-009-0076-1

Molecules and Cells

Carbachol Regulates Pacemaker Activities in Cultured Interstitial Cells of Cajal from the Mouse Small Intestine

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We studied the effect of carbachol on pacemaker currents in cultured interstitial cells of Cajal (ICC) from the mouse small intestine by muscarinic stimulation using a whole cell patch clamp technique and Ca^{2+}-imaging. ICC generated periodic pacemaker potentials in the current-clamp mode and generated spontaneous inward pacemaker currents at a holding potential of -70 mV. Exposure to carbachol depolarized the membrane and produced tonic inward pacemaker currents with a decrease in the frequency and amplitude of the pacemaker currents. The effects of carbachol were blocked by 1-dimethyl-4-diphenylacetoxypiperidinium, a muscarinic M_3 receptor antagonist, but not by methotramine, a muscarinic M_2 receptor antagonist. Intracellular GDP-β-S suppressed the carbachol-induced effects. Carbachol-induced effects were blocked by external Na^+-free solution and by flufenamic acid, a non-selective cation channel blocker, and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor in the endoplasmic reticulum. However, carbachol still produced tonic inward pacemaker currents with the removal of external Ca^{2+}. In recording of intracellular Ca^{2+} concentrations using fluo 3-AM dye, carbachol increased intracellular Ca^{2+} concentrations with increasing of Ca^{2+} oscillations. These results suggest that carbachol modulates the pacemaker activity of ICC through the activation of non-selective cation channels via muscarinic M_3 receptors by a G-protein dependent intracellular Ca^{2+} release mechanism.

INTRODUCTION

Interstitial cells of Cajal (ICC) are pacemaker cells of the gastrointestinal system and have multifunctional roles. ICC produce pacemaker potentials and regulate slow wave propagation, transmit neuronal signals to the smooth muscle and act as mechanoreceptors (Hirst and Edwards, 2004; Sanders et al., 2006; Suzuki, 2000; Won et al., 2005). Impairment of ICC is implicated in diverse motility disorders (Streutker et al., 2007), which indicates that ICC have an important role in regulating gastrointestinal motility. Slow waves generated by pacemaker potentials of ICC determine the frequency and the timing of smooth muscle contractions (Szurszewski, 1987). Numerous chemical agents such as neurotransmitters, hormones, endogenous substances, and drugs affect the frequency and the configurations of slow waves that determine the excitability of smooth muscles (Olsson and Holmgren, 2001). In addition to several receptors, neurotransmitters are found in ICC as determined in immunohistochemical and pharmacological studies (Choi et al., 2006; Ward and Sanders, 2006). Acetylcholine (ACh) is a major excitatory neurotransmitter in the gastrointestinal system. Muscarinic receptor stimulation by ACh induces membrane depolarization and excitatory junction potential (EJP) (Huizinga, 1984; Inoue and Chen, 1993). Furthermore, ACh modulates non-selective cation channels or K^+ channels in single gastrointestinal smooth muscle cells (Jun et al., 2001; Sims, 1992; Vogalis and Sanders, 1990). The activation of nonselective cation channels or the inhibition of K^+ channels is responsible for membrane depolarization and mechanical contractions. Although the effects and the signal mechanism of muscarinic stimulation by ACh have been investigated in various gastrointestinal smooth muscles, understanding of the action and signaling is very limited in ICC. Therefore, we investigated muscarinic receptor activation with the use of carbachol, a muscarinic receptor agonist, on the regulatory mechanisms of electrical pacemaker activities in cultured ICC from the mouse small intestine.

MATERIALS AND METHODS

Preparation of cells

All experiments were performed according to the Guiding Principles for the Care and Use of Animals approved by the Ethics Committee of Chosun University and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals

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Received November 4, 2008; revised March 17, 2009; accepted March 24, 2009; published online May 15, 2009

Keywords: carbachol, interstitial cells of Cajal, intestine, muscarinic receptor

used and the suffering of the animals.

Balb/C mice (8-13 days old) of either sex were anesthetized with ether and were sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and were opened along the mesenteric border. The luminal contents were washed away 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 intestinal muscle were equilibrated in Ca^{2+}-free Hank's solution containing 5.36 mM KCl, 125 mM NaCl, 0.34 mM NaOH, 0.44 mM Na_2HCO_3, 10 mM glucose, 2.9 mM sucrose, 11 mM HEPES for 30 min, and the cells were dispersed with an enzyme solution containing 1.3 mg/ml collagenase (Worthington Biochemical, USA), 2 mg/ml bovine serum albumin (Sigma, USA), 2 mg/ml trypsin inhibitor (Sigma) and 0.27 mg/ml ATP. Cells were plated onto sterile glass coverslips coated with murine collagen (2.5 μg/ml, Falcon/BD, USA) in 35 mm culture dishes. The cells were then cultured at 37°C in a 5% CO_2 incubator in SMGM (smooth muscle growth medium; Clonetics, USA) supplemented with 2% antibiotics/antimycotics (Gibco, USA) and murine stem cell factor (SCF, 5 ng/ml; Sigma). Interstitial cells of Cajal (ICC) were identified immunologically with the use of 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 after 2-3 days in culture. The reason that spontaneous inward currents from small groups of cells are more robust and regular than from single cells, we recorded from small clusters of ICC. Currents or potentials were amplified by Axopatch 1-D and 200B (Axon Instruments, USA). A 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 the data were displayed on an oscilloscope, a computer monitor and a pen recorder (Gould 2200, Gould, USA). 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 containing 5 mM KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, and 10 mM HEPES, adjusted to pH 7.2 with Tris. NaCl was replaced with equimolar *N*-methyl-$_D$-glucamine (NMDG) for making Na^+-free solution and $CaCl_2$ was omitted in bath solution for Ca^{2+}-free solution. The pipette solution contained 20 mM K-aspartate, 120 mM KCl, 5 mM $MgCl_2$, 2.7 mM K_2ATP, 0.1 mM Na_2GTP, 2.5 mM creatine phosphate disodium, 5 mM HEPES, 0.1 mM EGTA, adjusted to pH 7.2 with Tris.

The drugs used were carbachol, methoctramine, 1-dimethyl-4-diphenylacetoxypiperidinium (4-DAMP), GDP-β-S, flufenamic acid and thapsigargin. All drugs were purchased from Sigma.

Measurement of the intracellular Ca^{2+} concentration

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were monitored by using fluo-3/AM, which was initially dissolved in dimethyl sulfoxide and stored at -20°C. Cultured ICC on coverslips (25 mm) were rinsed twice with a bath solution (5 mM: KCl, 135 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$ and 10 mM HEPES, adjusted to pH 7.4 with Tris). The coverslips were then incubated in the bath solution containing 5 μM fluo-3 with 5% CO_2 at 37°C for 5 min, rinsed two more times with the bath solution, mounted on a perfusion chamber, and were scanned every 0.4 s with a Nikon Eclipse TE200 inverted microscope equipped with a Perkin-Elmer Ultraview confocal scanner and a Hamamatsu Orca ER 12-bit CCD camera (× 200). Fluorescence was excited at a wavelength of 488 nm, and emitted light was observed at 515 nm. During scanning of the Ca^{2+} imaging, the temperature of the perfusion chamber containing the cultured ICC was kept at 30°C. Variations of the intracellular Ca^{2+} fluorescence emission intensity were expressed as F1/F0, where F0 is the intensity of the first imaging.

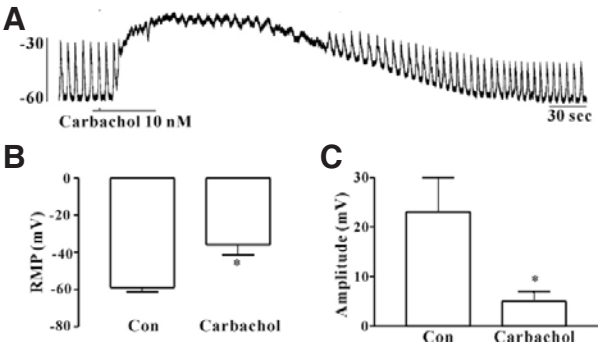

Fig. 1. The depolarization of pacemaker potential by carbachol in cultured ICC from the mouse small intestine. (A) Pacemaker potentials of ICC exposed to carbachol (10 nM) in the current-clamping mode (I = 0). Carbachol caused membrane depolarization and decreased amplitude of pacemaker potentials. Responses to carbachol are summarized in (B) and (C). The bars represent mean values ± SE. *P < 0.05, significantly different from the untreated control.

Statistical analysis

Data are expressed as the mean ± standard error. Differences in the data were evaluated by use of the Student's *t*-test. P values less than 0.05 are considered 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 carbachol on pacemaker activities in cultured ICC

Under the current clamp mode (I = 0), ICC generated spontaneous pacemaker potentials. Exposure to carbachol (10 nM) produced depolarization of the resting membrane potential with a decrease of the amplitude of the pacemaker potential (Fig. 1A). Under control conditions with the current clamp mode, resting potentials and the amplitude of pacemaker potential were -58 ± 3 mV and 23 ± 7 mV. In the presence of carbachol, the resting membrane potential and the amplitude of pacemaker potentials were -36 ± 2 mV and 5 ± 2 mV, respectively (n = 7, Figs. 1B and 1C). Under a voltage clamp at a holding potential of -70 mV, exposure to carbachol induced tonic inward currents (resting currents) and reduced the frequency and amplitude of pacemaker currents in a concentration-dependent manner (Figs. 2A-2D). The summarized values and a bar graph of the effects of carbachol on pacemaker currents are shown in Figs. 2E-2G (n = 5).

Effect of muscarinic receptor antagonists in the carbachol - induced responses in cultured ICC

Muscarinic antagonists were used to identify the receptor subtypes of carbachol. 4-DAMP, a muscarinic M_3 receptor antagonist and methoctramine, a muscarinic M_2 receptor antagonist,

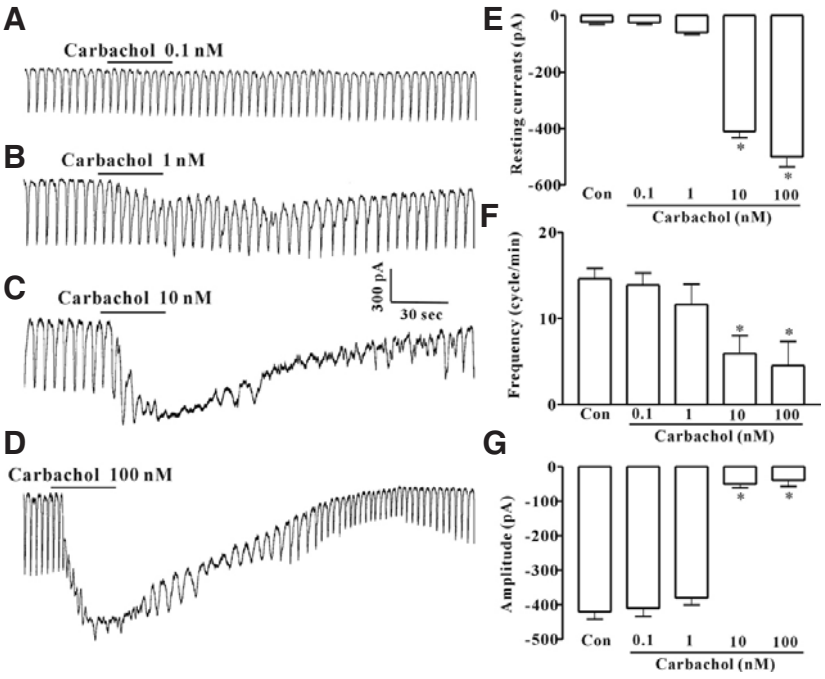

Fig. 2. The tonic inward pacemaker currents by carbachol in cultured ICC from the mouse small intestine. Pacemaker currents of ICC recorded at a holding potential of -70 mV and exposed to various concentrations of carbachol (from 0.1 to 100 nM). (A-D) Carbachol caused a concentration-dependent increase in tonic inward currents and decrease in the frequency and amplitude of pacemaker currents. Responses to carbachol are summarized in (E-G). The bars represent mean values ± SE. **P* < 0.05, significantly different from the untreated control. The dotted lines indicate zero current levels; Con, Control.

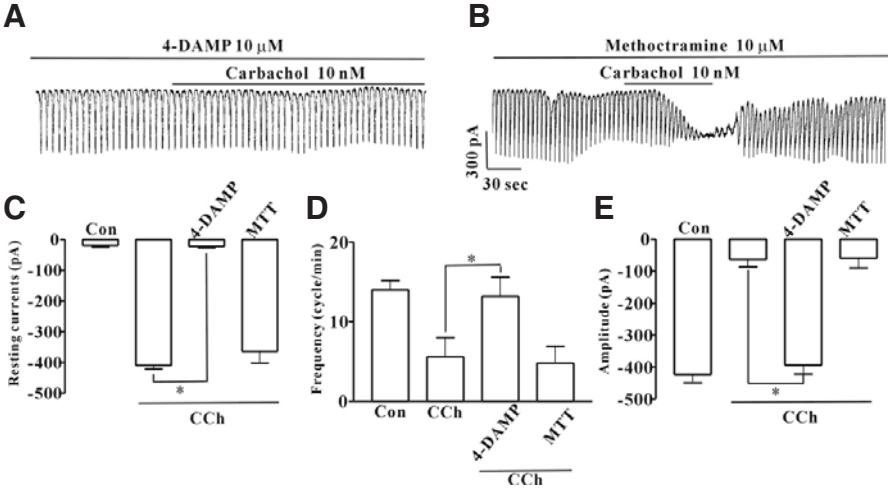

Fig. 3. The blocking effect of a muscarinic M_3 receptor antagonist on the carbachol-induced responses on pacemaker currents in cultured ICC from the mouse small intestine. (A) In the presence of 4-DAMP (10 μM), a muscarinic M_3 receptor antagonist, the effects of carbachol on pacemaker currents were completely blocked. (B) Methoctramine (10 μM), a muscarinic M_2 receptor antagonist, did not block the carbachol-induced responses on pacemaker currents. Responses to carbachol (CCh) in the presence of 4-DAMP or methoctramine (MTT) are summarized in (C)-(E). The bars represent mean values ± SE. **P* < 0.05, significantly different from treatment with carbachol alone. The dotted lines indicate zero current levels.

were used. Treatment with 4-DAMP or methoctramine had no effect on pacemaker currents. Treatment with 4-DAMP (10 μM) almost blocked the carbachol (10 nM)-induced responses on pacemaker currents (Fig. 3A). However, in the presence of methoctramine (10 μM), the carbachol-induced responses on pacemaker currents were still evident (Fig. 3B). Under control conditions at a holding potential of -70 mV, the resting currents, frequency and amplitude of pacemaker currents were -19 ± 6 pA, 14 ± 1.2 cycles/min and -423 ± 26 pA (n = 11). In the presence of 4-DAMP, the resting currents, frequency and amplitude of pacemaker currents with carbachol treatment were -22 ± 5 pA, 13.2 ± 2.4 cycles/min and -394 ± 28 pA, respectively (n = 6). In the presence of methoctramine the resting currents, frequency and amplitude of pacemaker currents with carbachol treatment were -365 ± 37 pA, 4.8 ± 2.1 mV cycles/min and -59 ± 31 pA, respectively (n = 5, Figs. 3C-3E). These results indicate that the modulation of pacemaker currents by carbachol is mediated by muscarinic M_3 receptors.

Involvement of G proteins in the carbachol- induced responses in cultured ICC

The effects of GDP-β-S, a nonhydrolysable guanosine 5′-diphosphate analogue, which permanently inactivates GTP binding proteins, was examined to determine whether G-proteins are involved in the carbachol-induced effects in ICC. When GDP-β-S (1 mM) was in the pipette, the carbachol-induced responses were significantly suppressed (Fig. 4A). Under control conditions at a holding potential of -70 mV, the resting currents, frequency and amplitude of pacemaker currents were -23 ± 9 pA, 14.6 ± 1.5 cycles/min and 422 ± 25 pA, respectively. In the presence of carbachol, the resting currents, frequency and amplitude of pacemaker currents were -359 ± 36

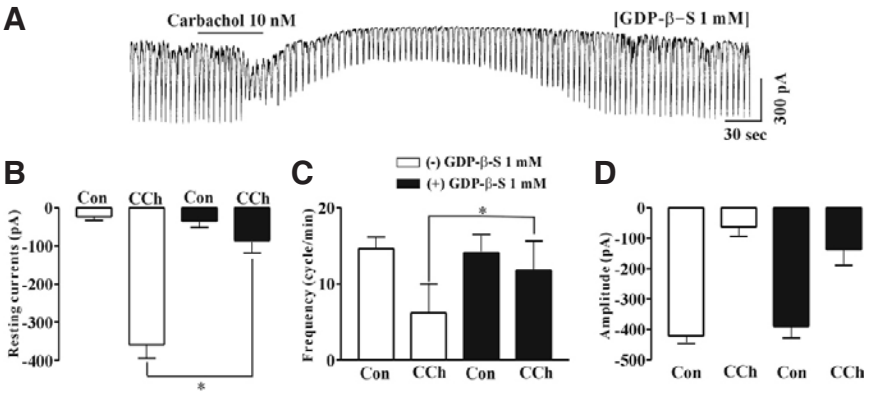

Fig. 4. The blocking effect of GDP-β-S on the response to carbachol on pacemaker currents in cultured ICC from the mouse small intestine. (A) Pacemaker currents from ICC exposed to carbachol (10 nM) in the presence of GDP-β-S (1 mM) in the pipette. GDP-β-S significantly blocked the carbachol-induced responses on pacemaker currents. The blocking effects of GDP-β-S on carbachol (CCh) action are summarized in (B, C, and D). The dotted lines indicate zero current levels. Bars represent mean values ± SE. *$P < 0.05$, significantly different from treatment with carbachol alone. Con, control.

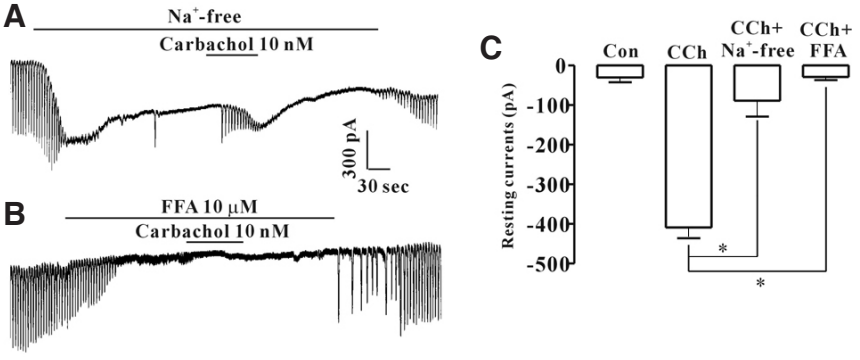

Fig. 5. The blocking effect of an external Na^+ -free solution or flufenamic acid, an NSCC channel blocker, on the carbachol-induced responses on pacemaker currents in cultured ICC from the mouse small intesetine. (A) The use of an external Na^+-free solution abolished the generation of pacemaker currents. Under these conditions, carbachol (10 nM) did not produce tonic inward currents. (B) Flufenamic acid (10 μM) abolished the generation of pacemaker currents and blocked the carbachol-induced tonic inward currents. Responses to carbachol (CCh) in the external Na^+-free solution and in the presence of flufenamic acid (FFA) are summarized in (C). The bars represent mean values ± SE. *$P < 0.05$, significantly different from carbachol treatment alone. The dotted lines indicate zero current levels. Con, control.

pA, 6.2 ± 3.8 cycles/min and -63 ± 31 pA, respectively. In the presence of GDP-β-S in the pipette, the carbachol-induced responses were suppressed; the resting currents, frequency and amplitude of pacemaker currents were -86 ± 32 pA, 11.8 ± 3.8 cycles/min and -137 ± 52 pA, respectively (n = 5, Figs. 4B-4D). These values are significantly different from those obtained with the use of carbachol alone. These results indicate that G-proteins have an essential role in the carbachol-induced responses in ICC.

Effects of an external Na^+-free solution or non-selective cation channel blocker on the carbachol -induced responses in cultured ICC

To determine the characteristic of the tonic inward currents induced by carbachol, we tested the effects of carbachol 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 currents. Under this condition, the tonic inward currents induced by carbachol (10 nM) were reduced (Fig. 5A). Under normal conditions, the resting currents of the tonic inward currents induced by carbachol was -409 ± 27 pA, and in presence of external Na^+-free solution, the resting currents was -89 ± 40 pA (n = 5, Fig. 5C). Together, flufenamic acid (10 μM) abolished the generation of pacemaker currents and blocked the carbachol (10 nM)-induced tonic inward currents (Fig. 5B). In the presence of flufenamic acid, the resting currents of tonic inward currents induced by carbachol was -29 ± 8 pA (n = 5, Fig. 5C). These results suggest that the carbachol-induced tonic inward currents are mediated by NSCCs.

A Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum suppress carbachol-induced responses in cultured ICC

To investigate the role of external Ca^{2+} or internal Ca^{2+}, exposure of ICC to carbachol was tested under external Ca^{2+}-free conditions and in the presence of thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum. The use of an external Ca^{2+}-free solution completely inhibited the pacemaker currents in the voltage clamp mode at a holding potential of -70 mV. In this condition, carbachol (10 nM)-induced tonic inward currents were still evident (n = 5, Fig. 6A). The value of the resting currents with carbachol treatment in the Ca^{2+}-free solution was not significantly different when compared with a control value obtained in normal solution (Fig. 6C). However, treatment with thapsigargin (5 μM) inhibited the pacemaker currents of the ICC and blocked the carbachol (10 nM)-induced tonic inward currents (Fig. 6B). In the presence of thapsigargin, the value of the resting currents with carbachol treatment with was significantly different from the value obtained with carbachol treatment in the absence of thapsigargin (n = 5, Fig. 6C).

Effects of intracellular Ca^{2+} intensity by carbachol in cultured ICC

As intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in ICC are considered the primary mechanism for the pacemaker activity in GI activity (Ward et al., 2002), we examined the effect of carbachol on $[Ca^{2+}]_i$ oscillations in ICC. In this study, we measured spontaneous $[Ca^{2+}]_i$ oscillations of ICC that are connected with cell clusters. Spontaneous $[Ca^{2+}]_i$ oscillations were observed in many ICC (low magnification; data not shown), which were loaded with fluo3-AM (Fig. 7A), and spontaneous regular $[Ca^{2+}]_i$

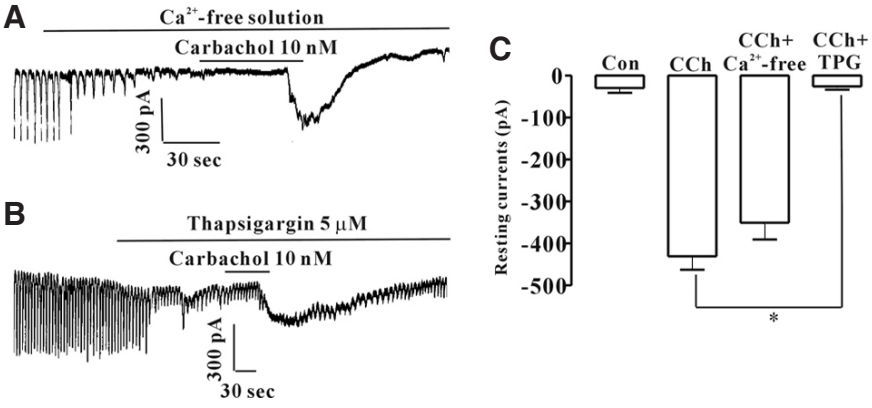

Fig. 6. The effect of an external Ca^{2+}-free solution or thapsigargin, a Ca^{2+} ATPase inhibitor of the endoplasmic reticulum, on the carbachol-induced responses on pacemaker currents in cultured ICC from the mouse small intestine. (A) The use of an external Ca^{2+}-free solution abolished the generation of pacemaker currents. Under these conditions, the carbachol (10 nM)-induced tonic inward currents were not blocked. (B) Thapsigargin (5 μM) abolished the generation of pacemaker currents and blocked the carbachol (10 nM)-induced tonic inward currents. Responses to carbachol (CCh) in the external Ca^{2+}- free solution and in the presence of thapsigargin (TPG) are summarized in (C). The bars represent mean values ± SE. *$P < 0.05$, significantly different from carbachol treatment alone. The dotted lines indicate zero current levels. Con, control.

oscillations were seen in a time series (Fig. 7D). Especially, the frequency of $[Ca^{2+}]_i$ oscillation was about 8-10 cycles/min. The difference of frequency between $[Ca^{2+}]_i$ oscillation and pacemaker activity is the reason that we scanned every 0.4 s for recording of $[Ca^{2+}]_i$. In the presence of carbachol (10 nM), the basal points of $[Ca^{2+}]_i$ oscillations increased but the peak points of $[Ca^{2+}]_i$ oscillations were slightly decreased (Fig. 7B and 7C). The data of the time series are shown in Fig. 7D. These results suggest that the action of carbachol in ICC may involve the regulation of spontaneous $[Ca^{2+}]_i$ oscillations.

DISCUSSION

Acetylcholine (ACh) depolarizes the membrane potential of slow waves and contracts gastrointestinal smooth muscle (Hirst et al., 2002). The main cause of membrane depolarization by ACh is due to the activation of NSCCs by muscarinic receptor stimulation. That leads Ca^{2+} influx through voltage-dependent Ca^{2+} channel activation and induces smooth muscle contractions (Unno et al., 2006). NSCCs conduct net inward currents predominantly by Na^+ under physiological conditions. The removal of external Na^+ decreased the amplitude of NSCC currents by muscarinic stimulation. Pharmacologically divalent cations such as Cd^{2+}, Ni^{2+}, Co^{2+}, and Mn^{2+} inhibit the muscarinic NSCC currents (Inoue, 1991; Kuriyama et al., 1998). ICC generate spontaneous inward pacemaker currents, which are mainly mediated by the periodic activation of NSCC (Koh et al., 1998; Thomsen et al., 1998). Reducing the concentration of external Na^+ abolished the generation of pacemaker currents. The external application of divalent cations and NSCC blockers such as Gd^{3+} and flufenamic acid also inhibited the generation of pacemaker currents (Jun et al., 2004). Transient receptor potential (TRP) channels are components of NSCC. Classical TRP (TRPC) mRNA is detected in ICC by RT-PCR and the expressed TRPC4 currents are similar to pacemaker currents of ICC (Epperson et al., 2000; Walker et al., 2002). Recently, it was reported that melastatin-type TRP7 (TRPM7) channels are involved in the generation of pacemaker currents in cultured ICC of the mouse small intestine. The knockdown of TRPM7 was accomplished by the use of siRNA-suppressed spontaneous pacemaker currents in cultured ICC (Kim et al., 2005). In cultured gastric ICC, ACh or carbachol produced membrane depolarization as seen in intact gastrointestinal smooth muscle and produced tonic inward pacemaker currents (Kim et al., 2003). However, the nature of the tonic inward currents was not defined. In the present study, carbachol depolarized the pacemaker potentials and produced tonic inward currents with reduced frequency and amplitude of pacemaker currents in the cultured intestinal ICC. The carbachol-induced tonic inward currents were blocked under a reducing external Na^+-free solution and with pretreatment with flufenamic acid. Interestingly, Na^+-free solution firstly generated the inward current and then the inward current returned the baseline. We cannot explain the exact mechanism of inward current generation by Na^+-free solution and just suspect that this may be due to the washout of intracellular Na^+ etc. Substance P is also a major excitatory neurotransmitter in the gastrointestinal tract that depolarizes the membrane potential and activates NSCC such as ACh (Zagorodnyuk et al., 1994). We have previously reported that substance P produced tonic inward currents that were mediated by NSCC activation in cultured intestinal ICC (Jun et al., 2004). Therefore, our results suggest that carbachol activates NSCC in ICC in a similar manner as substance P.

A G-protein-mediated signal transduction pathway is believed to play a key role in the gating process of the muscarinic-receptor-activated cationic current (Inoue and Isenberg, 1990a). Internally applied guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S), a nonhydrolyzable analogue of guanosine 5′-triphosphate (GTP) that permanently inactivates GTP-binding proteins, can induce similar cationic currents in gastrointestinal smooth muscle cells and the activation of muscarinic receptor-activated cationic currents can be inhibited by guanosine 5′-diphosphate (GDP-β-S), a nonhydrolysable guanosine 5′-diphosphate(GDP) analogue that permanently inactivates GTP-binding proteins (Unno et al., 2006). In the present study, when GDP-β-S was present in the pipette, the carbachol-induced effects on pacemaker currents were significantly suppressed. These results suggest that G-proteins are involved in modulating pacemaker currents by muscarinic receptor activation.

Muscarinic receptors are composed of five subtypes from M_1 to M_5. Gastrointestinal smooth muscles express the M_2 and M_3 subtypes of the muscarinic receptors. M_2 receptors are more distributed than M_3 receptors in the ratio of 80% M_2 to 20% M_3 (Sanders, 1998). In molecular studies, mRNA of M_2 and M_3 receptors were detected by RT-PCR from isolated ICC (Epperson et al., 2000). Carbachol-induced effects on pacemaker currents were blocked by a muscarinic M_3 receptor blocker in cultured gastric ICC but not by an M_2 receptor blocker (Kim et al., 2003). In the present study, we also found that 4-DAMP, a muscarinic M_3 receptor antagonist, inhibited the

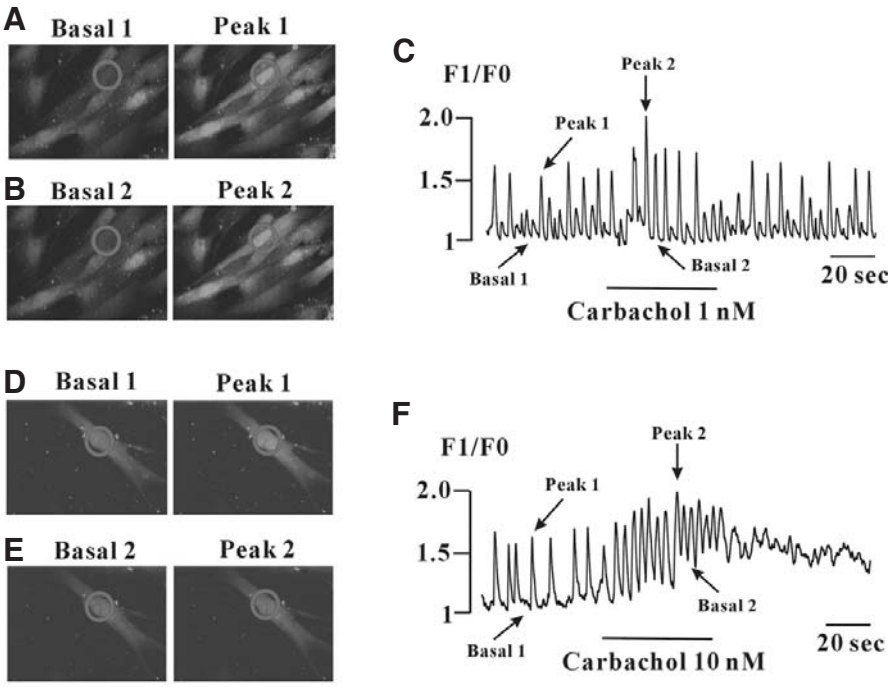

Fig. 7. The regulation of carbachol on spontaneous Ca^{2+} oscillation in cultured ICC from the mouse small intestine. (A) Sequential fluorescence intensity images of Fluo-3 loaded cultured ICC under normal conditions. (B, C) Sequential fluorescence intensity images of Fluo-3 loaded cultured ICC in the presence of carbachol (10 nM). (D) Fluorescence intensity changes plotted in the red marker of (A, B, and C). In the presence of carbachol (10 nM), the basal points of $[Ca^{2+}]_i$ oscillations increased but the peak points of $[Ca^{2+}]_i$ oscillations were slightly decreased. The image of basal 1, 2, and 3 and peak 1, 2, and 3 points in (A, B, and C) acquired from indicators in (D).

carbachol-induced effects significantly in cultured intestinal ICC. However, methoctramine, a M_2 receptor blocker, did not inhibit the carbachol-induced effects. Therefore, it seems that muscarinic M_3 receptors may be involved in the regulation of pacemaker currents in intestinal ICC.

Extracellular Ca^{2+} and intracellular Ca^{2+} have obligatory roles in muscarinic NSCC activation in gastrointestinal smooth muscle. When Ca^{2+} is removed from the external solution (Ca^{2+}-free solution) or when Ca^{2+} buffers are loaded into the cells, the amplitude of muscarinic NSC currents are decreased (Inoue and Isenberg, 1990b; Sakamoto et al., 2006), which indicates that activation of NSCCs is Ca^{2+} dependent. In addition, the generation of pacemaker currents in ICC is dependent upon extracellular and intracellular Ca^{2+}. The removal of extracellular Ca^{2+} abolishes pacemaker activity in ICC (Torihashi et al., 2002). The generation of pacemaker currents in ICC is initiated by inositol 1,4,5-triphosphate (IP_3) receptor-activated Ca^{2+} release from the endoplasmic reticulum (Ward et al., 2002). Furthermore, the activation of muscarinic M_3 receptors is coupled with phosphatidylinositol turnover. The activation of muscarinic M_3 receptor stimulates phospholipase C (PLC) and generates IP_3 and diacylglycerol (DAG). In turn, IP_3 increases intracellular Ca^{2+} from the endoplasmic reticulum and DAG activates protein kinase C (Sanders, 1998). In gastric ICC, the use of a PLC inhibitor blocked carbachol-induced tonic inward currents. However, a protein kinase C inhibitor did not block carbachol-induced tonic inward currents (Kim et al., 2003). In the present study, the carbachol-induced tonic inward currents were still induced by the removal of extracellular Ca^{2+}. Thapsigargin, a Ca^{2+} ATPase inhibitor that acts on the endoplasmic reticulum, blocked the carbachol-induced tonic inward currents. Therefore, our results suggest that IP_3-dependent intracellular Ca^{2+} release by muscarinic receptor activation is essential to the carbachol-induced tonic currents rather than Ca^{2+} influx from the extracellular space.

Intracellular Ca^{2+} oscillations are the primary mechanism to generate pacemaker currents. Intracellular Ca^{2+} oscillations are well matched with periodic pacemaker currents. Recently, Nakayama et al. (2005) suggested that sulfonylurea receptor (SUR) subunit can modulate the pacemaker activity of ICC by regulating of $[Ca^{2+}]_i$ oscillations and also our previous report showed PGE_2 inhibited pacemaker currents by decreasing intracellular Ca^{2+} oscillations in cultured ICC (Choi et al., 2006). In this study, treatment with carbachol in ICC initially increased the oscillating Ca^{2+} wave frequency and the basal point of $[Ca^{2+}]_i$ levels and were followed by a decrease of the peak point and oscillating Ca^{2+} wave frequency. However, the $[Ca^{2+}]_i$ intensity was broadly increased as with the action of tonic inward currents reversely. We think that the reduced frequency of pacemaker currents after treatment with carbachol is related with these changes of intracellular Ca^{2+} oscillations. Koh et al. (2002) reported that spontaneous NSCC activities are reduced at a high intracellular Ca^{2+} concentration in cultured ICC. Therefore, it seems that the reduced frequency and the fused tonic inward currents are due to an increase of intracellular Ca^{2+} by carbachol.

In conclusion, our results show that the modulation of pacemaker currents by carbachol is mediated through the activation of non-selective cation channels via muscarinic M_3 receptors by a G-protein dependent intracellular Ca^{2+} release mechanism.

ACKNOWLEDGMENT

This work was supported by research fund from Chosun University 2006.

REFERENCES

Choi, S., Yeum, C.H., Chang, I.Y., You, H.J., Park, J.S., Jeong, H.S., So, I., Kim, K.W., and Jun, J.Y. (2006). Activating of ATP-dependent K^+ channels comprised of K_{ir} 6.2 and SUR 2B by PGE_2 through EP_2 receptor in cultured interstitial cells of Cajal from murine small intestine. Cell. Physiol. Biochem. *18*, 187-198.

Epperson, A., Hatton, W.J., Callaghan, B., Doherty, P., Walker, R.L., Sanders, K.M., Ward, S.M., and Horowitz, B. (2000). Molecular markers expressed in cultured and freshly isolated interstitial

cells of Cajal. Am. J. Physiol. Cell. Physiol. *279*, C529-C539.
Hirst, G.D., and Edwards, F.R. (2004). Role of interstitial cells of Cajal in the control of gastric motility. J. Pharmacol. Sci. *96*, 1-10.
Hirst, G.D., Dickens, E.J., and Edwards, F.R. (2002). Pacemaker shift in the gastric antrum of guinea-pigs produced by excitatory vagal stimulation involves intramuscular interstitial cells. J. Physiol. *541*, 917-928.
Huizinga, J.D., Chang, G., Diamant, N.E., and El-Sharkawy, T.Y. (1984). Electrophysiological basis of excitation of canine colonic circular muscle by cholinergic agents and substance P. J. Pharmacol. Exp. Ther. *231*, 692-699.
Inoue, R. (1991). Effect of external Cd^{2+} and other divalent cations on carbachol-activated non-selective cation channels in guinea-pig ileum. J. Physiol. *442*, 447-463.
Inoue, R., and Chen, S. (1993). Physiology of muscarinic receptor-operated nonselective cation channels in guinea-pig ileal smooth muscle. EXS. *66*, 261-268.
Inoue, R., and Isenberg, G. (1990a). Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. J. Physiol. *424*, 73-92.
Inoue, R., and Isenberg, G. (1990b). Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. Am. J. Physiol. *258*, C1173-C1178.
Jun, J.Y., Kong, I.D., Koh, S.D., Wang, X.Y., Perrino, B.A., Ward, S.M., and Sanders, K.M. (2001). Regulation of ATP-sensitive K^+ channels by protein kinase C in murine colonic myocytes. Am. J. Physiol. Cell. Physiol. *281*, C857-C64.
Jun, J.Y., Choi, S., Yeum, C.H., Chang, I.Y., You, H.J., Park, C.K., Kim, M.Y., Kong, I.D., Kim, M.J., Lee, K.P., et al. (2004). Substance P induces inward current and regulates pacemaker currents through tachykinin NK1 receptor in cultured interstitial cells of Cajal of murine small intestine. Eur. J. Pharmacol. *495*, 35-42.
Kim, T.W., Koh, S.D., Ordög, T., Ward, S.M., and Sanders, K.M. (2003). Muscarinic regulation of pacemaker frequency in murine gastric interstitial cells of Cajal. J. Physiol. *546*, 415-425.
Kim, B.J., Lim, H.H., Yang, D.K., Jun, J.Y., Chang, I.Y., Park, C.S., So, I., Stanfield, P.R., and Kim, K.W. (2005). Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. Gastroenterology *129*, 1504-1517.
Koh, S.D., Sanders, K.M., and Ward, S.M. (1998). Spontaneous electrical rhythmicity in cultured interstitial cells of cajal from the murine small intestine. J. Physiol. *513*, 203-213.
Koh, S.D., Jun, J.Y., Kim, T.W., and Sanders, K.M. (2002). A Ca^{2+}-inhibited non-selective cation conductance contributes to pacemaker currents in mouse interstitial cell of Cajal. J. Physiol. *540*, 803-814.
Kuriyama, H., Kitamura, K., Itoh, T., and Inoue, R. (1998). Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. Physiol. Rev. *78*, 811-920.
Nakayama, S., Ohya, S., Liu, H.N., Watanabe, T., Furuzono, S., Wang, J., Nishzawa, Y., Aoyama, M., Murase, N., Matsubara, T., et al. (2005). Sulphonylurea receptors differently modulate ICC pacemaker Ca^{2+} activity and smooth muscle contractility. J. Cell. Sci. *118*, 4163-4173.
Olsson, C., and Holmgren, S. (2001). The control of gut motility. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. *128*, 481-503.
Sakamoto, T., Unno, T., Matsuyama, H., Uchiyama, M., Hattori, M., Nishimura, M., and Komori, S. (2006). Characterization of muscarinic receptor-mediated cationic currents in longitudinal smooth muscle cells of mouse small intestine. J. Pharmacol. Sci. *100*, 215-226.
Sanders, K.M. (1998). G protein-coupled receptors in gastrointestinal physiology. IV. Neural regulation of gastrointestinal smooth muscle. Am. J. Physiol. *275*, G1-G7.
Sanders, K.M., Koh, S.D., and Ward, S.M. (2006). Interstitial cells of Cajal as pacemakers in the gastrointestinal tract. Annu. Rev. Physiol. *68*, 307-343.
Sims, S.M. (1992). Cholinergic activation of a non-selective cation current in canine gastric smooth muscle is associated with contraction. J. Physiol. *449*, 377-398.
Streutker, C.J., Huizinga, J.D., Driman, D.K., and Riddell, R.H. (2007). Interstitial cells of Cajal in health and disease. Part I: normal ICC structure and function with associated motility disorders. Histopathology. *50*, 176-189.
Suzuki, H. (2000). Cellular mechanisms of myogenic activity in gastric smooth muscle. Jpn. J. Physiol. *50*, 289-301.
Szurszewski, J.H. (1987). Electrical basis for gastrointestinal motility. In Physiology of the Gastrointestinal Tract, L.R. Johnson, ed., 2nd ed. (New York, USA; Raven Press), pp. 383-422.
Thomsen, L., Robinson, T.L., Lee, J.C., Farraway, L.A., Hughes, M.J., Andrews, D.W., and Huizinga, J.D. (1998). Interstitial cells of Cajal generate a rhythmic pacemaker current. Nat. Med. *4*, 848-851.
Torihashi, S., Fujimoto, T., Trost, C., and Nakayama, S. (2002). 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.
Unno, T., Matsuyama, H., Okamoto, H., Sakamoto, T., Yamamoto, M., Tanahashi, Y., Yan, H.D., and Komori, S. (2006). Muscarinic cationic current in gastrointestinal smooth muscles: signal transduction and role in contraction. Auton. Autacoid. Pharmacol. *26*, 203-217.
Vogalis, F., and Sanders, K.M. (1990). Cholinergic stimulation activates a non-selective cation current in canine pyloric circular muscle cells. J. Physiol. *429*, 223-236.
Walker, R.L., Koh, S.D., Sergeant, G.P., Sanders, K.M., and Horowitz, B. (2002). TRPC4 currents have properties similar to the pacemaker current in interstitial cells of Cajal. Am. J. Physiol. Cell. Physiol. *283*, C1637-C1645.
Ward, S.M., and Sanders, K.M. (2006). Involvement of intramuscular interstitial cells of Cajal in neuroeffector transmission in the gastrointestinal tract. J. Physiol. *576*, 675-682.
Ward, S.M., Ordog, T., Koh, S.D., Baker, S.A., Jun, J.Y., Amberg, G., Monaghan, K., and Sanders, K.M. (2002). Pacemaking in interstitial cells of Cajal depends upon calcium handling by endoplasmic reticulum and mitochondria. J. Physiol. *525*, 355-361.
Won, K.J., Sanders, K.M., and Ward, S.M. (2005). Interstitial cells of Cajal mediate mechanosensitive responses in the stomach. Proc. Natl. Acad. Sci. USA *102*, 14913-14918.
Zagorodnyuk, V., Santicioli, P., and Maggi, C.A. (1994). Different Ca^{2+} influx pathways mediate tachykinin receptor-induced contraction in circular muscle of guinea-pig colon. Eur. J. Pharmacol. *255*, 9-15.