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# The role of the RhoA/ROCK pathway in gender-dependent differences in gastric smooth muscle contraction

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**Abstract** Gender-related differences in various gastric functions and diseases have been reported, with women having a higher prevalence of gastrointestinal disturbances than men. The aim of this study was to investigate sex-dependent differences in activation of the Rho-associated protein kinase (ROCK; RhoA/Rho kinase) pathway and muscle contraction in the stomach using single gastric smooth muscle cells (GSMC) from male and female Sprague–Dawley rats. Expression of ROCK1 and ROCK2 protein and acetylcholine (ACh)-induced activation of RhoA and ROCK were measured using a specifically designed enzyme-linked immunosorbent assay and activity assay kits, respectively. Contraction of a single GSMC was measured by scanning micrometry in the presence or absence of the ROCK inhibitor Y27632 dihydrochloride. ACh-induced activation of RhoA and ROCK and subsequent contraction were greater in male rats than in female rats but neither was related to differences in the expression of ROCK1 or ROCK2 or total RhoA amount. Most important, Y27632 inhibited and abolished differences in ACh-induced contraction in both sexes. In conclusion, increased ACh-induced contraction in the GSMC of male rats is attributable to greater RhoA/ROCK activation independent of differences in the expression of ROCK isoforms or total RhoA.

**Keywords** Gastrointestinal system · Smooth muscle · Contraction · Rho kinase

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## Introduction

An accumulating body of evidence suggests that sex can affect gastrointestinal (GI) motility. It has been shown that women have a longer gastric half-emptying time for solids as compared to men [1] and that accordingly women are more likely to be affected by gastroparesis—a chronic motility disorder which is defined as delayed gastric emptying of solids and liquids in the absence of obstruction [2, 3]. Women have also been shown to be more likely to complain of GI disturbances, such as nausea, vomiting, bloating, and constipation, than men [4, 5].

Studies on colonic motility have demonstrated that healthy men have shorter GI transit times than healthy women [6–8]. Rao et al. performed 24-h ambulatory colonic manometry in healthy humans and demonstrated that the female subjects had significantly less pressure activity in the colon during daytime hours than the male subjects [9]. Moreover, the healthy men were found to have higher minimum and maximum basal anal sphincter pressures, higher anal pressures during maximum conscious sphincter contraction, lower rectal volumes required to cause an anal relaxation, and higher volumes to induce a desire to defecate. These results suggest that healthy men have stronger anal sphincter pressures than women [10].

Epidemiological data on the sex ratio in inflammatory bowel syndrome suggest that this syndrome is predominantly diagnosed in women, with a female to male sex ratio ranging from 2:1 to 4:1 [11]. Interestingly, most of the extraintestinal diseases associated with this syndrome also share this female predominance, such as fibromyalgia [12], migraine [13], other functional GI disorders (e.g., functional dyspepsia [14]), chronic pelvic pain [15], chronic fatigue syndrome [16], and depression [17].

In the physiological context, smooth muscle cells (SMC) are considered the final effectors responsible for producing fine and delicate GI tract movements. Contraction of smooth muscle is regulated by both $Ca^{2+}$-dependent and $Ca^{2+}$-independent ($Ca^{2+}$ sensitization) mechanisms [18]. An increase in intracellular $Ca^{2+}$ levels leads to myosin light chain (MLC) kinase activation, resulting in an increase in $MLC_{20}$ phosphorylation and thus smooth muscle contraction. Importantly, $MLC_{20}$ phosphorylation can also be increased through inhibition of MLC phosphatase, which enhances smooth muscle force generation without a change in intracellular $Ca^{2+}$ level [19]. Rho-associated protein kinase (ROCK), a serine/threonine kinase and an important downstream effector of the small G protein RhoA, has been found to be an important factor in developing smooth muscle tone. Activation of the RhoA/ROCK pathway maintains the level of $MLC_{20}$ phosphorylation, the essential step in smooth muscle contraction, via the inhibition of MLC phosphatase activity by phosphorylation of the MLC phosphatase target subunit (MYPT1). The importance of the RhoA/ROCK pathway has been demonstrated in the pathogenesis of cardiovascular disorders and as a target in the development of new drugs, such as fasudil, a Rho-kinase inhibitor [20–22].

Sex differences in signaling pathways within vascular muscle contribute to differences in contractile function, as exemplified in male mice, whose arteries contract to a greater magnitude in response to selected agonists than those of female mice [23]. This difference in contractile response is due to agonist-specific activation of the signaling pathways that regulate the $Ca^{2+}$ sensitivity of contractile proteins. Most importantly, the RhoA/ROCK pathway has been found to be upregulated in male vascular smooth muscle relative to that of females [23]. Whether gender-related differences in GI motility are due to differential activation of the RhoA/ROCK pathway—and thus smooth muscle contraction—in the sexes is not known. The aim of this study was to test the hypothesis that there is a sex-related difference in gastric muscle cell contraction which may result from differences in the activation of the RhoA/ROCK pathway. Since motility disorders are the major characteristics of many GI disturbances, the findings of this study may be of considerable importance in improving our understanding of the cause of their disproportionate prevalence among women. They may also pave the way for a better understanding of the role of the RhoA/ROCK pathway in smooth muscle contraction and thereby contribute to the development of novel therapeutic approaches for treatment of GI disorders.

## Materials and methods

Animals   Young mature male and female Sprague–Dawley rats (approx. 12 weeks of age, 250–300 g) were provided by the animal house of the Jordan University of Science and Technology. Mature female rats were randomly selected regardless of the stage of the ovarian cycle. The average data from all mature female rats should roughly represent the average changes in gastric smooth muscle contraction during different stages of the ovarian cycle. Considering the short ovarian cycle (every 4–5 days) and estrous stage (12 h) on the whole, any possible fluctuations in estrogen levels at the specific stages of the ovarian cycle should cancel out. Rats were euthanized by $CO_2$ inhalation and the stomach rapidly excised. All procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Jordan University of Science and Technology.

Preparation of dispersed gastric SMC   Smooth muscle cells were isolated from the circular muscle layer of the rat stomach by sequential enzymatic digestion, filtration, and centrifugation, as described previously [24]. Briefly, strips of circular muscle free of mucosa were dissected from all regions of the stomach and incubated at 31 °C for 30 min in HEPES medium (pH was adjusted to 7.4) containing 120 mM NaCl, 4 mM KCl, 2.0 mM $CaCl_2$, 2.6 mM $KH_2PO_4$, 0.6 mM $MgCl_2$, 25 mM HEPES, 14 mM glucose, 2.1 % Eagle's essential amino acid mixture, 0.1 % collagenase, and 0.01 % soybean trypsin inhibitor. The tissue was continuously gassed with 100 % oxygen during the entire isolation procedure. The partly digested strips were then washed twice with 50 ml of enzyme-free medium and the muscle cells allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through a 500-µm Nitex mesh (precision woven open-mesh monofilament fabric with 500-µm pores; Amazon) and centrifuged twice at 350 $g$ for 10 min to remove broken cells and organelles. The harvested cells were counted in a hemocytometer; it was estimated that 95 % of the cells excluded trypan blue. The cell isolation procedure consistently yielded spindle-shaped and viable GSMC that showed significant contraction in response to contractile stimuli. All of the experiments were performed within 2–3 h of cell dispersion.

Identification of SMC   The identity of the gastric cells isolated was confirmed as SMC by immunohistochemical staining of paraffin-embedded rat gastric muscle using anti-calponin antibody ab46794 (Abcam, Cambridge, MA) at a 1:100 dilution.

Expression of ROCK1 and ROCK2 protein by enzyme-linked immunosorbent assay   The membranes of the collected GSMC were broken down by being subjected to repeated freeze–thaw cycles. After centrifugation of the lysates (20,000 $g$, 10 min, 4 °C), the protein concentrations of the supernatant were determined using a DC Protein Assay kit from Bio-Rad Laboratories (Hercules, CA). Samples of equal amounts of proteins were quantitated for ROCK1 and ROCK2 protein using the respective enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions [ROCK1 ELISA kit (MBS909842); MyBioSource, San Diego, CA; ROCK2 ELISA kit (CSB-EL020059RA); Cusabio Biotech, Newark, DE]. Briefly, the protein concentration of the supernatants of the lysed cells was measured and then adjusted where needed so that all samples had the same protein concentration; they were then added to 96-well plates precoated with ROCK1 (ROCK1 ELISA) or ROCK2 (ROCK2 ELISA) and incubated for 2 h at 37 °C, following which 100 μL of Biotin antibody specific for ROCK1 (ROCK1 ELISA) or for ROCK2 (ROCK2 ELISA) was added to each well and the plates incubated for 1 h at 37 °C. After washing, 100 μL of horseradish peroxidase–avidin was added to each well and the plates incubated for 1 h at 37 °C. Optical density was determined using the BioTek a microplate reader (BioTek Instruments, Winooski, VT) at 450 nm.

Measurement of ROCK activity   ROCK activity was analyzed by enzyme immunoassay with Cell Biolabs' 96-well ROCK activity assay kit (STA-416; Cell Biolabs Inc., San Diego, CA). All experiments were carried out according to the manufacture's protocol using 10 μL of protein lysate. The total starting protein concentration in each sample was 1 mg/mL. In brief, the protein concentration of the supernatants of the lysed cells was measured and then adjusted where needed so that all samples had the same protein concentration. The samples were then added (90 μL/well) to 96-well plates precoated with substrates corresponding to the C-terminus of MYPT1, which contains a threonine residue that may be phosphorylated by ROCK, and 10 μL of kinase reaction buffer was added to initiate the kinase reaction. The mixture was allowed to incubate for 60 min at 30 °C. After the plates were washed with the washing buffer provided in the kit, 100 μL of diluted anti-phospho-MYPT1 (Thr[696]) antibody was added to each well and the plates incubated at room temperature for 1 h. The specific detector antibody was horseradish peroxidase-conjugated anti-phospho-MYPT1 (incubated at room temperature for 1 h). The amount of phosphorylated substrates was measured by binding with horseradish peroxidase-conjugated anti-phospho-MYPT1, which catalyzes the conversion of tetramethylbenzidine to a yellow solution. The results were quantified using a BioTek microplate reader at 450 nm.

Measurement of active and total RhoA   Levels of activated and total RhoA were determined using the RhoA G-LISA™ Activation Assay kit (BK124) and the Total RhoA ELISA kit (BK150) (Cytoskeleton Inc., Denver, CO), respectively, according to the manufacturer's instructions, using 10 μL of protein lysate. The total starting protein concentration for all samples was 1 mg/mL. Briefly, GSMC were lysed using the cell lysis buffer provided and the protease inhibitor cocktail provided in the kits. Lysates were quantified using the DC Protein Assay kit from Bio-Rad by measuring the absorbance at 750 nm on the BioTek microplate reader. Lysates were diluted to 0.5 mg/mL using lysis buffer and then loaded onto to the G-LISA (activated RhoA) or ELISA (total RhoA) plate for protein analysis. The results were obtained as absorbance values at 490 nm. For total RhoA, the results were converted to total protein levels using a standard curve obtained with each test.

Measurement of contraction in dispersed SMC   Contraction in freshly dispersed GSMC was determined by scanning micrometry [25]. Aliquots (0.4 ml) of cells containing approximately $10^4$ cells/ml were prepared and categorized into aliquots of cells from either male or female rats. Cells were stimulated with acetylcholine (ACh; 0.1 μM) for 10 min at room temperature in the presence or absence of the ROCK inhibitor Y27632 (Santa Cruz Biotechnology, Dallas, TX), and the reaction was terminated with the addition of 1 % acrolein at a final concentration of 0.1 %. Acrolein kills and fixes cells without affecting cell length. The cells were viewed using a 10× or 20× objective on an inverted Nikon TMS-f microscope (Nikon Corp., Tokyo, Japan), and cell images were acquired using a Canon digital camera (Canon Inc., Tokyo, Japan) and Image J acquisition software. The resting cell length was determined in control experiments in which muscle cells were not treated with ACh. The mean length of at least 50 muscle cells from each group was measured by image J software. The contractile response to ACh was defined as the decrease in the average length of at least 50 cells and expressed as the percentage of change in length relative to the average resting length.

Materials   All chemicals, other than those whose suppliers are specifically mentioned above, were obtained from Sigma (St. Louis, MO) USA. The stock solution of Y27632 was prepared in dimethylsulfoxide.

## Statistical analysis

Each experiment was performed on GSMC that were harvested from at least five to ten different rats of each sex. Statistical analysis of all experiments was performed using Prism 5.0 software (GraphPad Software, San Diego, CA).

The unpaired Student's *t* test was used to reveal significant differences between two means, and one-way analysis of variance followed by Fisher's post hoc analysis was performed for the comparison of more than two means. A $P < 0.05$ was required for statistical significance in all the experiments. All data are shown as the mean ± standard error of the mean.

## Results

### Smooth muscle identification

Freshly isolated GSMC observed under a phase-contrast microscope were of variable length and had a spindle-shaped morphology. Some cells were at rest while others were at different phases of contraction (Fig. 1a). The length of resting cells from male and female rats was 113.53 ± 4 and 114.39 ± 3 μm, respectively. The smooth muscle identity of the rat gastric cells was verified by immunohistostaining with anti–calponin antibody (Fig. 1b), which showed that more than 95 % of cells stained positive for calponin (h1-calponin is specific to differentiated SMC [26]).

### ROCK activity

Treatment of freshly dispersed male or female GSMC with 0.1 μM ACh, a Gαq/13-coupled receptor agonist, for
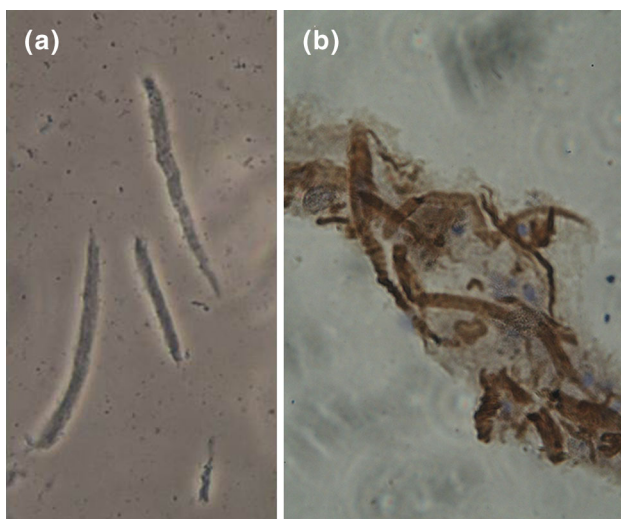


**Fig. 1** Identification of rat gastric smooth muscle cells (GSMC). **a** Freshly isolated GSMC were of variable length and had a *spindle-shaped* form by phase contrast microscopy. Cells were viewed under a 20× objective of an inverted Nikon TMS-f microscope, and the image was captured with a Canon digital camera. Average length of resting SMC isolated from male and female rats was 113.53 ± 4 and 114.39 ± 3 μm, respectively. **b** Immunohistochemical staining of paraffin-embedded rat smooth muscle using anti-h1-calponin antibody at 1/100 dilution
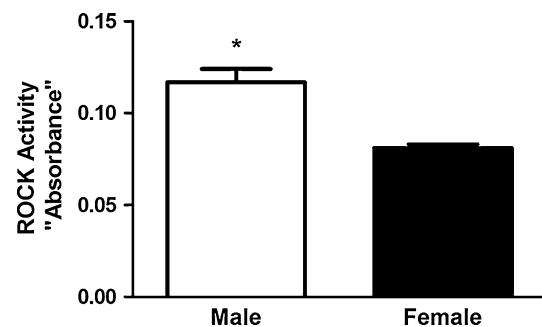
10 min significantly increased ROCK activity above the basal level. Importantly, ACh-induced ROCK activity was greater in male versus female GSMC ($P < 0.01$) (Fig. 2). Basal ROCK activity was similar in both male and female groups of cells (data not shown).
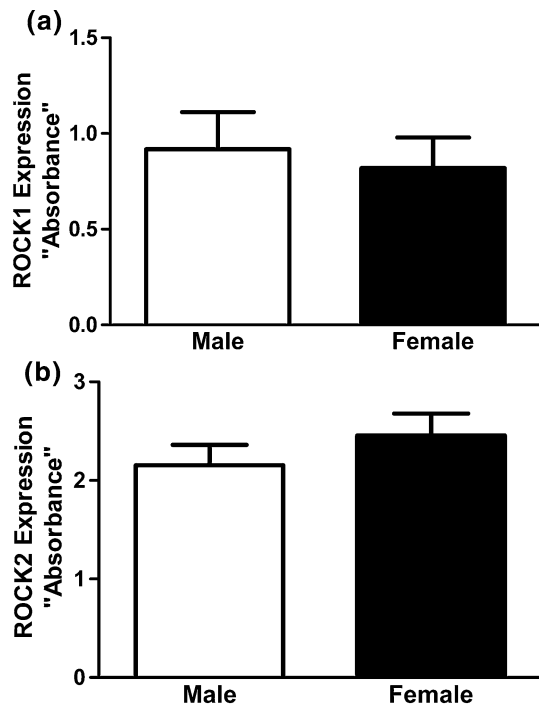
### ROCK expression

To determine whether the sex differences in ROCK activity correlates with its expression profile we compared the protein levels of ROCK1 and ROCK2, the predominant smooth muscle isoforms [27], in GSMC isolated from male and female rats, respectively, by ELISA. Despite the higher agonist-stimulated ROCK activity in male gastric muscle cells compared to female ones, the expression of both ROCK1 and ROCK2 protein was not different in the two groups of cells ($P > 0.05$) (Fig. 3a, b). This observation raises the possibility that gender differences in ROCK activity might be due to an effect on other upstream regulators of the enzyme, such as RhoA.

### RhoA activation

Treatment of freshly dispersed male or female GSMC with 0.1 μM ACh for 10 min significantly augmented RhoA activation above the basal level. Most importantly, ACh-induced RhoA activation was greater in male GSMC than in female ones ($P < 0.01$) (Fig. 4a). Basal RhoA activity was similar in the two groups of isolated from male and female rats (data not shown). Interestingly, despite the higher RhoA activation in male GSMC, total RhoA protein level (active and inactive forms) was similar in both male and female cells (Fig. 4b).



**Fig. 2** Acetylcholine (ACh)-induced activity of Rho-associated protein kinase (*ROCK*) in GSMC isolated from male and female rats. ROCK activity is expressed as absorbance at 450 nm. Treatment of GSMC with ACh (0.1 μM) significantly increased ROCK activity in the cells of both sexes. Note that the ACh-induced activation of ROCK was significantly higher in cells isolated from male rats than in those isolated from female rats (*$P < 0.01$ vs. female; $n = 10$ males, $n = 7$ females)

**Fig. 3** Expression of ROCK1 and ROCK2 proteins in GSMC of male and female rats. ROCK1 (**a**) and ROCK2 (**b**) protein expression level is expressed as absorbance at 450 nm. The values shown are representative of at least four independent experiments performed in triplicate. Note that there was no significant difference in both ROCK1 and ROCK2 protein levels in the GSMC of male and female rats ($P > 0.05$; $n = 11$ males, $n = 7$ females)

## Smooth muscle contraction

Freshly dispersed GSMC from male and female rats were treated with 0.1 μM ACh, and the decrease in muscle cell length was measured by scanning micrometry. The lengths of resting muscle cells from male and female rats were similar (data not shown). ACh caused both male and female muscle cells to contract, but male muscle cells showed a significantly greater contraction than female cells ($P < 0.01$) (Fig. 5). Preincubation of GSMC with the ROCK inhibitor Y27632, (1 μM) significantly reduced ACh-induced contraction in the muscles cells of both male and female rats ($P < 0.01$) (Fig. 6). Most importantly, Y27632 abolished the sex differences in ACh-induced contractions (Fig. 6).

## Discussion

The main findings of this study are:

- The contraction of GSMC was sex-dependent
- Contraction to ACh was greater in gastric muscle cells isolated from male rats than in those from female rats.
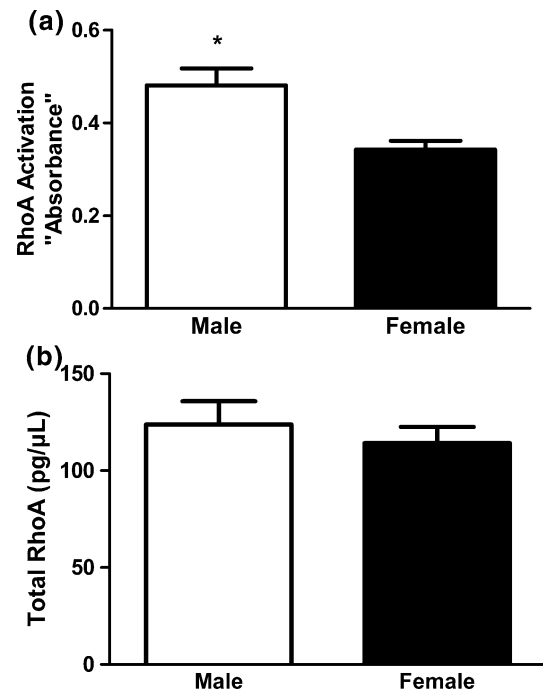


**Fig. 4** Acetylcholine-induced RhoA activation in GSMC of male and female rats. **a** Activated RhoA is expressed as absorbance at 450 nm. Analysis using the RhoA G-LISA™ Activation Assay kit revealed increased levels of activated RhoA in response to ACh (0.1 μM) in both male and female GSMC. Note that ACh-induced activation of RhoA was significantly greater in male cells versus female. **b** Total RhoA protein was similar in both male and female GSMC (*$P < 0.01$ vs. female, $n = 10$ males, $n = 7$ females)
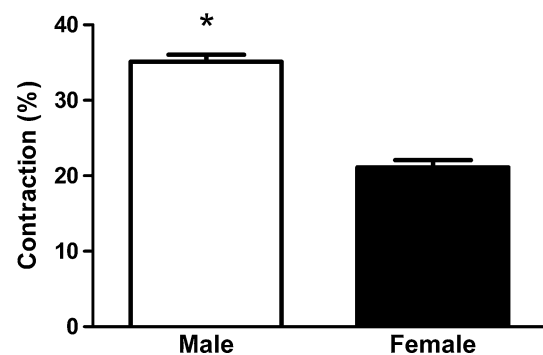


**Fig. 5** Acetylcholine-induced contraction of GSMC isolated from male and female rats. GSMC of male and female rats were stimulated with ACh (0.1 μM) and viewed under a Nikon microscope. Images of treated and non-treated single cells were acquired and cell contraction measured. ACh caused muscle cell contraction in GSMC from both male and female rats. Note that contraction in response to ACh was significantly greater in male cells than in female ones (*$P < 0.01$ vs. female; $n =$ at least 50 cells from 10 different rats of each sex)

In our study of GSMC isolated from male and female rats, ACh-induced activation of ROCK was higher in GSMC from male rats than in those from female rats. This increased activation of ROCK was associated with a greater activation of RhoA even though the total RhoA protein level was
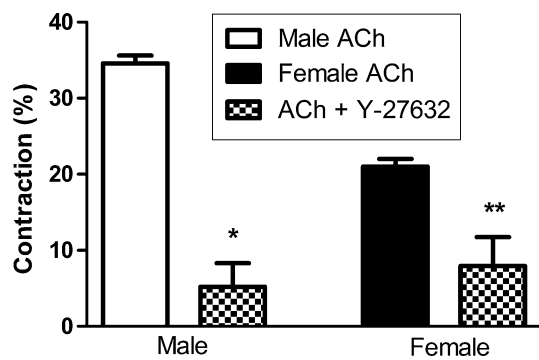
**Fig. 6** Effect of the ROCK inhibitor Y27632 on ACh-induced contraction in GSMC of male and female rats. GSMC of male and female rats were stimulated with ACh (0.1 μM) in the presence or absence of 1 μM Y27632 and then viewed under a Nikon microscope. Images of treated and non-treated single cells were acquired and cell contraction measured. The ROCK inhibitor significantly inhibited ACh-induced contraction in GSMC of both male and female rats. Importantly, Y27632-induced inhibition of gastric muscle contraction abolished the sex differences in the strength of ACh-induced contractions (*$P < 0.01$ vs. male ACh, **$P < 0.01$ vs. female ACh; $n =$ at least 50 cells from 10 different rats of each sex)

similar (active and inactive forms) in cells from both sexes. Despite the higher agonist-induced ROCK activation in male cells, the protein levels of both ROCK1 and ROCK2 were similar in male and female gastric muscle cells. Inhibition of ROCK abolished the sex-dependent differences in contraction induced by ACh. These results suggest that activation of RhoA and ROCK are regulated differently in GSMC from male and female rats.

Sex differences in smooth muscle function have been reported in several different tissue organs and in different species; for example, the authors of two separate vascular studies reported that the arteries of males have a greater myogenic tone than those of females [28, 29]. Further, it has also been suggested that sex differences in vascular reactivity may be related to differential regulation of the Rho/Rho kinase pathway [30, 31]. Chrissobolis et al. reported greater Rho kinase function and stronger vasodilator response to Y-27632 in the male cerebral circulation of rats than in that of female rats, but the measured protein levels of RhoA and Rho kinase were not different [30]. To the best of my knowledge, this is the first study to show differential activation of the RhoA/ROCK pathway and different degrees of contraction of single GSMC isolated from male and female rats. Because gender-related differences in gastric contraction may be due to differences in the various types of gastric cells, studying muscle contraction and regulation of the RhoA/ROCK pathway in multicellular preparations, as commonly attempted in previous studies, may be difficult and non-specific. To avoid the contribution of other non-muscle cell types, in the current study all experiments were performed using single SMC freshly isolated from the stomach of rats.

Various receptor agonists generate both initial/transient (<1 min) $Ca^{2+}$-dependent and sustained (>5 min) $Ca^{2+}$-independent contraction in GI SMC [32–34]. Recent studies have revealed that ROCK, a serine/threonine kinase downstream of the small G protein RhoA, is important in developing smooth muscle tone. It inhibits MLC phosphatase activity by phosphorylation of MYPT1. A decrease in MLC phosphatase activity maintains phosphorylation of myosin and therefore contributes to smooth muscle contraction at low concentrations of intracellular $Ca^{2+}$ [35].

In the experimental design of the present study, single GSMC were treated with the agonist for 10 min; consequently, the results of the kinase and contraction assays mostly represent differences in the sustained phase of contraction. Gender differences in the initial phase of contraction, such as changes in $Ca^{2+}$ level and/or in the activity of various kinases (e.g., MLC kinase), should be tested in future studies.

Consistent with the results of many previous studies, the treatment of muscle cells with ACh significantly enhanced ROCK activity in the cells from both males and females [36, 37]. Moreover, the results presented here support previously reported findings on the vasculature and confirm that ACh-induced activation of ROCK is higher in males [30]. Two ROCK isoforms have been identified; ROCK1 (or β isoform) and ROCK2 (or α isoform). The ROCK assay kit used in this study uses MYPT1 Thr$^{696}$ as a probe for the kinase which can be phosphorylated by both ROCK isoforms, so it does not distinguish betweeen the activities of the ROCK1 and ROCK2 isoforms. The expression levels of both kinase isoforms were measured in order to determine whether they correlated with the respective activation pattern or not. Despite the differences in ROCK activation in GSMC from both genders, expression of the two isoforms of the enzyme was similar in single GSMC from males and females, indicating that the differences in the expression of either ROCK1 or ROCK2 did not account for the greater activity of ROCK in GSMC isolated from males. The results reported here raise the possibility that sex-linked increased ROCK activity in males might be due to differences in the activation of other upstream regulators of the enzyme, such as RhoA. The inactive form of RhoA (RhoA.GDP) is present in the cytosol bound to a guanine dissociation inhibitor (GDI). Activation of RhoA, such as via ACh-stimulated muscarinic receptors (M3), is mediated by various Rho-specific guanine nucleotide exchange factors (RhoGEFs) which in turn promote the exchange of GDP for GTP. Upon GTP binding and activation, RhoA interacts with and stimulates the activity of downstream effectors, including ROCK. Active RhoA (GTP-bound) is in turn inactivated by GTPase-activating proteins (GAPs) which hydrolyze GTP to GDP [33]. Parallel to these ROCK findings are those showing that ACh-induced activation of

RhoA was greater in male GSMC than in female ones. However, despite this greater ACh-induced RhoA activation in male GSMC, total RhoA levels, including both active (GTP-bound) and inactive (GDP-bound) forms, were similar in both the male and female cells. These findings suggest that the observed sex differences in RhoA activation may be related to other regulators of Rho, such as GDI inhibitors, RhoGEFs, and GAPs, which control the balance between active and inactive RhoA. The role of these regulatory proteins in this reported gender-linked RhoA activation needs to be tested in future studies.

Noteworthy, ACh-induced muscle cell contraction was greater in male gastric cells than in female ones. Most importantly, ROCK inhibitor Y27632 abolished the sex difference in agonist-induced contractions. Inhibition of ROCK normalized contractions to ACh in GSMC from male and female rats, demonstrating that the enhanced ROCK activity in the male stomach mediates the sex difference. Although higher concentrations of Y27632 also inhibited PKC, previous studies confirmed that Y27632 at 1 μM is highly selective for Rho kinase [38, 39].

In conclusion, the results of this study demonstrate that activation of RhoA and ROCK in response to ACh is greater in the male stomach than in the female one, with the result that male stomachs undergo greater contractions. The exact mechanisms by which the RhoA/ROCK pathway is differentially regulated in the sexes require further investigations. Sex differences are clearly present in the GI system, with the RhoA/ROCK pathway playing a crucial role in GI functionality. Further understanding of the role of the RhoA/ROCK pathway in modulating normal physiological and pathophysiological functions of the GI tract will enable the development of more effective and sex-tailored treatments for GI disturbances.

**Compliance with ethical standards**

**Conflict of interest** The author declares that there are no conflicts of interest.

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