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RESEARCH PAPER

Contribution of Rho-kinase to membrane excitability of murine colonic smooth muscle

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BACKGROUND AND PURPOSE

The Rho-kinase pathway regulates agonist-induced contractions in several smooth muscles, including the intestine, urinary bladder and uterus, via dynamic changes in the Ca^{2+} sensitivity of the contractile apparatus. However, there is evidence that Rho-kinase also modulates other cellular effectors such as ion channels.

EXPERIMENTAL APPROACH

We examined the regulation of colonic smooth muscle excitability by Rho-kinase using conventional microelectrode recording, isometric force measurements and patch-clamp techniques.

KEY RESULTS

The Rho-kinase inhibitors, Y-27632 and H-1152, decreased nerve-evoked on- and off-contractions elicited at a range of frequencies and durations. The Rho-kinase inhibitors decreased the spontaneous contractions and the responses to carbachol and substance P independently of neuronal inputs, suggesting Y-27632 acts directly on smooth muscle. The Rho-kinase inhibitors significantly reduced the depolarization in response to carbachol, an effect that cannot be due to regulation of Ca^{2+} sensitization. Patch-clamp experiments showed that Rho-kinase inhibitors reduce GTPγS-activated non-selective cation currents.

CONCLUSIONS AND IMPLICATIONS

The Rho-kinase inhibitors decreased contractions evoked by nerve stimulation, carbachol and substance P. These effects were not solely due to inhibition of the Ca^{2+} sensitization pathway, as the Rho-kinase inhibitors also inhibited the non-selective cation conductances activated by excitatory transmitters. Thus, Rho-kinase may regulate smooth muscle excitability mechanisms by regulating non-selective cation channels as well as changing the Ca^{2+} sensitivity of the contractile apparatus.

Abbreviations

AUC, area under the curve; CCh, carbachol; EFS, electrical field stimulation; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; H-1152 (S)-(+)-(2-methyl-5-isoquinolinyl) sulphonylhomopiperazine2HCl; HA-1077, (5-isoquinolinesulphonyl) homopiperazine 2HCl; $MLC_{20,}$ myosin regulatory light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NSCC, non-selective cation channels; SMC, smooth muscle cells; TTX, tetrodotoxin; Y-27632, (R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl) cyclohexanecarboxanecarb-oxamide 2HCl

Introduction

Smooth muscle contraction is primarily regulated by phosphorylation of the 20 kDa myosin regulatory light chain (MLC_{20}). The degree of phosphorylation is dependent on the balance between myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) activity. Recent studies have revealed the importance of the small GTP-binding protein, Rho-A and the RhoA-dependent

serine-threonine kinase, Rho-kinase, in regulating the activity of MLCP and increasing the sensitivity of the contractile machinery (Ohama *et al.*, 2003; Somlyo and Somlyo, 2003; Rattan and Patel, 2008). Stimulation of receptors coupled to heteromeric G proteins results in activation of Rho-A, which in turn stimulates Rho-kinase. The non-catalytic subunit of MLCP is phosphorylated by Rho-kinase thus inactivating it. Therefore, MLCK phosphorylation of MLC_{20} is augmented leading to a greater force of contractions at a certain level of $[Ca^{2+}]_i$ (Ohama *et al.*, 2003; 2007; Somlyo and Somlyo, 2003; Rattan and Patel, 2008).

Several studies have examined the contribution of Rho-kinase to agonist-induced contractions, basal tone and pathological conditions such as pulmonary hypertension using Rho-kinase inhibitors, such as (R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl) cyclohexanecarboxanecarb-oxamide 2HCl (Y-27632), H-1152 and (5-isoquinolinesulphonyl) homopiperazine 2HCl (HA-1077) (Jarajapu and Knot, 2005; Rattan and Patel, 2008; Kizub *et al.*, 2010). The selectivity of these inhibitors has been examined through their inhibitory effects on, not only Rho-kinase, but other enzymes such as protein kinase C and MLCK (Eto *et al.*, 2001; Rattan and Patel, 2008). The contribution of the RhoA/Rho-kinase pathway to agonist-induced contractions has been demonstrated in several smooth muscle preparations including the intestine, urinary bladder and uterus (Jezior *et al.*, 2001; Friel *et al.*, 2005; Al-Jarallah *et al.*, 2008; Rattan and Patel, 2008). In the colon, carbachol (CCh)-induced contractions are significantly reduced by pretreatment with Rho-kinase inhibitors such as Y-27632, suggesting an important contribution of Ca^{2+} sensitization to agonist-induced contractions (Al-Jarallah *et al.*, 2008). Rho-kinase also plays an important role in vascular smooth muscle with particular reference to pulmonary hypertension (Jarajapu and Knot, 2005; Oka *et al.*, 2008; Kizub *et al.*, 2010). For example, Rho-kinase contributes to increased Ca^{2+} sensitivity of arterial myofilaments in diabetic rats (Kizub *et al.*, 2010). Therefore, it has been suggested that specific RhoA/Rho-kinase inhibitors may have an important therapeutic value for treating vascular conditions such as pulmonary hypertension (Oka *et al.*, 2008).

There is accumulating evidence that Rho-kinase may be involved in the regulation of certain ion channels (Pochynyuk *et al.*, 2006; Villalba *et al.*, 2008). Activation of Rho-kinase by Rho-A can stimulate phosphatidylinositol-4-phosphate 5-kinase leading to increases in PIP_2 levels that then promote the insertion of epithelial Na^+ channels into the plasma membrane (Pochynyuk *et al.*, 2006). In rat penile arteries, the nifedipine-resistant Ca^{2+} influx evoked by phenylalanine was inhibited by Y-27632. This influx was proposed to be via non-selective cation currents (NSCC) that were regulated by Rho-kinase (Villalba *et al.*, 2008).

However, the contribution of Rho-kinase to colonic excitability has not been investigated. Furthermore, no studies have clearly investigated the involvement of Rho-kinase in regulating ion channels in gastrointestinal smooth muscles. In this study we demonstrated the effects of Rho-kinase on membrane potential and ionic currents in murine colonic smooth muscle.

Methods

Animals

BALB/c mice were anaesthetized with isoflurane and killed by cervical dislocation. Colons were removed from the animals through a midline abdominal incision. The animals were maintained and the experiments performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Institutional Animal Use and Care Committee at the University of Nevada approved all procedures used.

Mechanical responses and nerve stimulation

Segments of the proximal colon from 1 cm distal to the ileocecal sphincter were removed through a midline abdominal incision and opened along the mesenteric border. Luminal contents were removed by washing with Krebs–Ringer bicarbonate solution (KRB, see solutions), and the cleaned tissue sheets were pinned down onto a Sylgard base with the mucosa facing up. The mucosa was removed by sharp dissection leaving the tunica muscularis and remnants of the submucosa.

Mechanical responses were performed using standard organ-bath techniques. Strips of muscle (10×5 mm) were cut from the tunica muscularis in the longitudinal direction by sharp dissection. The muscles were attached longitudinally with sutures to a fixed mount within the organ bath and to an isometric strain gauge (World Precision Instruments, Sarasota, FL, USA). The muscles were immersed in oxygenated KRB and maintained at 37.5 ± 0.5°C. The muscles were set at resting tension by applying 0.1–0.3 g of basal tension and then allowed to equilibrate for 1–2 h in fresh KRB solution. Contractions in the longitudinal muscle layer were monitored, digitized and stored using Axoscope software (Axon Instruments, Foster City, CA, USA). Contractions were quantified by calculations of area above the baseline using the pClamp software (v9.02, Axon instruments). For electrical field stimulation (EFS) experiments, parameters were 150 V and 0.3 ms with various stimulation frequencies and durations. The area under the curve (AUC) was determined as the integral values above the baseline of a selected area for 5 min recordings ($Mn \cdot min^{-1}$). The AUC for the tissues exposed to the drugs tested were compared with that for tissues under control conditions during an equivalent period of time. Drugs were diluted to the desired concentrations and applied to the muscles by switching the perfusion to the drug-containing solution.

Intracellular microelectrode recordings

After the mucosa was removed with fine-tipped forceps, strips of proximal colon (1 cm in length × 0.5 cm in width) were taken from the region 1–2 cm from the ileocecal sphincter and pinned to a Sylgard (Dow Corning Corp., Midland, MI, USA) elastomer-coated recording chamber with the mucosal side of the circular muscle facing upwards. Smooth muscle cells (SMC) were impaled with glass microelectrodes filled with 3 M KCl and having electrical resistances of 80–100 MΩ. Transmembrane potentials were measured with a standard high input impedance amplifier (WPI Duo 773, Sarasota, FL, USA). Electrical signals were recorded by a computer running

AxoScope data acquisition software (Axon Instruments) and analysed by Clampfit (v9.02, Axon Instruments). All experiments were performed in the presence of wortmannin (10 μM) to reduce movement and facilitate impalements of cells for extended periods of time.

Preparation of isolated colonic SMC

Colons were cut open along the longitudinal axis, pinned out in a Sylgard-lined dish, and washed with Ca^{2+}-free phosphate-buffered saline containing (mM): 125 NaCl, 5.36 KCl, 15.5 $NaHCO_3$, 0.336 Na_2HPO_4, 0.44 KH_2PO_4, 10 glucose, 2.9 sucrose and 11 HEPES and adjusted to pH 7.4 with NaOH. Mucosa and submucosa were removed with fine-tipped forceps. Pieces of muscle were incubated for 30–40 min at 37°C in a Ca^{2+}-free solution (1 mL) containing 2 mg collagenase (Worthington Biochemical, Lakewood, NJ, USA), 4 mg trypsin inhibitor, 4 mg fatty acid-free bovine serum albumin, 1 mg papain and 0.3 mg dithiothreitol (Sigma-Aldrich, St. Louis, MO, USA). After enzymatic treatment, the muscles were washed with Ca^{2+}-free solution and agitated gently to create a cell suspension. Dispersed SMC were stored at 4°C in Ca^{2+}-free solution for up to 6 h. Cells were transferred from the refrigerator to the recording chamber. Drops of the cell suspensions were placed on the bottom of a 300 μL chamber mounted on an inverted microscope and allowed to adhere to the bottom of the chamber for 5 min before recording.

Voltage-clamp methods

Whole cell voltage-clamp techniques were used to record membrane currents from dissociated SMC. Membrane currents were amplified by an Axopatch 1D (Axon Instruments) and digitized with an analogue-to-digital converter (Digidata 1200, Axon Instruments). Data were collected at 5 kHz, filtered at 2 kHz via Bessel filter and digitized online with pCLAMP software. The data were analysed with the use of Clampfit software (version 9.2, Axon Instruments). Pipette resistances were 1–4 MΩ. Perforated whole cell patch-clamp techniques were performed using amphotericin B (see solutions) for recording L-type Ca^{2+} currents. When analysing L-type Ca^{2+} currents, the linear leak current was subtracted digitally. Standard whole cell patch-clamp recordings were performed when dialysing with guanosine 5′-3-*O*-(thio)triphosphate (GTPγS). All experiments were performed at room temperature (between 22°C and 25°C).

Solutions

For contractile and conventional microelectrode recordings, the strips were exposed to KRB with the following composition (mM): 118.5 NaCl, 4.5 KCl, 1.2 $MgCl_2$, 23.8 $NaHCO_3$, 1.2 KH_2PO_4, 11.0 dextrose and 2.4 $CaCl_2$.

In order to measure L-type Ca^{2+} currents, colonic SMC were bathed in a Ca^{2+}-containing physiological salt solution (CaPSS) containing (mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, 10 HEPES, adjusted to pH 7.4 with Tris. The patch pipettes were filled with the following solution (in mM): 135 CsCl, 0.1 EGTA, 0.1 Na_2GTP, 3 MgATP, 10 glucose, 2.5 creatine phosphate disodium and 10 HEPES. This solution was adjusted to pH 7.2 with Tris. Amphotericin B was dissolved in dimethyl sulphoxide as a stock solution (0.08 mg·μL^{-1}) and added to the pipette solution (0.4 mg·mL^{-1}).

In experiments designed to directly activate non-selective cation channels (independently of muscarinic activation), the pipette solution contained (in mM): 80 CsCl, 1 Mg-ATP, 0.0005 GTPγS, 5 creatine, 5 glucose, 10 HEPES, 10 BAPTA, 4.6 $CaCl_2$, pH adjusted to 7.2 with CsOH. CaPSS was perfused during seal formation and approximately 3 min was allowed for equilibration. Then CaPSS was replaced with the following external solution (in mM): 120 CsCl, 12 D-glucose, 10 HEPES and pH adjusted to 7.4 using CsOH (total Cs^+ 124 mM). All drug and molecular target nomenclature conforms to that described in Alexander *et al*. (2009).

Statistical analysis

Data are reported as means ± SEM. *N* is the number of mice used and *n* refers to the number of cells in patch-clamp experiments. Statistical significance was evaluated by Student's *t*-test. *P* values less than 0·05 were considered significant.

Materials

Y-27632((R)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl) cyclohexanecarboxanecarb-oxamide, 2HCl), H-1152 ((S)-(+)-(2-methyl-5-isoquinolinyl) sulphonylhomopiperazine, 2HCl) and HA-1077 were obtained from Calbiochem (San Diego, CA, USA). Tetrodotoxin (TTX), GTPγS, amphotericin B and wortmannin were obtained from Sigma (St. Louis, MO, USA).

Results

Rho-kinase inhibition reduced nerve-stimulated and CCh-induced contractions in colonic smooth muscle

We performed isometric force measurements to examine the effects of Y-27632 on nerve-stimulated contractions in murine colonic smooth muscle. Increasing the frequency of EFS at a fixed duration (from 5 to 30 Hz, duration 30 s) increased the amplitude of on-contractions, which reached a plateau/peak during EFS [Figure 1A (see inset), Ca and Da]. On-contractions are the result of cholinergic neurotransmitter release from nerve terminals, and they are blocked by atropine (Komori and Suzuki, 1986). Off-contractions, evoked upon cessation of EFS, also increased in amplitude with increased frequency of EFS (Figure 1A, Cb and Db). Mechanisms underlying off-contractions have not been clearly elucidated. However, previous studies have suggested that these responses are due to release of non-cholinergic excitatory peptides, such as substance P and neurokinin A (Shuttleworth *et al*., 1993). Pretreatment with the Rho-kinase inhibitor, Y-27632 (1 μM) caused a significant decrease in the amplitudes of nerve-stimulated on- and off-contractions (data not shown). An increase in the concentration of Y-27632 to 10 μM caused a further decrease in on- and off-contractions elicited by EFS at all frequencies (Figure 1B and C, $N = 13$, $P < 0.005$). Under control conditions, application of the muscarinic receptor agonist, CCh (1 μM), induced a prolonged contraction, which slowly declined over 5 min (Figure 1A). Pretreatment with Y-27632 (10 μM) caused a dramatic decrease in the amplitude of CCh-induced contractions (Figure 1A and B).

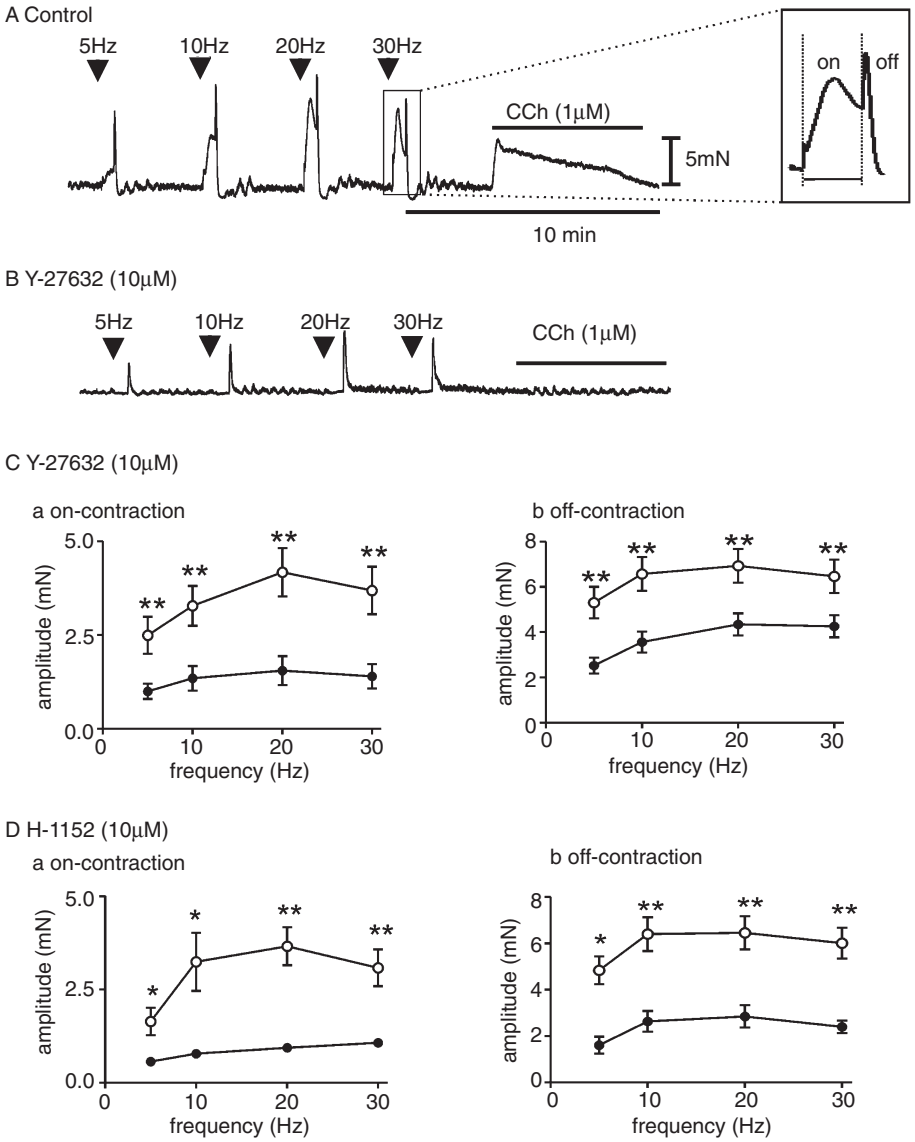

Figure 1

Rho-kinase inhibition reduced the amplitude of nerve-stimulated on- and off-contractions. (A) Under control conditions, increasing the frequency of EFS (5, 10, 20 and 30 Hz for 30 s) increased the amplitude of on- and off-contractions in colonic smooth muscle. (see expanded trace illustrating on and off regions of contraction). (B) Pretreatment with Y-27632 (10 μM) dramatically decreased on- and off-contractile amplitude. (Ca and Cb) Summarized graphs show the effect of Y-27632 (10 μM) on on- and off-contractions evoked at increasing frequencies ($N = 13$, $^{**}P < 0.005$). Open symbols, control; solid symbols, in the presence of Y-27632. (Da and Db) Summarized graphs show the effects of H-1152 (10 μM) on nerve-stimulated on- and off-contractions at increasing frequencies ($N = 6$, $^{*}P < 0.05$, $^{**}P < 0.005$). Open symbols, control; solid symbols, in the presence of H-1152.

Several Rho-kinase inhibitors are available with different selectivities and potencies (Rattan and Patel, 2008). For example, in the rat internal anal sphincter, H-1152 and Y-27632 were the most potent, but HA-1077 and the Rho-kinase II inhibitor were significantly less potent. Therefore, we tested whether H-1152 had effects similar to Y-27632 on nerve- and agonist-induced contractions and excitability. On-contractions evoked by EFS (5 to 30 Hz, duration 30 s) were significantly reduced by H-1152 (10 μM) (Figure 1Da, $N = 6$, $P < 0.005$). Off-contractions were also significantly reduced by H-1152 (Figure 1Db, $N = 6$, $P < 0.005$). As with Y-27632 (10 μM), CCh-induced contractions were suppressed by H-1152 (10 μM) (data not shown).

We also tested the effect of EFS duration on contractility before and after Y-27632. Under control conditions, increasing the duration of the EFS (1, 5, 10 and 20 s; at a fixed frequency of 5 Hz) increased on- and off-contractions (Figure 2A). Pretreatment with Y-27632 (5 μM), significantly reduced the amplitude of both phases of the contractile response (Figure 2B–D, $N = 4$, $P < 0.05$). Rho-kinase inhibitors may affect smooth muscle contraction by reducing excitatory neurotransmitter release and/or by Rho-kinase

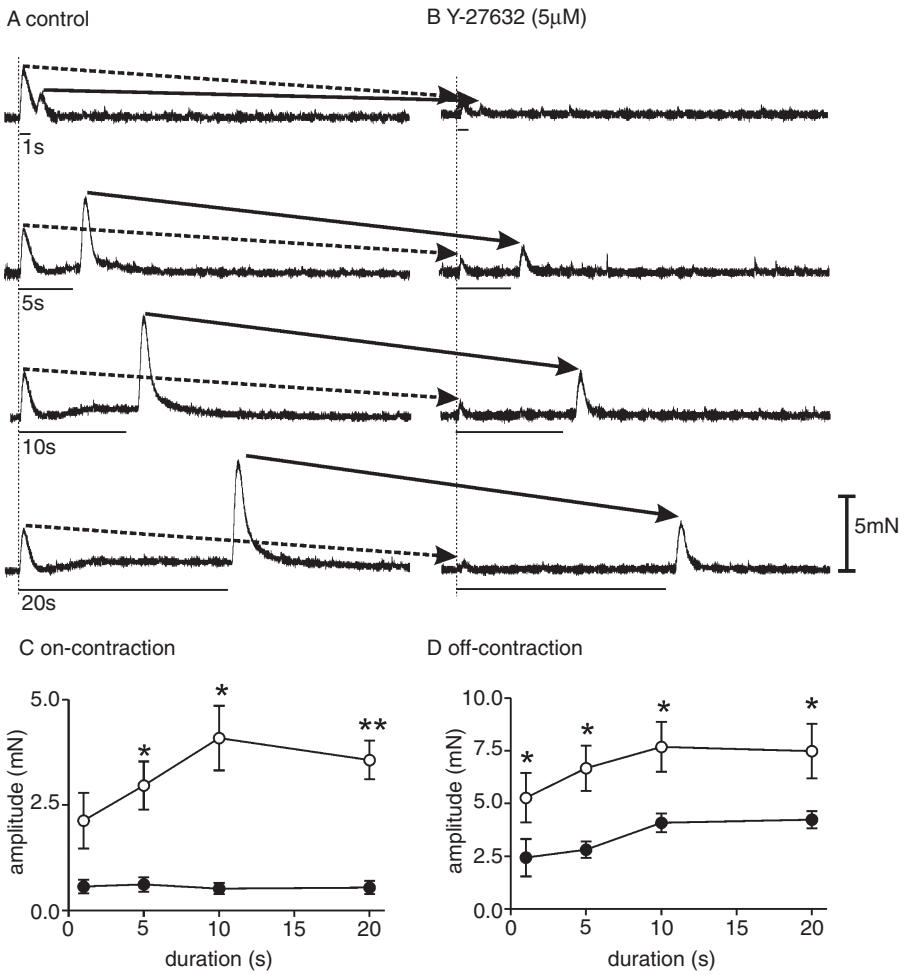

Figure 2

Y-27632 reduced the amplitude of nerve-stimulated on- and off-contractions evoked at increasing durations. (A) Under control conditions, duration of EFS at a fixed frequency (1, 5, 10 and 20 s at 5 Hz) increased the amplitude of on- and off-contractions in colonic smooth muscle. (B) Pretreatment with Y-27632 (5 μM) significantly reduced the amplitude of both on- and off-contractions. (C) Summarized graph shows the effect of Y-27632 (5 μM) on the amplitude of on-contractions evoked at increasing durations of EFS compared with control ($N = 4$, $^*P < 0.05$, $^{**}P < 0.005$). (D) Summarized graph shows the effect of Y-27632 (5 μM) on off-contractions evoked following the cessation of EFS at increasing durations ($N = 4$, $^*P < 0.05$). Open symbols, control; solid symbols, in the presence of Y-27632.

pathways in smooth muscle. Therefore, we performed additional contractile experiments in the presence of TTX as TTX blocks nerve axonal action potentials. TTX (1 μM) increased the amplitude of spontaneous phasic contractions (tabulated as AUC), confirming that spontaneous activity of enteric inhibitory neurones regulates the basal excitability of murine colonic smooth muscles (Figure 3A and B; Spencer *et al.*, 1998). Application of Y-27632 (10 μM) in the presence of TTX (1 μM) decreased the contractile response to CCh (1 μM) (Figure 3B–D, $N = 4$, $P < 0.05$). We also examined the effects of Y-27632 (10 μM) on responses to a range of CCh concentrations (0.05–10 μM). Contractile responses to CCh were significantly reduced by Y-27632 (10 μM). The EC_{50} of CCh response in the presence of Y-27632 increased to 9.8 mM compared with CCh alone (EC_{50} = 313 nM), and the greatest effect was observed at 1 μM CCh (Figure 3E, $N = 5$). Therefore, 1 μM CCh was used for subsequent experiments.

Release of substance P from intrinsic enteric neurones may underlie rebound or off-contractions elicited at cessation of EFS in colonic smooth muscle (Ward *et al.*, 1992). Figure 1Cb, 1Db, 2B and 2D show that the amplitudes of the off-contractions were also decreased by Rho-kinase inhibitors. Therefore, we tested whether contractions to substance P were also affected by Rho-kinase inhibitors. In the presence of TTX (1 μM), substance P (500 nM) increased peak contraction (4.9 ± 1.1 to 6.7 ± 0.5 mN, $n = 4$, $P < 0.05$) and AUC (19.9 ± 4.4 to 40.8 ± 4.5 mN·min^{-1}, $P < 0.05$). Pretreatment with Y-27632 (10 μM) diminished the peak contraction to substance P (4.8 ± 1.1 mN to 1.8 ± 0.6 mN, $n = 4$, $P < 0.05$) and AUC (16.2 ± 6.0 to 3.6 ± 0.7 mN·min^{-1}, $P < 0.05$) in the presence of TTX. Additional application of substance P did

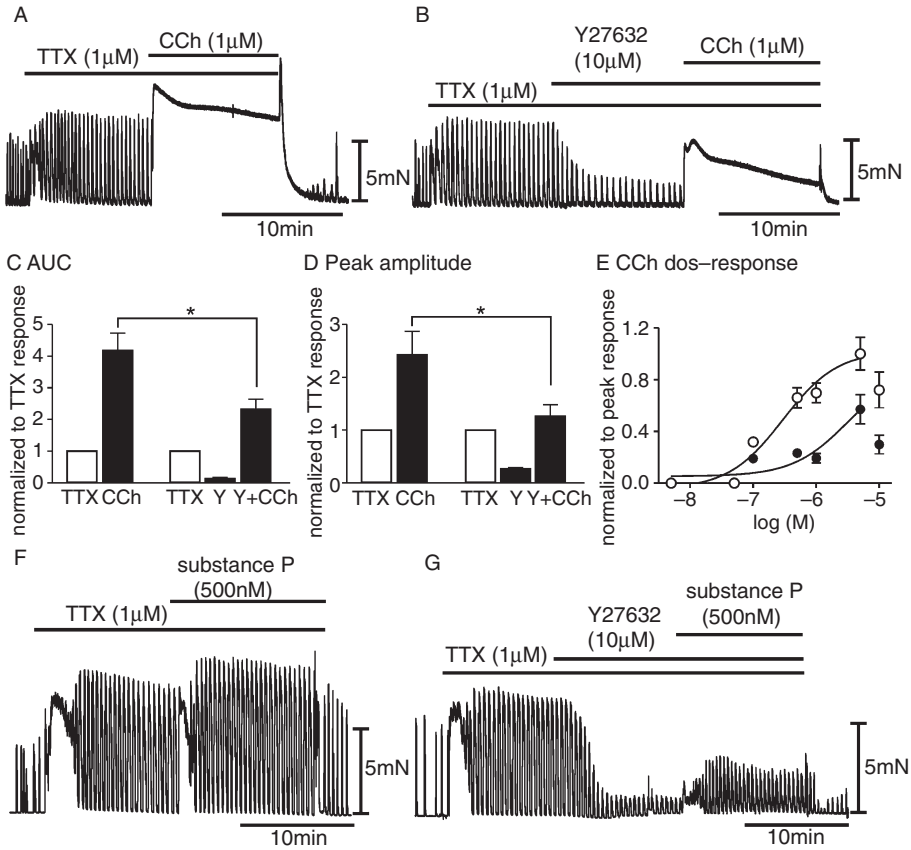

Figure 3

Y-27632 decreased the contractions induced by CCh and substance P independently of neuronal influences. (A) Representative mechanical trace illustrates that in the presence of TTX (1 μM), exposure to CCh (1 μM) results in an increase in contractility in mouse colonic smooth muscle. (B) Representative trace shows how application of Y-27632 (10 μM) causes a decrease in the CCh-evoked contraction in murine colonic smooth muscle. (C) Summary graph shows a significant decrease in AUC following treatment with CCh (1 μM) in the presence of Y-27632 (10 μM) normalized to TTX response ($N = 4$, $^*P < 0.05$). (D) Summarized graph shows a significant decrease in the peak amplitude of CCh-induced contractions in the presence of Y-27632 (10 μM) normalized to TTX response ($N = 4$, $^*P < 0.05$). (E) Summarized graph shows the effect of Y-27632 (10 μM) on the contractile responses to increasing concentrations of CCh (0.05–10 μM) ($N = 5$). The peak contractions in response to CCh were normalized to the maximal responses at 1 μM. The averaged responses were fit with a sigmoidal function (points at 10 μM were omitted from the fit). Values for r^2 were 0.96 and 0.90 in control and in the presence of Y-27632 respectively. Open symbols, control; solid symbols, in the presence of Y-27632 (10 μM). (F) Representative mechanical trace illustrates that in the presence of TTX (1 μM), exposure to substance P (500 nM) results in an increase in contractile amplitude in murine colonic smooth muscle ($N = 4$, $P < 0.05$). (G) Pretreatment with Y-27632 (10 μM) significantly decreased substance P contractile amplitude ($N = 4$, $P < 0.05$).

not significantly increase the peak contraction (1.8 ± 0.6 to 2.0 ± 0.5 mN, $n = 4$) and AUC (3.6 ± 0.7 to 6.5 ± 2.2 mN·min^{-1}).

Rho-kinase inhibition significantly reduced CCh-induced depolarization in colonic smooth muscle

The decrease in CCh-induced contractions by Y-27632 could be due to effects on membrane potential, so we performed intracellular microelectrode recordings. CCh induced depolarization in murine colonic smooth muscles in the presence of TTX (1 μM)

(Figure 4A and Ca, $N = 7$). Pretreatment with Y-27632 (10 μM) in the presence of TTX (1 μM), significantly reduced the depolarization evoked by CCh 1 μM (Figure 4B and Ca; $N = 7$, $P < 0.001$), but membrane potential was not significantly affected by Y-27632 alone. HA-1077 (10 μM) and H-1152 (10 μM) had similar effects to Y-27632 on CCh-induced depolarization (see Figure 4Cb and Cc; $N = 6$).

Y-27632 did not significantly effect KCl-induced contractions

It is possible that the effects of Rho-kinase inhibitors on contractile responses could be due to inhibitory effects of these compounds on L-type Ca^{2+} channels. Therefore, we tested the effects of the Rho-kinase inhibitors on contractile responses to KCl. If Y-27632 blocked L-type Ca^{2+} channels, then KCl-induced contractions should be reduced by this compound. Y-27632 (10 μM) had no effect on contractions evoked by 20, 40 and 60 mM KCl (Figure 5, $N = 6$). These

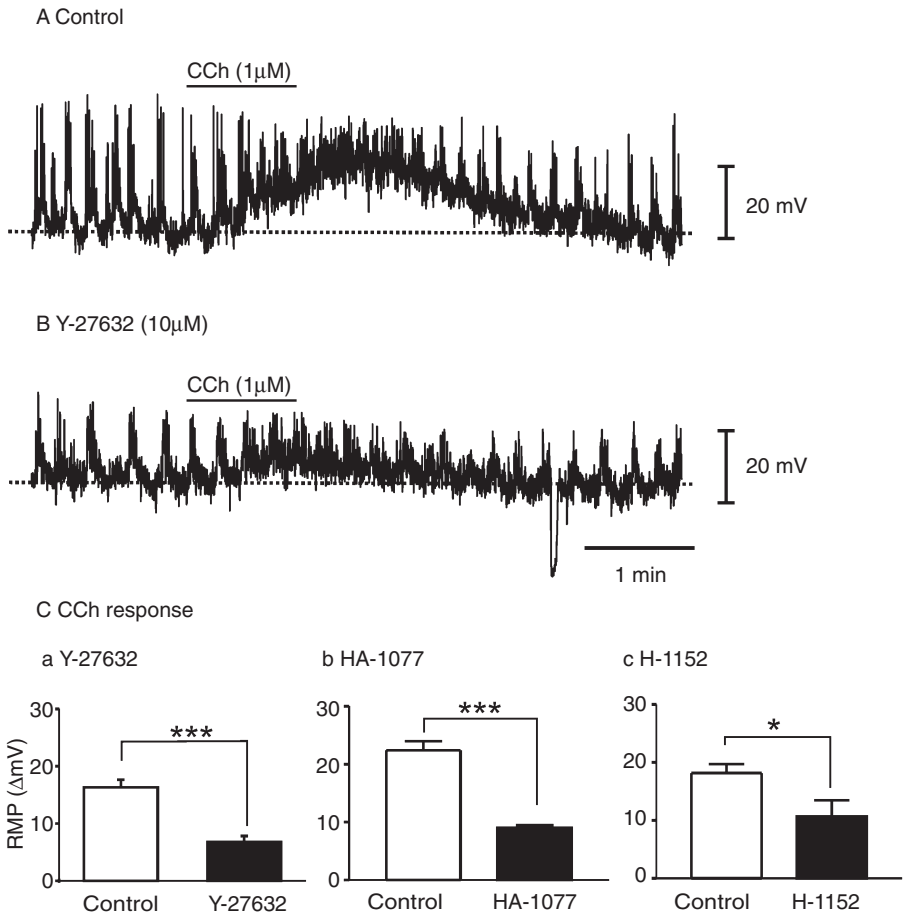

Figure 4

Rho-kinase inhibition reduced CCh-induced depolarization in murine colonic smooth muscle. (A) Representative trace illustrates a typical CCh-induced depolarization in colonic smooth muscle. (B) Pretreatment with Y-27632 (10 μM) suppressed the CCh-induced depolarization (1 μM). (C) Summary graphs show that (Ca) Y27632 (10 μM) (Cb) HA-1077 (10 μM) or (Cc) H-1152 (10 μM) caused a significant decrease in RMP change upon CCh (1 μM) application (Y-27632, $N = 7$, ***$P < 0.001$; HA-1077, $N = 6$, ***$P < 0.001$; H-1152, $N = 6$, *$P < 0.05$). TTX (1 μM) was added at the beginning of each experiment.

findings suggest that the effects of Y-27632 on spontaneous and CCh-induced contractions are not via inhibition of L-type Ca^{2+} channels.

Y-27632 reduced GTPγS-evoked currents

We also tested the effects of Y-27632 more directly on L-type Ca^{2+} currents using patch-clamp techniques. During whole cell recording with a pipette solution containing CsCl (140 mM) to reduce outward current (see Methods), current responses were elicited under voltage clamp by ramp depolarizations from –80 mV to +80 mV (500 ms every 1 min). Inward currents at negative potentials have been previously shown to be due to L-type Ca^{2+} channels (Koh *et al.*, 2001). Y-27632 (5 μM) had no effect on L-type Ca^{2+} currents (Figure 6A, $n = 10$ cells from $N = 9$ mice).

We also examined the effects of Rho-kinase inhibitors on NSCC activated by CCh (Benham *et al.*, 1985; Inoue, 1991). Muscarinic activation of NSCC results from GTP-binding protein mediated signal transduction. Therefore, analogues of GTP that are resistant to hydrolysis, such as GTPγS, are often used to activate this conductance (e.g. Kim *et al.*, 2007). In the present experiments, a 'noisy' inward current was elicited at a holding potential of –50 mV in cells dialysed with GTPγS (Figure 6B). Y-27632 (5 μM or 10 μM) reduced the GTPγS-evoked current [Figure 6B and C, Y-27632 (10 μM), $n = 4$ cells from $N = 3$ mice; Y-27632 (5 μM), $n = 4$ from $N = 4$ mice, data not shown]. Figure 6C shows the current evoked by ramp depolarization from –120 mV to +80 mV before and in the presence of Y-27632 (10 μM) [see red trace (b)]. Y-27632 reduced the amplitude of the GTPγS-evoked current [*see black trace (a)*]. The effects of H-1152 on GTPγS-evoked current were also tested. Concentrations of 0.1 μM and 0.5 μM had no significant effects on NSCC (0.1 μM, $n = 5$ from $N = 2$ mice; 0.5 μM, $n = 4$ from $N = 2$ mice, data not shown), but 1 μM H1152 significantly reduced the NSCC (Figure 6D and E, $n = 5$ from $N = 2$ mice). These data suggest that NSCC may be regulated by Rho-kinase, and a reduction in NSCC by Y-27632 or H-1152 may account for the decrease in depolarization and contraction responses to CCh induced by Rho-kinase inhibitors.

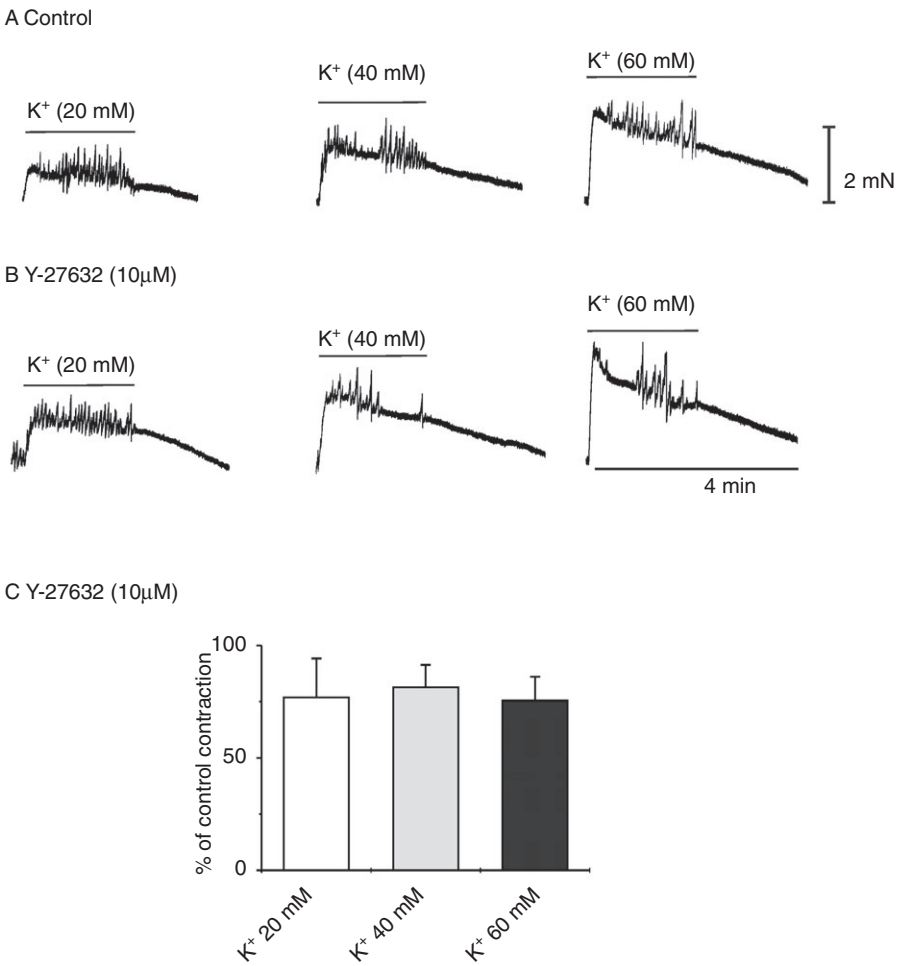

Figure 5

Y-27632 did not effect KCl-evoked contractions in colonic smooth muscle. (A) Under control conditions, increasing the external concentration of KCl from 5 mM to 20, 40 and 60 mM for 2 min, which is a classical stimulus for activation of L-type Ca^{2+} channels, evoked increasing amplitude of contractions in colonic smooth muscle. (B) Pretreatment with Y-27632 (10 μM) had no significant effect on KCl-evoked contractions (N = 6). (C) Summarized data show the effects of Y-27632 (10 μM) on the percentage of KCl-induced control contractions following pretreatment with TTX (1 μM) (N = 6).

Discussion and conclusions

Based on use of Rho-kinase inhibitors, previous studies have suggested that the Rho-kinase pathway regulates contractile responses of GI muscles to cholinergic stimulation (Somlyo and Somlyo, 2004; Al-Jarallah *et al.*, 2008; Rattan and Patel, 2008). These authors suggest that cholinergic responses depend upon increased Ca^{2+} sensitivity of the contractile apparatus due to Rho-kinase-dependent inhibition of MLCP. We have confirmed the effects of Rho-kinase inhibitors on cholinergic responses in the present study, and we showed that responses to enteric excitatory nerve stimulation and to exogenous CCh and substance P were greatly reduced by Rho-kinase inhibitors. Our findings, however, supplement previous studies by suggesting that regulation of Ca^{2+} sensitivity may not be the only way in which Rho-kinase regulates cholinergic responses. CCh via muscarinic receptors activates NSCC in colonic SMC (Sanders, 2008). The ensuing depolarization activates L-type Ca^{2+} channels and the increased flux of Ca^{2+} increases contractions (Sanders, 2008). We found that Rho-kinase inhibitors have no direct effect on L-type Ca^{2+} channels, but these compounds reduce the NSCC activated by CCh. Thus, at least a portion of the inhibitory effect of Rho-kinase inhibitors on cholinergic responses appears to be due to the reduced excitability of colonic smooth muscles.

In several smooth muscle preparations, Y-27632 significantly reduced EFS-induced nerve-stimulated contractions (Büyükafşar and Levent, 2003; Wibberley *et al.*, 2003; Fernandes *et al.*, 2006). In the murine gastric fundus, Y-27632 (100 μM) reduced neurotransmitter release and caused relaxation (Büyükafşar and Levent, 2003). In others studies, murine and guinea-pig tracheal preparations were loaded with [^{3}H]-choline, and Y-27632 (10 μM) was shown to increase significantly the outflow of radioactivity following EFS stimulation, suggesting this compound increases the release of ACh. Despite increased transmitter release, the net

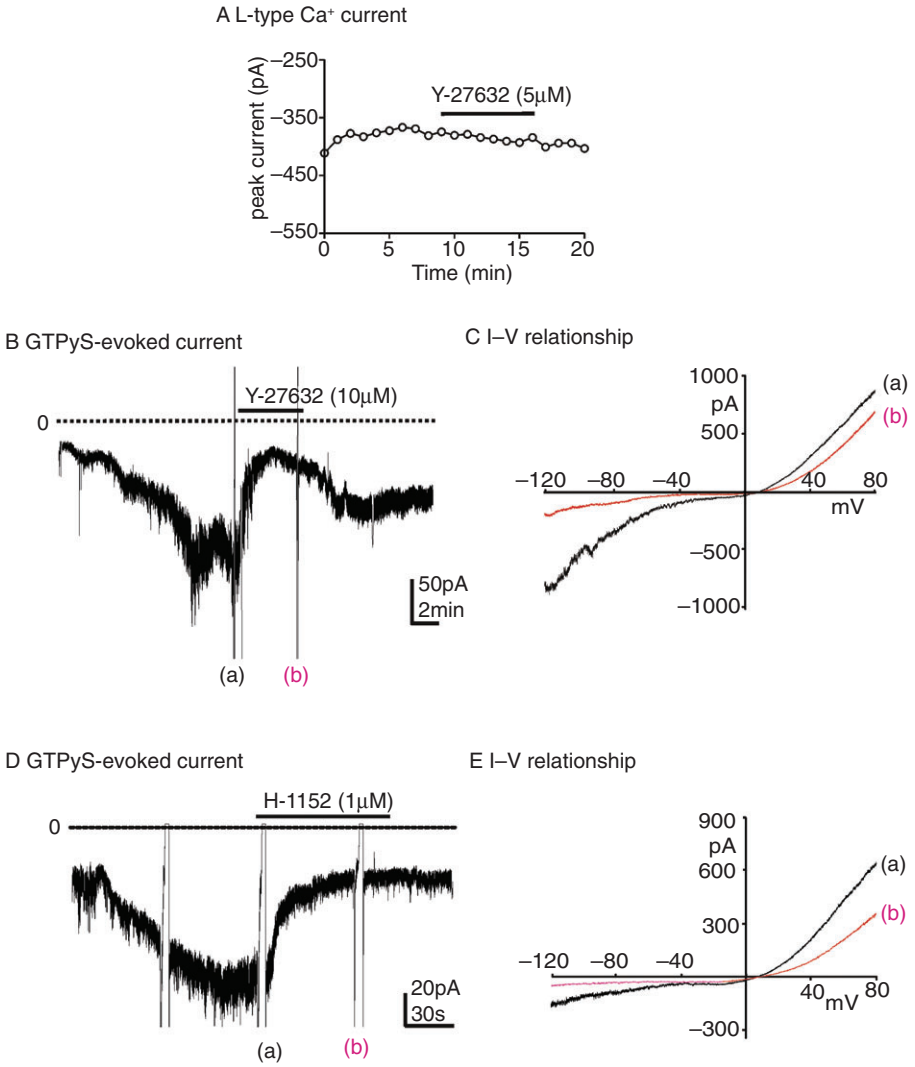

Figure 6

Rho-kinase inhibitors reduced GTPγS-activated NSCC. (A) Time course of peak L-type Ca^{2+} currents evoked by step depolarizations to 0 mV from a holding potential of –80 mV every 1 min illustrating no effect was induced by application of Y-27632 (5 μM) ($n = 10$ from $N = 9$ mice). (B) When whole cell configuration was achieved using a pipette solution containing GTPγS (0.5 mM), a 'noisy' inward current was gradually activated over 2–4 min. Application of Y-27632 (10 μM) significantly reduced this current ($n = 4$ from $N = 3$ mice). (C) Steady-state I–V relationship obtained by applying a slow 6 s duration voltage ramp from –120 to 80 mV before and following a 5 min application of Y-27632 (10 μM). (D) Similar to Y-27632 (10 μM), H-1152 (1 μm) considerably reduced GTPγS-evoked currents. (E) The I–V trace obtained from the slow ramp depolarization shows a significant reduction in currents evoked at positive and negative potentials in the presence of H-1152 (1 μM) (red trace, b) compared with the control (black trace, a) ($n = 5$ from $N = 2$ mice).

effect of Y-27632 was inhibition of CCh- and nerve-evoked contractions, leading to the conclusion that the dominant effects of Y-27632 were postjunctional (Fernandes *et al.*, 2006). In the present study TTX was used to examine the inhibitory effects of Rho-kinase on smooth muscle contractions in the absence of the spontaneous activity of enteric neurones. Under these conditions, Y-27632 suppressed nerve-evoked and CCh-induced contractions, suggesting this compound acts directly on postjunctional cells. However, our results contrast with the findings of Ihara *et al.* (2009), who reported that Y-27632 had no effect on CCh-induced contractions in circular muscles of murine colon. The reason for the differences between these studies is not clear. We note that tissues of much smaller dimension were used by Ihara *et al.* and these muscles did not display spontaneous contractile activity. We found that spontaneous phasic contractions are common in colonic muscles and this activity was also reduced by Y-27632. Whatever process(es) suppressed spontaneous activity in the muscles used by Ihara and coworkers (Ihara *et al.*, 2009) might also have muted any effects of Rho-kinase on the responses.

Ca^{2+} entry, via L-type Ca^{2+} channels, is a major factor required for spontaneous contractions and agonist responses in colonic muscles, because the L-type Ca^{2+} channel blocker,

nifedipine, inhibits contractions (Wegener *et al.*, 2006). We characterized the effects of Y-27632 and/or H-1152 on KCl-evoked contractions and L-type Ca^{2+} currents and found that Rho-kinase inhibitors did not affect L-type Ca^{2+} currents. Thus, the inhibitory effects of Rho-kinase inhibitors are not mediated directly through inhibition of L-type Ca^{2+} channels.

L-type Ca^{2+} channels are regulated largely by transmembrane potentials in GI muscles (Sanders, 2008). Therefore, any ion channel that affects membrane potential or blocks membrane potential responses to agonists could affect Ca^{2+} entry and contractile responses. It is well known that a major postjunctional effect of excitatory neurotransmitters is activation of NSCC (Benham *et al.*, 1985; Inoue, 1991; So and Kim, 2003; Unno *et al.*, 2003; Tsvilovskyy *et al.*, 2009), and a recent study suggested that Rho-kinase might regulate specific NSCC channels (Villalba *et al.*, 2008). Therefore, we hypothesized that Rho-kinase may regulate NSCC involved in depolarization responses to cholinergic stimulation. Dialysis with GTPγS stimulates CCh-like NSCC through activation of multiple G proteins (Helliwell and Large, 1997; Dresviannikov *et al.*, 2006). In the present study, currents activated by GTPγS currents were inhibited by Y-27632, suggesting that decreased depolarization by CCh in the presence of Y-27632 could be due to inhibition of NSCC. The exact mechanism by which Rho-kinase regulates NSCC is currently unknown. However, it has been suggested that Rho-kinase may regulate ion channel activity through several different mechanisms including actin reorganization, crosstalk with protein kinases involved in specific intracellular pathways or through direct phosphorylation of ion channel subunits (Jin, 2009; Luykenaar *et al.*, 2009). Further investigation is required to delineate the molecular mechanisms by which Rho-kinase modulates NSCC.

Unlike the on-contractions elicited by nerve stimulation, far less investigation has been directed at the mechanism(s) for off-contractions. Some authors have suggested that post-stimulus synthesis of eicosanoids is involved, because non-steroidal anti-inflammatory drugs, such as indomethacin, suppress rebound excitation (Ward *et al.*, 1992). However, indomethacin can also inhibit L-type Ca^{2+} channels, and this effect must be controlled for when interpreting the involvement of eicosanoids in rebound excitation (Sawdy *et al.*, 1998). In the present study we showed that off-contractions were also suppressed by Rho-kinase inhibitors although the extent of this inhibition was significantly less than for on-contractions. These findings could be due to inhibition of substance P-activated NSCC (Lee *et al.*, 1995; D'Antonio *et al.*, 2009). However, further studies are required to understand the mechanism of Rho-kinase inhibition of off-contractions.

In summary, Rho-kinase inhibitors were shown to reduce EFS-evoked on-contractions, CCh-induced contractions and CCh-induced depolarization. Furthermore GTPγS-activated NSCC were attenuated by Y-27632. Therefore, Rho-kinase may be involved in not only the Rho-kinase/MLCP pathway contributing to Ca^{2+} sensitization but also direct regulation of NSCC.

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Conflicts of interest

None.

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