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Actin Microfilament Involved in Regulation of Pacemaking Activity in Cultured Interstitial Cells of Cajal from Murine Intestine

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Abstract The present study investigated the effect of actin microfilament structure on pacemaker currents and calcium oscillation in cultured murine intestinal interstitial cells of Cajal (ICCs) by whole-cell patch-clamp technique and calcium imaging technique. Cytochalasin B, a disruptor of actin microfilaments, decreased the amplitude and frequency of pacemaker currents from 491.32 ± 160.33 pA and 11.73 ± 0.79 cycles/min to 233.12 ± 92.00 pA and 10.29 ± 0.76 cycles/min. Cytochalasin B also decreased the amplitude and frequency of calcium oscillation from 0.32 ± 0.08 (ΔF/F0) and 2.75 ± 0.17 cycles/min to 0.02 ± 0.01 (ΔF/F0) and 1.20 ± 0.08 cycles/min. Phalloidin, a stabilizer of actin microfilaments, increased the amplitude and frequency of pacemaker currents from 751.79 ± 282.82 pA and 13.93 ± 1.00 cycles/min to 1234.34 ± 607.83 pA and 14.68 ± 1.00 cycles/min. Phalloidin also increased the amplitude and frequency of calcium oscillation from 0.26 ± 0.01 (ΔF/F0) and 2.27 ± 0.18 cycles/min to 0.43 ± 0.03 (ΔF/F0) and 2.87 ± 0.07 cycles/min. 2-Aminoethoxydiphenyl borane (2-APB), an IP_3 receptor blocker, suppressed both pacemaker currents and calcium oscillations. 2-APB also blocked the phalloidin-induced increase in pacemaker currents and calcium oscillation. Ryanodine, an inhibitor of calcium-induced calcium release, did not affect pacemaker current but suppressed calcium oscillations. Ryanodine had no effect on altering phalloidin-induced increases in pacemaker current and calcium oscillation. These results suggest that actin microfilaments regulate pacemaker activity via the IP_3-induced calcium release signaling pathway.

Keywords Patch clamp technique · Calcium imaging technique · Interstitial cells of Cajal · Actin microfilament · Pacemaker current · Calcium oscillation

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The gastrointestinal (GI) tract shows spontaneous mechanical contractions mediated by the periodic generation of electrical pacemaker potentials, the basic determinant of GI smooth muscle activity (Szurszewski 1987). Numerous physiological studies have demonstrated that electrical pacemaker and motor neurotransmission activity in the stomach is dependent on the function of the interstitial cells of Cajal (ICCs) (Huizinga et al. 1995; Sanders 1996; Sanders et al. 1999). ICCs generate spontaneous electrical slow wave activity, conducting a rhythmic impulse to smooth muscle cells and mediate the propagation of the slow waves (Sanders 1996; Der-Silaphet et al. 1998; Ward and Sanders 2001). Previous researchers have reported that Ca^{2+}-dependent plasma membrane ion channels are responsible for mediating ICCs pacemaker potential (Huizinga et al. 2002; Walker et al. 2002), and spontaneous Ca^{2+} activity in ICCs is considered the primary mechanism for rhythmic ICCs pacemaker activity. The oscillations in intracellular calcium ($[Ca^{2+}]_i$) observed in ICCs can periodically activate plasma membrane Ca^{2+}-dependent ion channels, thereby generating pacemaker potentials. Two families of intracellular Ca^{2+}

release channels (including the inositol 1,4,5-trisphosphate receptor types 1–3 and ryanodine receptor types 1–3) along with several intracellular messengers and modulators (including cyclic adenosine diphosphate ribose and nicotinic acid adenine dinucleotide phosphate) have a widespread messenger role in shaping Ca^{2+} signals in many cell types (Galione and Churchill 2002; Masgrau et al. 2003). Several lines of evidence now suggest that the inositol 1,4,5-trisphosphate receptor (IP_3R) plays an important role in generating spontaneous electrical activity in GI pacemaker cells (Suzuki et al. 2000; Malysz et al. 2001), and the ryanodine receptor has been suggested to be involved in NO-induced Ca^{2+} transients in colonic ICCs (Publicover et al. 1993).

The actomyosin cytoskeleton is a large network of structural, motor, and signaling proteins that coordinate a plethora of cellular functions including cell division, adhesion, and migration. The activity of various transport proteins as well as ion- and water-permeable channels have been shown to be dependent on the extent of actin polymerization (Jessen and Hoffmann 1992; Cantiello et al. 1993; Prat et al. 1993). Epithelial ion channels can interact directly or indirectly with the cytoskeleton, which serves to maintain the polarized expression of ion channels within specific membrane domains and to regulate ion channel activity (Mazzochi et al. 2006). Gravante et al. (2004) reported the pacemaker channel HCN1 directly binds to a ubiquitous isoform of filamin A in cardiac myocytes, and this suggests that the cytoskeleton may contribute to modulating channel activity.

ICCs also possess three components of the cytoskeleton in mice and humans (Faussone-Pellegrini and Thuneberg 1999). The lack of full-length dystrophin, a membrane-associated cytoskeletal protein, is known to be associated with ultrastructural modifications of gastric ICCs, most of which can be interpreted as a marker of new membrane formation, altered Ca^{2+} handling, and defective generation and regeneration of slow-wave activity (Vannucchi et al. 2004).

However, the effect of the cytoskeleton on ICC electrical pacemaker activity in the GI tract has not been investigated. Thus, in the present study, the effects of cytochalasin B and phalloidin, regulators of actin microfilaments (one of the three major cytoskeletal components) on pacemaker activity, were investigated in cultured intestinal ICCs of mice by means of patch clamping and Ca^{2+} fluorescence techniques.

Methods

Preparation of Cells

BALB/c mice (7–13 days old) of either sex were obtained from the Experimental Animal Center of the Chinese Academy of Science. Animals were killed by cervical dislocation. The small intestine was dissected from 1 cm below the pyloric ring to the cecum, and luminal contents were washed out with Krebs-Ringer bicarbonate (KRB). Tissues were pinned to the base of a silica gel dish and the mucosae removed. Small strips of intestinal muscle were incubated at 37°C for 23 min in enzyme solution comprising the following: 1.3 mg/ml collagenase (type II, Worthington), 2 mg/ml bovine serum albumin (Sino-American Hua Mei Biotechnology Company of Beijing, China), 2 mg/ml trypsin inhibitor (Amresco, Solon, OH) and 0.27 mg/ml adenosine triphosphate (ATP; Sigma Aldrich, St. Louis, MO). The muscle strips were then washed three times to remove the enzyme before being triturated through blunt pipettes. The resulting cell suspension was plated on murine collagen-coated (2.5 μg/ml) sterile glass coverslips in culture dishes. Cells were allowed to settle for 30 min before adding smooth muscle cell basal medium (SMBM) (Clonetics Corp, San Diego, CA) supplemented with 2% antibiotics/antimycotics (Gibco Invitrogen, Grand Island, NY) and murine stem cell factor (5 ng/ml; Sigma Aldrich). Cells were then incubated at 37°C in a 5% CO_2 incubator (Zhang et al. 2006).

All experimental protocols were approved by the local animal care committee and conformed with the *Guide for the Care and Use of Laboratory Animals* published by the Science and Technology Commission of PRC (STCC Publication 2, revised 1988).

Ca^{2+} Fluorescent Imaging

Cultured ICCs (48–72 h) were loaded with the Ca^{2+}-sensitive indicator Fluo-3AM (8 μM) and Pluronic F-127 (0.03%) for 2 h at 37°C and washed for a further 30 min in physiological salt solution for de-esterification. A microscope (Olympus BX51, Japan) equipped with a Hamamatsu 1394 Orca-ER CCD camera (Hamamatsu, Japan) was used to monitor and record digital images. Simple PCI 6 software (Compix Inc.) was used for data analysis. Cells were illuminated at 488 nm and emission light of 515–565 nm was detected. Fluorescence intensity was expressed as a fluorescence ratio (F/F0), elucidating Ca^{2+} fluorescence intensity from its arbitrary resting fluorescence intensity (F0). All recordings were made at room temperature with digital images recorded every second.

Patch Clamp Experiments

The whole-cell patch clamp technique was used to record inward currents (voltage clamp) and membrane potential (current clamp) from cultured ICCs (48–72 h). Glass pipettes with a resistance of 3–5 MΩ were used to form giga seals. Electric signals were amplified with an EPC-10 patch

clamp amplifier (HEKA Instruments, Lambrecht/Pfalz, Germany) and digitized with a PCI-16 A/D converter (HEKA Instruments). Data were analyzed by Origin 6.0 and Sigmaplot 2.0. All recordings were made at 30°C.

Solutions and Drugs

The KRB solution comprised (mM): KCl 4.7, NaCl 117, $CaCl_2$ 2.6, Glucose 11, $MgSO_4$ 1.2, KH_2PO_4 1.2, and $NaHCO_3$ 25; it was bubbled with 95% O_2/5%CO_2 (final pH 7.4). Physiological salt solution comprised (mM): KCl 4.5, NaCl 135, $CaCl_2$ 2, glucose 10, $MgCl_2$ 1, and 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid (HEPES) 10 (final pH adjusted to 7.4 with Tris). The pipette solution comprised (mM): KCl 140, $MgCl_2$ 5, K_2ATP 2.7, Na_2GTP 0.1, creatine phosphate (disodium salt) 2.5, HEPES 5, and ethylene glycol tetraacetic acid (EGTA) 0.1 (final pH adjusted to 7.2 with Tris).

2-Aminoethoxydiphenyl borane (2-APB) and ryanodine were dissolved in dimethyl sulfoxide to provide stock solutions stored at −20°C until use. EGTA was dissolved in distilled water. Ryanodine was purchased from Merck (Darmstadt, Germany). Fluo-3AM and Pluronic F-127 were purchased from AnaSpec. All other drugs/agents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

Statistical Analysis and Presentation

Data are expressed as mean ± standard error of the mean. Differences between data sets were evaluated by paired *t*-tests. *P* values of < 0.05 were considered significant. The "n" values reported correspond to the number of cells analyzed in each experiment.

Results

Effect of Cytochalasin B on Pacemaker Current and Calcium Oscillation

Under whole-cell voltage clamp mode, the spontaneous rhythmic inward pacemaker currents were recorded at −60 mV of holding potential. Cytochalasin B or D and phalloidin are the most common chemical compound to depolymerize and stabilize actin microfilaments (Brown and Spudich 1979; Allingham et al. 2006; Mazzochi et al. 2006). Therefore, in this study, we used cytochalasin B and phalloidin as a disruptor and stabilizer of actin microfilaments. Addition of 20 μM cytochalasin B to the external perfusion solution significantly suppressed pacemaker currents (Fig. 1Aa). The amplitude and frequency of pacemaker currents were found to be significantly reduced, from 491.32 ± 160.33 pA and 11.73 ± 0.79 cycles/min recorded in control cells to 233.12 ± 92.00 pA and 10.29 ± 0.76 cycles/min after cytochalasin B treatment, respectively (Fig. 1Ab and c, $n = 6$, $P < 0.05$).

Calcium oscillations are vital for the generation of pacemaker currents (Sanders et al. 2000) and the cytoskeleton is known to regulate Ca^{2+} influx and release from intracellular calcium stores (Rosado and Sage 2000; Bose and Thomas 2009). To investigate the mechanisms of actin microfilaments on pacemaker currents, we tested the effect of cytochalasin B on periodic $[Ca^{2+}]_i$ oscillation. Cultured ICCs (48–72 h) preloaded with fluo-3, a high-affinity Ca^{2+}-sensitive indicator, exhibited spontaneous periodic calcium oscillation. Addition of 20 μM cytochalasin B into the external solution significantly suppressed the spontaneous Ca^{2+} oscillations. The amplitude (ΔF/F0) and frequency of calcium oscillations were significantly decreased, from 0.32 ± 0.08 and 2.75 ± 0.17 cycles/min at baseline to 0.02 ± 0.01 and 1.20 ± 0.08 cycles/min after cytochalasin B administration, respectively (Fig. 1B, $n = 6$, $P < 0.05$). Basal fluorescence intensity was not affected by addition of cytochalasin B.

Effect of Phalloidin on Pacemaker Current and Calcium Oscillations

Administering the actin microfilament stabilizer phalloidin (20 μM) to the external perfusion solution significantly potentiated pacemaker currents (Fig. 2Aa). The amplitude and frequency of pacemaker currents were enhanced from 751.79 ± 282.82 pA and 13.93 ± 1.00 cycles/min in control cells, to 1234.34 ± 607.83 pA and 14.68 ± 1.00 cycles/min after phalloidin addition, respectively (Fig. 2Ab and c, $n = 6$, $P < 0.05$). Moreover, addition of 20 μM phalloidin to the external solution significantly potentiated spontaneous calcium oscillations. The amplitude (ΔF/F0) and frequency of calcium oscillations were increased from 0.26 ± 0.01 and 2.27 ± 0.18 cycles/min in control cells, to 0.43 ± 0.03 and 2.87 ± 0.07 cycles/min after drug administration, respectively (Fig. 2B, $n = 6$, $P < 0.05$). The basal fluorescence intensity was not affected by addition of phalloidin.

Effect of 2-APB on Phalloidin-induced Increase in Pacemaker Currents and Calcium Oscillation

IP_3R plays a key role in generating spontaneous electrical activity in GI pacemaker cells (Suzuki et al. 2000; Malysz et al. 2001); we thus administered an IP_3R inhibitor, 2-APB, to observe its impact on pacemaker currents. Application of 2-APB completely abolished pacemaker currents and blocked the phalloidin-induced increase in

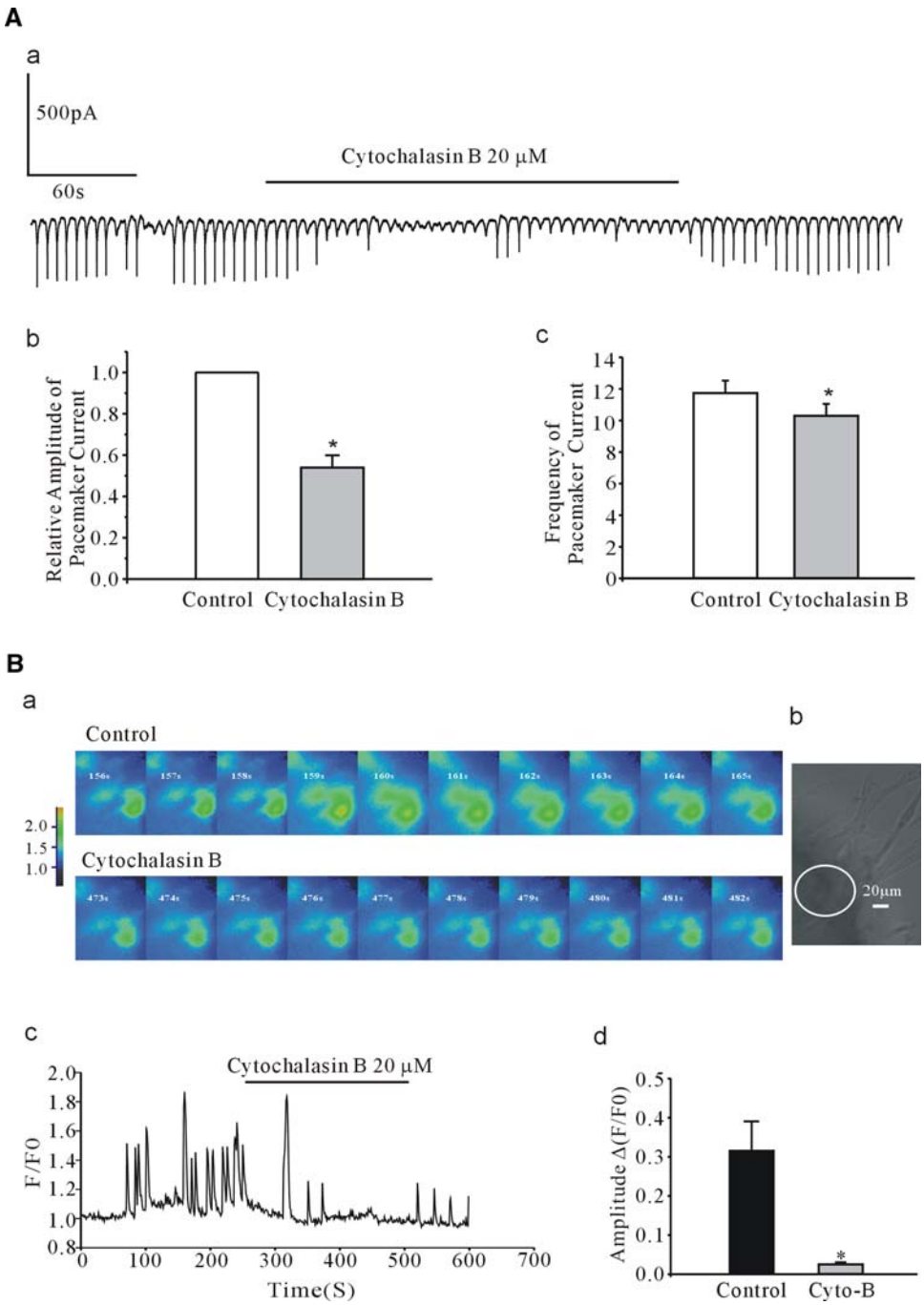

Fig. 1 Effect of cytochalasin B on pacemaker activity in cultured murine small intestine ICCs. **A** The effect of cytochalasin B on pacemaker currents. **Aa** Raw trace of cytochalasin B–induced inhibition. **Ab, Ac** Summary of the amplitude and frequency of pacemaker currents recorded (* $P < 0.05$ vs. control, $n = 6$). **B** Effect of cytochalasin B on calcium oscillations. **Ba** Pseudo-color ratio images of the fluo-3 fluorescence intensity recorded in the square regions (white line in (**Bb**)). **Bc, Bd** Raw trace of calcium oscillations is shown and calcium oscillation amplitude is summarized, respectively (* $P < 0.05$ vs. control, $n = 6$)

pacemaker currents (Fig. 3A, $n = 6$). Furthermore, 30 μM 2-APB significantly suppressed calcium oscillations, significantly reducing oscillation amplitude from 0.21 ± 0.04 to 0.09 ± 0.03 (Fig. 3B, $n = 6$, $P < 0.05$). The phalloidin-induced increase in calcium oscillations was almost completely blocked by the presence of 2-APB, maintaining ΔF/F0 values equal to that of control: 0.09 ± 0.03 vs. 0.09 ± 0.01 for control and phalloidin cells, respectively, (Fig. 3B, $n = 6$, $P > 0.05$).

Effect of Ryanodine on Phalloidin-induced Increase in Pacemaker Current and Calcium Oscillation

Ryanodine receptor–dependent calcium release is another intracellular calcium source, with ryanodine receptors reportedly involved in nitric oxide–induced Ca^{2+} transients in colonic ICCs (Publicover et al. 1993). We therefore tested the effect of administering ryanodine, an inhibitor of calcium-induced calcium release, on pacemaker currents

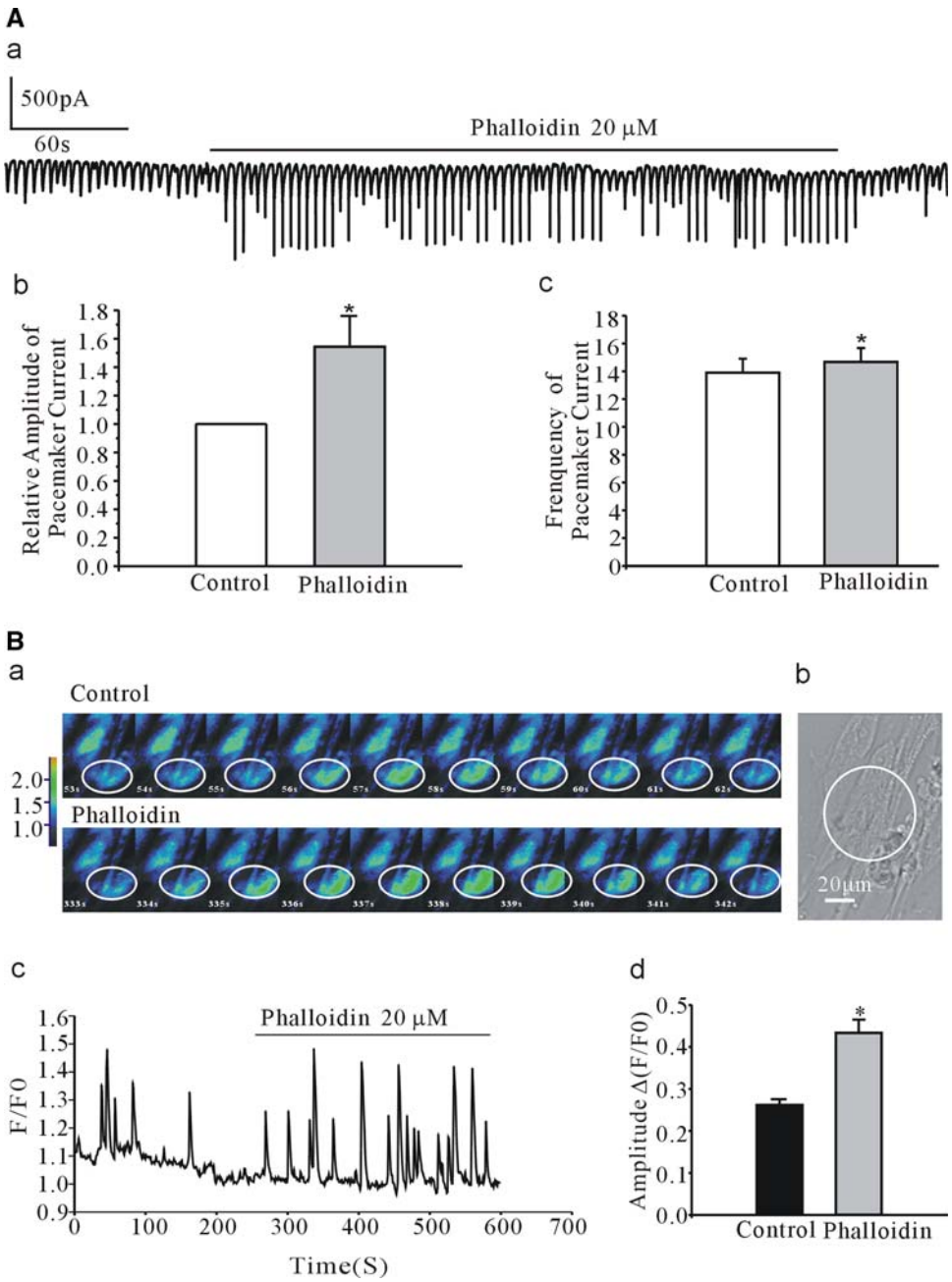

Fig. 2 Effect of phalloidin on pacemaker activity of cultured murine small intestine ICCs. **A** The effect of phalloidin on pacemaker currents. **Aa** Raw trace of phalloidin-induced increase in pacemaker currents. **Ab, Ac** Summary of the amplitude and frequency of pacemaker currents (* $P < 0.05$ vs. control, $n = 6$). **B** Effect of phalloidin on calcium oscillation. **Ba** Pseudo-color ratio images of the fluo-3 fluorescence intensity recorded in the square regions (white line in **Bb**). **Bc, Bd** Raw trace of calcium oscillations is shown and calcium oscillation amplitude is summarized, respectively (* $P < 0.05$ vs. control, $n = 6$)

and calcium oscillation. Addition of ryanodine was found to have no effect on the amplitude or frequency of pacemaker currents, nor did it block phalloidin-induced increases in pacemaker currents (Fig. 4A, $n = 6$, $P > 0.05$). Observing its effects on Ca^{2+} oscillations, 100 μM ryanodine significantly reduced oscillation amplitude from 0.28 ± 0.07 to 0.09 ± 0.01 in control and ryanodine-treated cells respectively (Fig. 4B, $n = 6$, $P < 0.05$). However, ryanodine did not inhibit the phalloidin-induced increase in calcium oscillation, but rather significantly enhanced it from 0.09 ± 0.01 to 0.24 ± 0.07 in control vs. phalloidin-treated cells, respectively (Fig. 4B, $n = 6$, $P < 0.05$).

Discussion

ICCs are fundamental components of the pacemaker apparatus of the GI tract. The generation of pacemaker currents by ICCs provides the basis for slow wave activity in GI muscles. The primary inward current responsible for regulating pacemaker activity in ICCs is suggested to be independent of voltage (Koh et al. 1998), but rather dependent upon intracellular Ca^{2+} handling (Ward et al. 2000). ICCs possess three vital cytoskeletal components: microfilament, microtubules and intermediate filament in mice and humans (Faussone-Pellegrini and Thuneberg

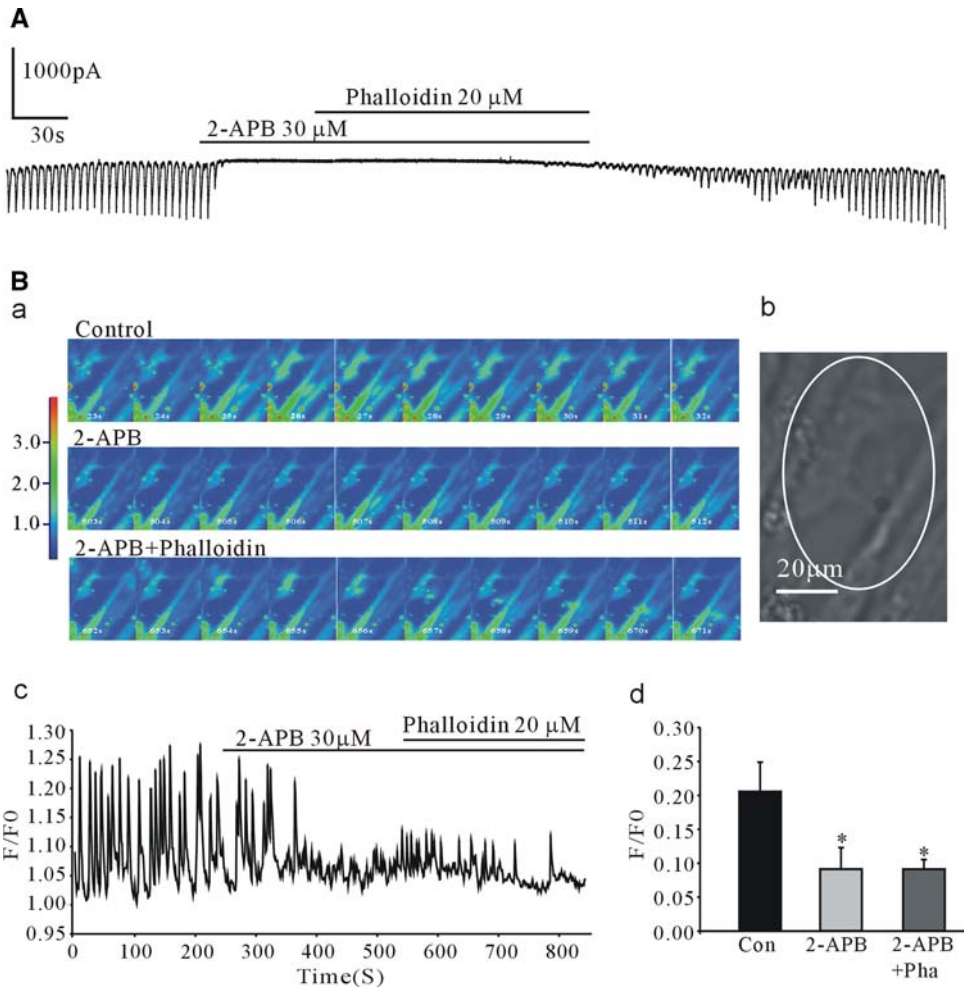

Fig. 3 Effect of 2-APB on phalloidin-induced increases in pacemaker current (**A**) and calcium oscillations (**B**). **Ba** Pseudo-color ratio images of the fluo-3 fluorescence intensity recorded in the square regions (white line in (**Bb**)). **Bc, Bd** Raw trace shown and calcium oscillation amplitude summarized, respectively (* $P < 0.05$ vs. control, $n = 6$)

1999). Many reports demonstrate that transmembrane proteins (such as channel proteins) linked to the actomyosin cytoskeleton regulate cell function (Goel et al. 2005; Clark et al. 2006). The present study was focused on the relationship among pacemaker currents, $[Ca^{2+}]_i$, and actin microfilaments in cultured mouse ICCs. The main conclusion drawn from the present study is that actin microfilaments regulate pacemaker currents via the IP_3-mediated calcium release signaling pathway.

Pacemaker currents have been suggested to result from the opening of nonselective cationic channels (Koh et al. 2002), and it was suggested that TRPC4 and TRPM7 from the transient receptor potential (TRP) channel superfamily were responsible for regulating this pacemaker current in ICCs (Walker et al. 2002; Kim et al. 2005). Recent studies now suggest that the actomyosin cytoskeleton plays a prominent role in regulating TRP channel activity (Papakonstanti and Stournaras 2008). It was demonstrated that TRPC4, one of the molecular basis of I_{SOC} in endothelial cells, seemed to be tethered to the cytoskeleton through a dynamic interaction with protein 4.1 (Cioffi et al. 2003). TRPM7 were also proved to play a role in linking receptor-mediated signals to actomyosin remodeling and cell adhesion (Clark et al. 2006). One mechanism by which the actomyosin cytoskeleton may affect TRP channel function is through modulating the interactions between TRP channels and their regulatory proteins. For example, some TRP channels localized within the plasma membrane associate with proteins residing in the endoplasmic reticulum, and this interaction requires an intact actomyosin cytoskeleton (Mehta et al. 2003; López et al. 2006). Li et al. (2007) found that α-actinin, an actin-bundling protein, not only binds TRPP3 to the cytoskeleton, but also upregulates TRPP3 channel activity. The cytoskeleton plays an important role in regulating Ca^{2+} influx or releasing (Rosado and Sage 2000; Bose and Thomas 2009). Actin microfilaments exist in a dynamic equilibrium between monomeric and polymerized actin, in which the ratio of monomers to polymeric forms is influenced by a variety of extracellular stimuli (Clarka et al. 2008). By using cytochalasin B, an actin microfilament disruptor, and phalloidin, an actin microfilament stabilizer, we effectively breached this equilibrium between monomeric and polymerized actin to observe the effect of actin microfilaments

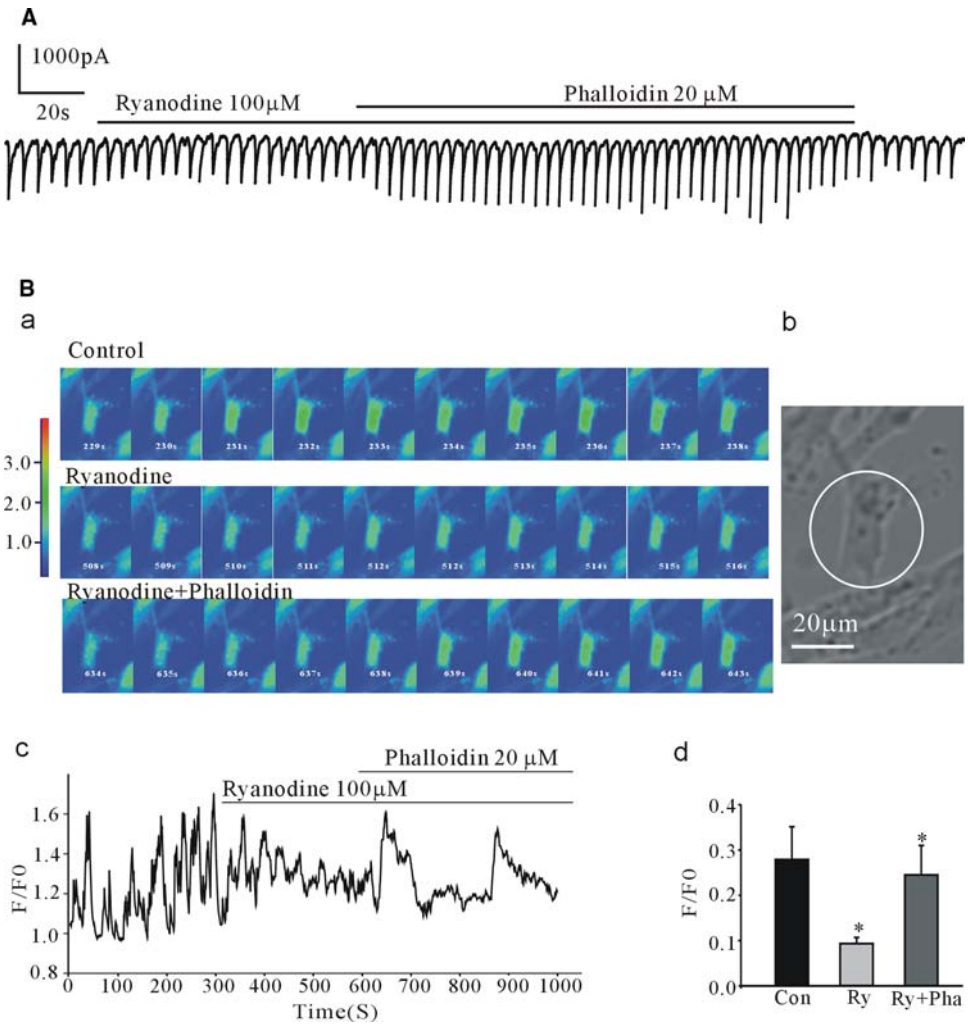

Fig. 4 Effect of ryanodine on phalloidin-induced increases in pacemaker currents (**A**) and calcium oscillation (**B**). **Ba** Pseudo-color ratio images of the fluo-3 fluorescence intensity recorded in the square regions (white line in (**Bb**)). **Bc, Bd** Raw trace shown and calcium oscillation amplitude summarized, respectively (* $P < 0.05$ vs. control, $n = 6$)

on pacemaker currents and calcium oscillation. We found that addition of cytochalasin B suppressed the amplitude and frequency of both pacemaker currents and calcium oscillations (Fig. 1), while phalloidin significantly increased the amplitude and frequency of pacemaker currents and calcium oscillation (Fig. 2). These results suggest that actin microfilaments are involved in the regulation of pacemaker currents via $[Ca^{2+}]_i$ handling. The equilibrium between monomeric and polymerized actin is vital for sufficient generation of pacemaker currents in ICCs.

Oscillations in $[Ca^{2+}]_i$ in ICCs periodically activate plasma membrane Ca^{2+}-dependent ion channels, thereby generating pacemaker potentials. Several investigators have concluded that the cellular event initiating the pacemaker currents are due to the localized release of Ca^{2+} from IP_3 receptors, as slow-wave formation is abolished in mice lacking IP_3 type 1 receptor (Suzuki et al. 2000; Ward et al. 2000; Malysz et al. 2001). It has already been suggested that a close association exists among IP_3 receptor-dependent Ca^{2+} stores, mitochondria, and ion channels in the plasma membrane, which together form a basic cellular structure termed the pacemaker unit in ICCs (Sanders et al. 2000; Koh et al. 2002). Recently, Bose and Thomas (2009) reported that disruption of cortical actin networks completely abolished IP_3 receptor-induced Ca^{2+} release in NG115-401L (401L) neuronal cells. Therefore, it is highly likely that actin microfilaments may regulate pacemaker activity via the IP_3-induced calcium release pathway in ICCs. To confirm this hypothesis, we investigated effect of phalloidin on calcium oscillation formation after pretreatment with 2-APB, an inhibitor of the IP_3 receptor. 2-APB suppressed pacemaker currents and calcium oscillations as well as blocking phalloidin-induced increases in pacemaker currents and calcium oscillation (Fig. 3). It was previously demonstrated that the ryanodine receptor (RyR) plays an essential role in generating pacemaker activity in gastric ICCs (Liu et al. 2005). Aoyama et al. (2004) reported that RyR3 is the predominant RyR subtype in ICCs and RyR can cooperate with IP_3R to generate pacemaker $[Ca^{2+}]_i$ oscillations in cell clusters from the small intestine. Therefore, understanding the relationship between $[Ca^{2+}]_i$ and RyR activity may be the key to

understanding the spontaneous rhythmic contractions of the small intestine. In the present study, we administered ryanodine, an inhibitor of calcium-induced calcium release, to observe its effect on the phalloidin-induced increase in pacemaker currents and calcium oscillations. In our hands, ryanodine could only significantly reduce calcium oscillations but had no effect on pacemaker currents (Fig. 4). The phalloidin-induced increase in pacemaker currents and calcium oscillation was also not inhibited when ryanodine was added (Fig. 4). Taken together, our results suggest that actin microfilaments regulate pacemaker activity via the release of calcium from IP_3-sensitive calcium stores in cultured intestinal ICCs from mice.

In summary, actin microfilaments are involved in the regulation of pacemaker currents via $[Ca^{2+}]_i$ handling. The integrity of actin microfilament is vital for sufficient generation of pacemaker currents in ICCs.

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