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Biochemical Pharmacology 69 (2005) 1483–1492
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Ca^{2+} sensitization and the regulation of contractility in rat anococcygeus and retractor penis muscle

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Received 22 December 2004; accepted 21 February 2005

Abstract

Stimulation of the RhoA/Rho-kinase (ROK) signaling represents a key step in the maintenance of agonist-induced contraction of smooth muscle. We aimed to demonstrate Ca^{2+} sensitization in rat anococcygeus and retractor penis muscles and to identify the molecular expression of major components of this pathway. Both anococcygeus and retractor penis showed a similar expression of RhoA, ROKα, and ROKβ at the protein level as well as the mRNA for RhoGEFs. Cumulative addition of the ROK inhibitors H-1152 (0.001–3 μM), Y-27632 (0.01–30 μM) or HA-1077 (0.01–30 μM) caused sustained relaxations of precontracted smooth muscle strips. Ca^{2+} sensitization induced by phenylephrine, norepinephrine and carbachol was markedly antagonized by all three ROK inhibitors. In addition, the contractile response to KCl-induced depolarization was highly sensitive to these ROK inhibitors. H-1152 was approximately 8–20 more potent than Y-27632 and HA-1077 to inhibit contraction. Electrical field stimulation (EFS, 1–32 Hz) caused transient contractions in both anococcygeus and retractor penis muscle, which were blocked by tetrodotoxin (1 μM), phentolamine (1 μM) or bretylium tosylate (30 μM). Similarly, H-1152 (0.1–1 μM), Y-27632 (1–10 μM) or HA-1077 (1–10 μM) significantly reduced EFS-evoked contractions in a concentration-dependent manner. The results indicate that the RhoA/ROK-mediated Ca^{2+} sensitization pathway is expressed in anococcygeus and retractor penis muscles and enhances contractions produced by receptor-dependent and independent mechanisms.

Keywords: Anococcygeus muscle; Retractor penis muscle; Rho-kinase; RhoGEFs; RhoA; Ca^{2+} sensitization

1. Introduction

It is widely accepted that the key signal to activate the contractile apparatus in smooth muscle is an increase in the intracellular Ca^{2+} concentration. The rise in intracellular Ca^{2+} promotes binding to calmodulin, which in turn activates the phosphorylation of myosin light chain (MLC) through MLC kinase, resulting in crossbridge cycling and force development [1–3]. Thus, contractile stimulation of smooth muscle with physiological agonists is primarily regulated by the level of MLC phosphorylation, which is determined by the balance of activity of the enzymes, MLC kinase and MLC phosphatase [4].

Several reports have revealed that smooth muscle contractile proteins could be sensitized to Ca^{2+}, representing a mechanism referred to as Ca^{2+} sensitization, by which excitatory G protein coupled-receptor (GPCR) agonists produce greater increases in force without a concomitant increase in intracellular Ca^{2+} [4]. This process has been shown to make a significant contribution to agonist-induced contraction under physiological conditions and is observed not only in vascular but also in other visceral smooth muscles. Indeed, receptor-dependent, G protein-mediated Ca^{2+} sensitization occurs in corpus cavernosum [5–7], urinary bladder [8], gastric fundus [9], ileal [10], myometrial [11], and tracheal smooth muscle [12]. Cellular signaling pathways involved in Ca^{2+} sensitization converge on an increase in MLC phosphorylation, which is mediated via inhibition of MLC phosphatase. The small G protein RhoA and one of its downstream effectors, Rho-kinase (ROK) have been shown to play important roles in this process [4,13,14]. RhoA activity is under the direct

Abbreviations: CCh, carbachol; EFS, electrical field stimulation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEFs, guanine nucleotide exchange factors; GPCR, G protein coupled-receptor; HA-1077, (5-isoquinolinesulfonyl)homopiperazine; H-1152, (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine; LARG, leukemia-associated RhoGEF; MLC, myosin light chain; NE, norepinephrine; PE, phenylephrine; RGS, regulator of G protein signaling domain; ROK, Rho-kinase; Y-27632, (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide

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doi:10.1016/j.bcp.2005.02.018

control of a large set of other regulatory proteins, including the guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP to activate RhoA [15–18]. In addition, based on the observations that ROK phosphorylates the regulatory subunit of MLC phosphatase [19], that recombinant constitutively-active ROK causes contraction in Triton X-100-skinned tissue [20], and that ROK inhibition antagonizes agonist-induced contraction of arterial smooth muscle [21], the RhoA/ROK system has emerged as an integral constituent of a signalling pathway leading to Ca^{2+} sensitization of the contractile apparatus.

The ROK inhibitors (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) and (5-isoquinolinesulfonyl)homopiperazine (HA-1077) have proven to be very useful in the identification of the relative role that Ca^{2+} sensitization plays in the regulation of smooth muscle contraction in different organ systems. These agents inhibit tonic contraction in vascular [21] and non-vascular [5–12] smooth muscle with a potency that is similar to the K_i values reported for inhibition of ROK activity in vitro. In fact, a recent study documenting the specificity of 28 commercially available kinase inhibitors indicates that Y-27632 and HA-1077 are highly selective for inhibition of ROK when used at the appropriate concentrations [22]. More recently, the compound (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-homopiperazine (H-1152) has been demonstrated to be a more potent and selective ROK inhibitor than Y-27632 and HA-1077, as shown by its competitive inhibition of ROK activity with respect to ATP, with a K_i value of 1.6 nM [23].

The anococcygeus and retractor penis muscle are part of the erectile machinery in male rodents [24]. The retractor penis muscle of the rat is essentially a bundle of smooth muscle extending from some fibers of the anococcygeus muscle which merge on the ventral surface of the rectum [25]. The relaxation of the retractor penis muscle stretches the sigmoid flexure and results in the protrusion of the penis, which is a pivotal step for erection in different species. The muscle is absent in primates and rabbits, and is vestigial in rats. Both the anococcygeus and the retractor penis muscles have a similar autonomic innervation, likely because of the contiguous nature of the two muscles, consisting of noradrenergic sympathetic motor fibers and non-adrenergic non-cholinergic parasympathetic inhibitory fibers [26–29].

Recently, Ayman et al. [30] reported that ROK-mediated Ca^{2+} sensitization is activated by receptor-dependent and independent mechanisms in the mouse anococcygeus. The study described in this report was designed to expand upon these findings by characterizing the Ca^{2+} sensitization pathway in both anococcygeus and retractor penis muscles. Since anatomic facts about these muscles may not provide adequate grounds for surmise about the nature of their contractile mechanisms, we sought to determine the functional and biochemical properties of the RhoA/ROK signaling in rat anococcygeus and retractor penis. For these purposes, we investigated the effects of H-1152, Y-27632 and HA-1077 on smooth muscle contractions evoked through both G-protein coupled and uncoupled mechanisms. Furthermore, we verified the expression of RhoA, ROKα and ROKβ by Western blot analysis as well as the mRNA expression of the regulator of G protein signaling domain (RGS)-containing RhoGEFs.

2. Materials and methods

2.1. Animals

The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, U.S.A.). All experiments were conducted in accordance with institutional guidelines and approved by the local committee on animal experiments. Experiments were performed on adult male Sprague–Dawley rats (250–275 g) obtained from Harlan Laboratories. The animals were housed two per cage on a 12 h light–dark cycle, and fed a standard chow diet with water ad lib.

2.2. Tissue preparation

The animals were anaesthetized with pentobarbital sodium (40 mg/kg, i.p.), killed by decapitation and exsanguinated. An inverted ''U''-shaped midline incision was made in the base of the scrotum with the lateral arms of the incision extending over the testicles. The fan-shaped insertion of the retractor penis muscle into the scrotal skin was identified and the muscle followed proximally to its origin in the ventral bar formed by the convergence of the paired anococcygeus muscle on the distal part of the terminal colon. The retractor penis muscle was dissected free of connective tissue, excised and cut longitudinally to provide two strips. To excise the anococcygeus muscle, the abdomen was opened in the midline and the pelvis split. While the colon was carefully lifted, the connective tissue was cut along the dorsal side, until the paired muscles could be seen passing from the vertebral column to the ventral side of the colon. The muscles were exposed, carefully freed of connective tissue and dissected out. The muscle strips were subsequently immersed in chilled Krebs solution of the following composition (mM): NaCl, 130; $NaHCO_3$, 14.9; dextrose, 5.5; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 7H_2O$, 1.17 and $CaCl_2 2H_2O$, 1.6.

2.3. Western blot analysis

The muscle strips were homogenized in a lysing buffer containing 40 mM HEPES, 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride. The tissue lysate was centrifuged at 10,000 × *g*

and the supernatant was collected. The protein concentration was determined using a BSA protein assay kit (Pierce Chemical). An aliquot of 50 μg protein from each sample was loaded per lane and resolved by 10 or 15% (for ROKα/ROKβ and RhoA, respectively) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions. Proteins were subsequently transferred onto nitrocellulose membranes (BioRad). Membranes were blocked by treatment with 5% milk in Tris–buffered saline containing 0.05% Tween 20, probed with antibodies against RhoA (1:500), ROKα (1:1000) or ROKβ (1:1000) and incubated with a horseradish peroxidase-conjugated second antibody. Immunoreactivity was detected by enhanced chemiluminescence autoradiography.

2.4. Semi-quantitative RT-PCR

Rat homologues of PDZ-RhoGEF, LARG and p115RhoGEF were identified by comparative genome analysis using publicly available rat, mouse and human data. Primers were designed with Primer3 program based on the known mRNA sequences for each gene. To exclude the possible contamination of genomic DNA, care was taken to ensure that the two primers for one gene were located at different exons. Sequences of forward and reverse primers were as follows:

PDZ-RhoGEF
Forward primer—GGACCCTCTTCGAGAACGCCAAA
Reverse primer—GGCAGCCACTTGTCCTTGTCAGG

LARG
Forward primer—GCCATGCGCGCTGGAGTACAAAC
Reverse primer—GCTCCAGGGGAATGAGGGGATGTC

p115RhoGEF
Forward primer—TCCGGACCAAGAGTGGGGACAAGA
Reverse primer—TACCCAGGCTTCCCTTCCGGTCTG

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)
Forward primer—TGCATCCTGCACCACCAACTGCTT
Reverse primer—CAGCCTTGGCAGCACCAGTGGAT

Total RNA (4 μg per reaction) extracted from tissue segments with TRIzol reagent (Invitrogen) was used for the first strand cDNA synthesis with superscript II kit (Invitrogen), according to manufacturer's specification. cDNA equal to 0.04 μg total RNA was used for each PCR reaction under the following conditions: 94 °C for 2 min and 22 (for GAPDH) or 30 (for RhoGEFs) cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, followed by 72 °C for 7 min. The reaction products were analyzed by electrophoresis on agarose gel and the expected product was extracted and verified by direct DNA sequencing. The gel images were recorded by video camera (Sony Video Camera Module CCD), connected to an IBM AT computer with a 512 × 512 pixel array imaging board with 256 gray levels. The PCR products were quantified by densitometric scanning of gel images using RFL Print software (BDI). Results were then expressed as the densitometric ratio of RhoGEF/GAPDH (%).

2.5. Measurement of contractile force

Strips of retractor penis and anococcygeus muscles were transferred to 4-ml myograph chambers (Danish Myograph Technology) containing Krebs solution at 37 °C continuously bubbled with a mixture of 95% O_2 and 5% CO_2, horizontally attached between two mounting clamps and connected to an isometric force transducer. Then, the tissues were stretched to a resting force of 10 mN and allowed to equilibrate for 60 min. Changes in isometric force were recorded using a PowerLab 8/SP™ data acquisition system (software Chart 5.0, ADInstruments). Electrical field stimulation (EFS) was applied in muscles placed between platinum pin electrodes attached to a stimulus splitter unit (Stimu-Splitter II), which was connected to a Grass S88 stimulator (Astro-Med Industrial Park). EFS was conducted at 50 V, 1 ms pulse width and trains of stimuli lasting 10 s at varying frequencies (1–32 Hz).

2.6. Experimental protocols

In order to verify the viability of the preparations, a K^+ solution (80 mM) was added to the organ baths at the end of the equilibration period. Cumulative concentration–response curves to H-1152 (0.001–3 μM), Y-27632 (0.01–30 μM) and HA-1077 (0.01–30 μM) were obtained in muscle strips contracted with carbachol (CCh, 10–30 μM), phenylephrine (PE, 1 μM) or KCl (80 mM). One concentration–response curve to a ROK inhibitor was obtained in each muscle. In order to negate any effects that might be produced by norepinephrine (NE) released from sympathetic nerves, the tissues were pre-exposed to bretylium tosylate (30 μM). In another set of experiments, concentration–response curves to CCh (0.01–100 μM) or NE (0.001–100 μM) were performed in absence and 30 min after the addition of the ROK inhibitors listed above. Electrically-evoked contractions were performed at supramaximum voltage in all strips. Frequency–response curves (1–32 Hz) were constructed in the absence and presence of ROK inhibitors. Hence, after completion of the control curves, the preparations were washed and curves were repeated 30 min after the addition of H-1152 (0.1–1 μM), Y-27632 (1–10 μM) or HA-1077 (1–10 μM).

2.7. Drugs and chemicals

Atropine, bretylium tosylate, carbachol, norepinephrine, phentolamine, phenylephrine and tetrodotoxin were purchased from Sigma Chemical Co. The compounds (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine (H-1152), (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632) and (5-isoquinolinesulfonyl)homopiperazine (HA-1077) were acquired from Calbiochem. The antibodies used to probe for RhoA, ROKα and ROKβ were obtained from BD Biosciences. All other reagents used were of analytical

grade. Stock solutions were prepared in deionized water and stored in aliquots at −20 °C; dilutions were made up immediately before use.

2.8. Statistical analysis

The magnitude of EFS-, NE- and CCh-induced contractions was given as percentage of the K^+ solution (80 mM)-induced contraction, which was taken as 100%. Relaxant responses were shown as percentages of the precontraction level. Quantitative data were expressed as mean ± S.E.M. of *n* experiments and the differences between two means were evaluated by the Student's two-tailed *t*-test for paired or unpaired observations, as appropriate. A probability of less than 0.05 was considered statistically significant. Analysis of variance (ANOVA) for repeated measurements was performed for the appropriate results and Bonferroni method was chosen as a post-test. A program package was used for the statistical analysis of all data (GraphPAD Software, version 3.00).

3. Results

3.1. Expression of RhoA, ROKα and ROKβ

Fig. 1 (panels a and b) demonstrates the protein expression of RhoA (a) as well as of ROKα and ROKβ (b) in retractor penis and anococcygeus muscle. The antibody against RhoA recognized a single protein with an approximate molecular mass of 21 kDa. Similarly, antibodies against ROKα and ROKβ revealed the expression of these kinases with approximate molecular weights of 180 and 160 kDa, respectively, as expected.

3.2. Expression of RGS-containing RhoGEFs

A RT-PCR assay was used to identify the mRNA expression of the RGS-containing RhoGEFs in the rat anococcygeus and retractor penis muscles, according to a methodology developed in our laboratory [31]. Fig. 1 (panels c and d) shows the electrophoretic visualization of the mRNA expression for PDZ-RhoGEF, p115RhoGEF and LARG in retractor penis and anococcygeus muscle. There were no significant differences with respect to RhoGEF expression between the two types of smooth muscle, as demonstrated by GAPDH normalized quantification of the PCR products.

3.3. Contractile effect of agonists in anococcygeus and retractor penis

The α-adrenergic agonist NE (0.001–100 μM) and the muscarinic agonist CCh (0.01–100 μM) produced sustained contractions in a concentration-dependent manner in rat anococcygeus and retractor penis muscle preparations ($n = 16$, each). The pEC_{50} and E_{max} values were not significantly different in the anococcygeus between the two agonists, being, respectively 5.96 ± 0.03 and 112 ± 3% for NE and 5.83 ± 0.09 and 107 ± 1% for CCh. On the other hand, the retractor penis muscle was more sensitive to NE (pEC_{50} and E_{max} values of 6.49 ± 0.05 and 124 ± 2%, respectively) than to CCh (pEC_{50} and E_{max} values of 5.64 ± 0.08 and 94 ± 1%, respectively). Addition of phentolamine (1 μM) or atropine (0.1 μM) caused parallel rightward shifts in the curves to NE (10-fold in anococcygeus and 28-fold in retractor penis muscle) and CCh (14-fold in anococcygeus and 8-fold in retractor penis muscle), in both muscle preparations ($n = 4$, each).

3.4. Effect of ROK inhibitors on agonist-induced contractions

To determine the involvement of the RhoA/ROK signaling pathway in rat anococcygeus and retractor penis muscle, we compared the inhibitory action of selective ROK inhibitors on contractions mainly dependent on receptor activation (PE, CCh) or MLC kinase (KCl). Contractions induced by KCl were evoked after treatment of the preparations with bretylium tosylate (30 μM) to inhibit possible effects of NE, which could be released from nerve terminals. The ROK inhibitors, H-1152 (0.001–3 μM), Y-27632 (0.01–30 μM) or HA-1077 (0.01–30 μM), were added cumulatively during the sustained phase of 80 mM KCl-, 1 μM PE- or 10–30 μM CCh-induced contraction. Application of each ROK inhibitor resulted in a concentration-dependent decrease in the force elevated by each constrictor (Fig. 2). The E_{max} values of the three compounds are summarized in Table 1. In the anococcygeus, Y-27632 and HA-1077 inhibited contractile responses equally, whereas the pEC_{50} of H-1152 was smaller regardless of the contractile agent employed (Fig. 3; $p < 0.01$). Fig. 3 also shows that although similar results were obtained in the retractor penis muscle, the pEC_{50} of Y-27632 against PE and CCh was smaller than that of HA-1077 ($p < 0.01$).

Pretreatment with increasing concentrations of H-1152 (0.1, 0.3 and 1 μM), Y-27632 (1, 3 and 10 μM) or HA-1077 (1, 3 and 10 μM) significantly attenuated both NE- and CCh-evoked contractions of anococcygeus and retractor penis muscle preparations, which are represented in Fig. 4 as the rightward shifts elicited by each concentration of the ROK inhibitors used ($n = 4$, each). The contractions induced by CCh were shown to be more sensitive to ROK inhibitors ($p < 0.01$) than those evoked by NE in both preparations.

3.5. Effect of ROK inhibitors on electrically-evoked contractions

Contractions of anococcygeus and retractor penis muscle strips induced by EFS at supramaximal voltage were

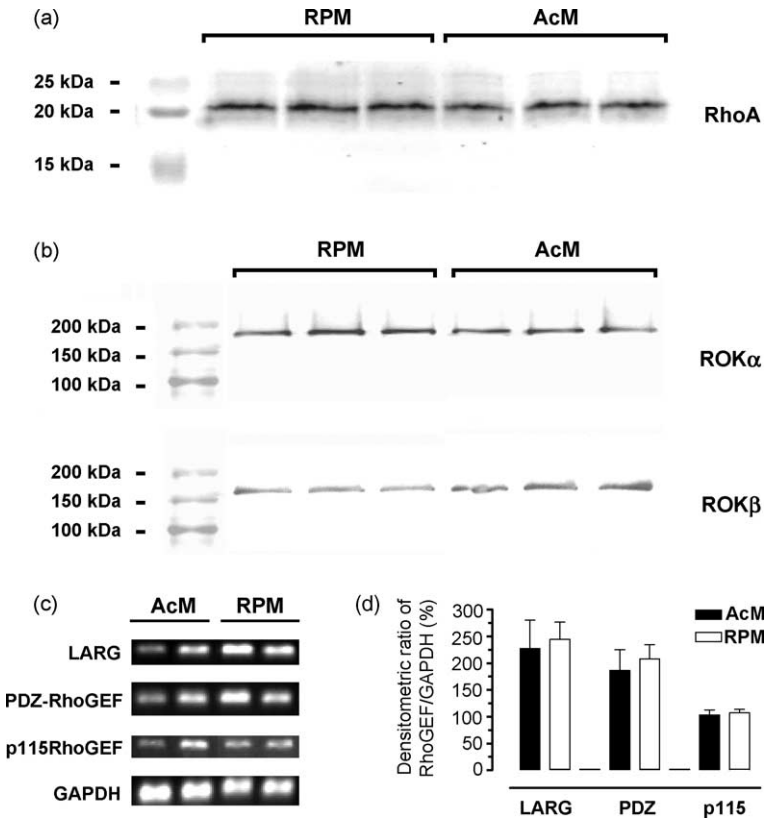

Fig. 1. Western blot analysis of RhoA (a), ROKα/ROKβ (b) and mRNA analysis (c and d) of p115RhoGEF, PDZ-RhoGEF and LARG in rat anococcygeus (AcM) and retractor penis muscles (RPM). Marks indicate the approximate molecular weight. Lanes represent RhoA, ROKα and ROKβ protein expression in isolated muscle strips from three different animals. Tissue extracts were subjected to SDS–PAGE followed by Western blotting with specific antibodies, as described in Section 2. The blots were detected with an advance chemiluminescence detection kit. Total RNA was isolated from crude homogenates of smooth muscle and expression of RGS-containing RhoGEFs mRNA was analyzed by semi-quantitative RT-PCR. (c) The electrophoretic visualization of the amplicons represents four sets of separate experiments; (d) GAPDH-normalized quantification of the PCR products.

frequency-dependent and consisted of rapidly developing and transient contractile responses. The mean amplitude values of anococcygeus contractions evoked by 1, 2, 4, 8, 16 and 32 Hz were 2 ± 1, 7 ± 1, 42 ± 2, 68 ± 2, 86 ± 2 and $96 \pm 2\%$, respectively ($n = 30$). In the retractor penis, the values of contraction for 1, 2, 4, 8, 16 and 32 Hz were 7 ± 1, 41 ± 1, 66 ± 2, 81 ± 1, 94 ± 1 and $103 \pm 1\%$, respectively ($n = 30$). The sodium channel blocker tetrodotoxin (1 μM; $n = 4$, each) and phentolamine (1 μM; $n = 4$, each) virtually abolished the contractions at all frequencies of stimulation in both tissues. Furthermore, the addition of bretylium tosylate (30 μM; $n = 4$, each) to deplete adrenergic fibers markedly reduced the EFS-induced contractions, whereas atropine (0.1 μM; $n = 4$, each) did not significantly affected these responses.

Addition of H-1152 (0.1, 0.3 and 1 μM; $n = 5$) to the bathing medium significantly inhibited EFS-evoked contractions of anococcygeus and retractor penis muscle in a concentration-dependent manner at all frequencies of stimulation (Figs. 5 and 6). Similar results were obtained with Y-27632 (1, 3 and 10 μM) and HA-1077 (1, 3 and 10 μM; Fig. 6).

4. Discussion

The main findings of the present study demonstrate that (1) RhoA, ROKα and ROKβ are expressed in the rat anococcygeus and retractor penis muscle; (2) RhoGEFs are expressed at the mRNA level in these tissues; and (3) the RhoA/ROK signaling pathway plays an important role in the maintenance of the contractile activity in rat anococcygeus and retractor penis muscle, supporting the original hypothesis.

Small GTPases of the Rho family are pivotal regulators of several aspects of cell behaviour [14]. In the smooth muscle, activation of RhoA leads to activation of ROK, which is a serine/threonine protein kinase that exists in two isoforms: ROKα (also called ROCK2) [32] and ROKβ (also known as p160ROCK or ROCK1) [33]. It is well established that one of the main substrates of ROK is MLC phosphatase, which is inactivated through ROK-mediated phosphorylation of its myosin binding subunit, causing an increase in MLC phosphorylation and smooth muscle contraction [4,13,14]. In the present investigation, expression of RhoA, ROKα and ROKβ was detected in rat anococcygeus and retractor penis muscles by Western

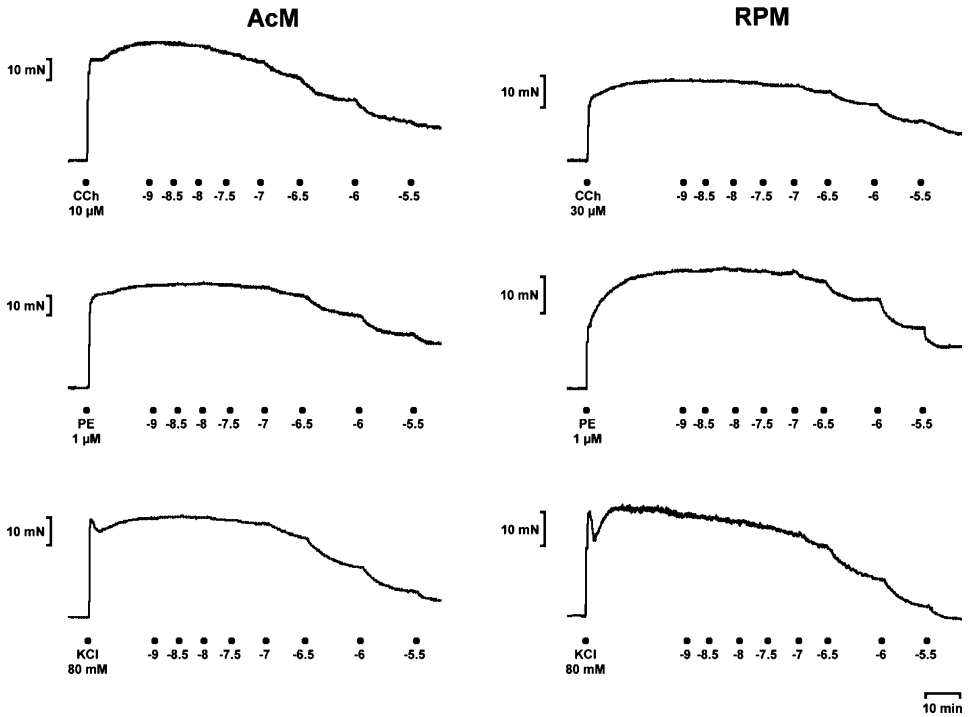

Fig. 2. Representative tracings showing the inhibitory effects of the ROK inhibitor H-1152 (0.001–3 μM) on the contractions induced by carbachol (CCh, 10–30 μM), phenylephrine (PE, 1 μM) or KCl (80 mM) in rat anococcygeus (AcM; left traces) and retractor penis muscle (RPM; right traces). Addition of H-1152 to the bathing medium caused sustained relaxations in a concentration-dependent manner. These are experimental tracings of five experiments.

blotting, thus providing support of a role for the RhoA/ROK signaling pathway in the contractile mechanism of these tissues.

In spite of the functional link between GPCRs and RhoA, the molecular mechanism by which heterotrimeric G proteins stimulate RhoA has just begun to be elucidated. In general, the functional activity of the Rho protein is tightly modulated by regulatory proteins, which include the RhoGEFs [4,13,14,16]. Available evidence indicates that a recently described family of GEFs, namely p115RhoGEF [34], PDZ-RhoGEF [35], and LARG (leukemia-associated RhoGEF) [36], contains an area of lim-

Table 1
Maximal response values of the ROK inhibitors (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine (H-1152, 0.001–3 μM), (*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632, 0.01–30 μM) and (5-isoquinolinesulfonyl)homopiperazine (HA-1077, 0.01–30 μM) on the established contractions induced by KCl (80 mM; $n = 12–13$), phenylephrine (PE, 1 μM; $n = 12–14$) and carbachol (10–30 μM; $n = 14$) in rat anococcygeus and retractor penis muscle strips

	Maximal response (%)[a]					
	Anococcygeus			Retractor penis		
	KCl	PE	CCh	KCl	PE	CCh
H-1152	93 ± 2	67 ± 2	80 ± 6	100 ± 4	56 ± 4	74 ± 6
Y-27632	100 ± 2	60 ± 7	71 ± 4	101 ± 3	60 ± 5	82 ± 5
HA-1077	89 ± 2*	55 ± 6	93 ± 3**	109 ± 2	60 ± 2	78 ± 5

[a] Experimental values of maximal response were calculated as the percentage of relaxation with respect to the level of contraction. Data represent the mean ± S.E.M. of *n* experiments.
* $p < 0.05$.
** $p < 0.01$ compared to Y-27632.

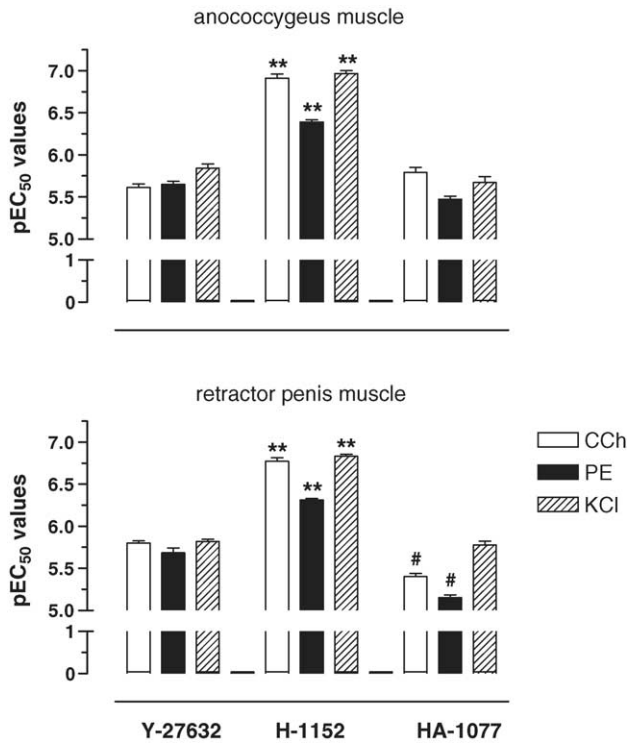

Fig. 3. Potency (pEC_{50}) values derived from concentration–response curves to the ROK inhibitors H-1152 (0.001–3 μM), Y-27632 (0.01–30 μM) and HA-1077 (0.01–30 μM) in rat anococcygeus (top panel) and retractor penis muscle (bottom panel) strips contracted with carbachol (CCh, 10–30 μM; $n = 14$, white bars), phenylephrine (PE, 1 μM; $n = 12–14$, black bars) or KCl (80 mM; $n = 12–13$, hatched bars). Data represent the mean ± S.E.M. of *n* experiments. ** $p < 0.01$ compared to Y-27632 and HA-1077 values; # $p < 0.01$ compared to Y-27632 values.

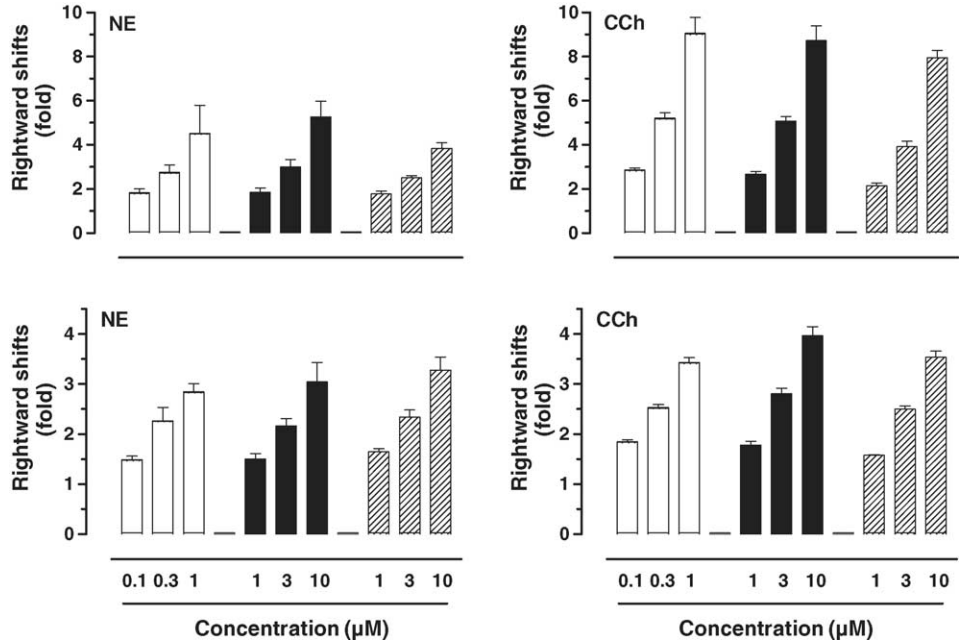

Fig. 4. Rightward shifts of the curves elicited by norepinephrine (NE, 0.001–100 μM) and carbachol (CCh, 0.01–100 μM) in the presence of increasing concentrations of H-1152 (0.1–1 μM; white bars), Y-27632 (1–10 μM; black bars) or HA-1077 (1–10 μM; hatched bars) in rat anococcygeus (top panels) and retractor penis muscle (bottom panels) preparations. Data are expressed as fold displacement to the right and represent the mean ± S.E.M. of four experiments.

ited similarity to a conserved region of regulator of G protein signaling (RGS) in the N-terminal region, which can transduce signals from GPCR to RhoA [15–18]. The findings of the present study demonstrated the mRNA expression of all three RGS domain-containing RhoGEFs in the rat anococcygeus and retractor penis, although their precise role in regulating smooth muscle contraction remains unclear.

This work used selective kinase inhibitors to identify the degree of participation of ROK-mediated Ca^{2+} sensitization in M3 muscarinic- and α-adrenoceptor-stimulated contraction of isolated strips of rat anococcygeus and

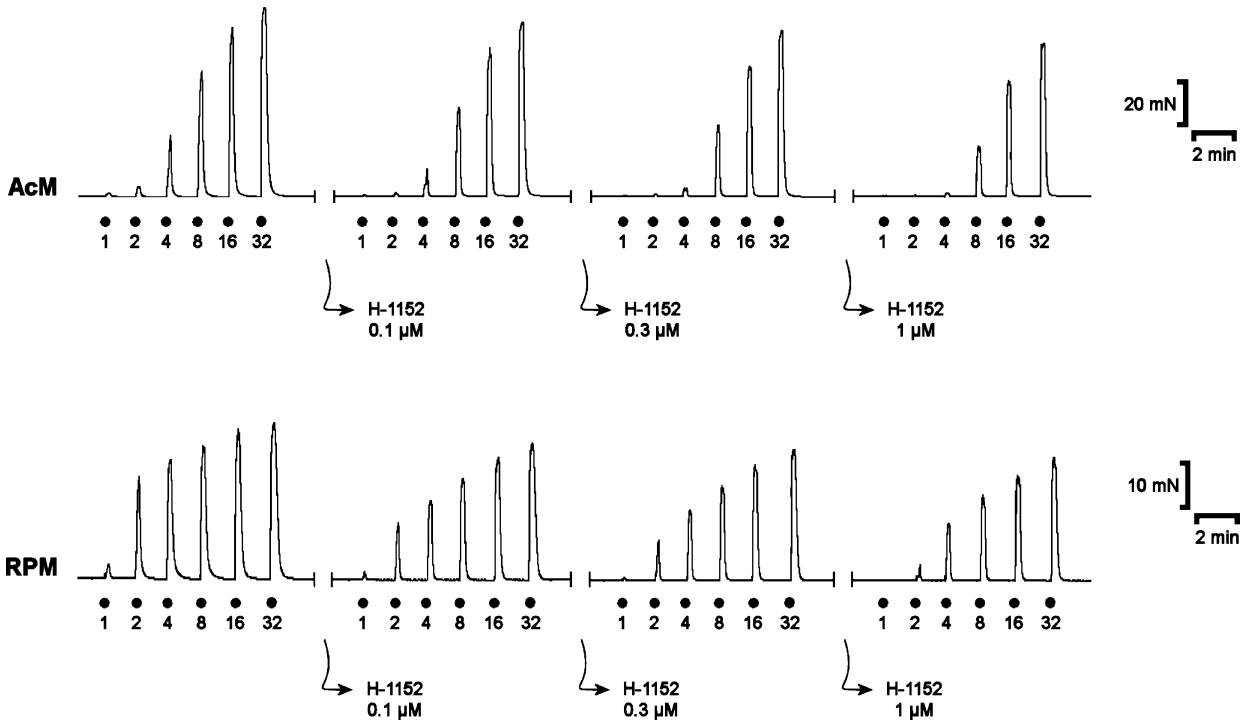

Fig. 5. Representative tracings showing the inhibitory effects of the ROK inhibitor H-1152 (0.1–1 μM) on electrically-induced contractions (1–32 Hz, 50 V; 10-s trains) of rat anococcygeus (AcM; top) and retractor penis muscle (RPM; bottom). Muscle strips were treated with each concentration of the inhibitor for 30 min prior to electrical field stimulation (see text for details). These are experimental tracings of five experiments.

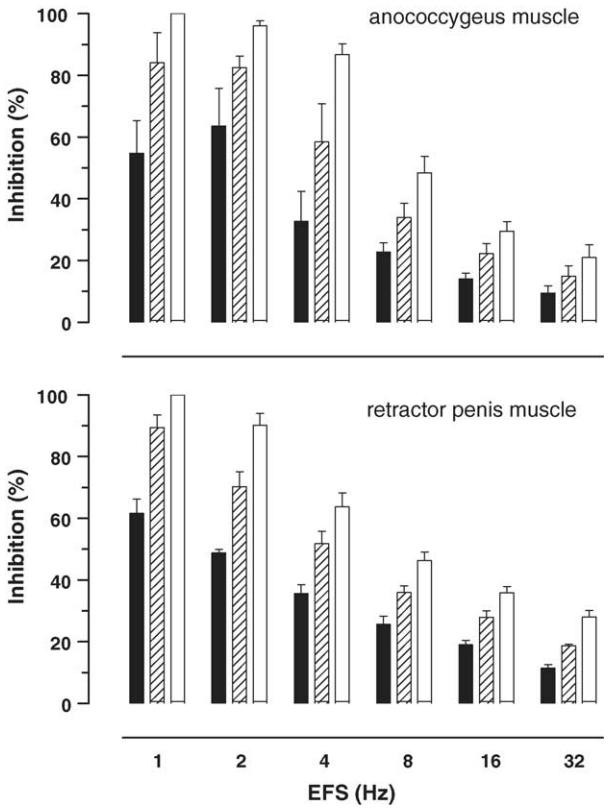

Fig. 6. Effects of the ROK inhibitor H-1152 (0.1–1 μM; $n = 5$) on the contractions induced by electrical field stimulation (EFS, 1–32 Hz) in rat anococcygeus (top panel) and retractor penis muscle (bottom panel) preparations. Experimental values of inhibition of contraction were obtained in presence of 0.1 μM (black bars), 0.3 μM (hatched bars) and 1 μM (white bars) of H-1152. Data were calculated relative to the contraction produced by each frequency of stimulation in control conditions (i.e., absence of inhibitor). Data represent the mean ± S.E.M. of n experiments.

retractor penis muscle. We examined the functional effects of the ROK inhibitors H-1152, Y-27632 and HA-1077, by applying either compound to strips precontracted with the muscarinic agonist CCh and the α_1-agonist PE. The contractions induced by these agents were inhibited by all three ROK inhibitors in a concentration-dependent manner, indicating that the RhoA/ROK pathway is involved in both muscarinic and adrenergic contraction of anococcygeus and retractor penis. However, we found that tonic force produced by either agonist was more sensitive to H-1152 than Y-27632 or HA-1077, since the former compound was approximately 8–20 times more potent than the later ones, as evidenced by their respective pEC_{50} values. The present data support the hypothesis that ROK participated in the contractile response in these tissues and indicates that H-1152 is a more effective inhibitor of ROK when compared to Y-27632 or HA-1077, which corroborates previous observations showing potency and selectivity of H-1152 to inhibit ROK activity [23]. Indeed, at 1 μM H-1152 produced a significantly greater shift in the curves to NE and CCh when compared to either Y-27632 or HA-1077.

Nevertheless, receptors for CCh and PE seem to be associated with RhoA/ROK signaling to a different degree. Although both sets of receptors are coupled to heterotrimeric $G_{\alpha q/11}$ proteins [37,38], the ROK inhibitors elicited relaxations to a different extent in both anococcygeus and retractor penis, in that when tone was elevated by PE, all ROK inhibitors produced a significantly less pronounced relaxation. This might suggest that excitatory M3 muscarinic receptors are coupled to the RhoA/ROK pathway in a different manner than are α_1-adrenoceptors. It is likely that PE causes excessive activation of ROK and thus the inhibitors induce a smaller relaxation. On the other hand, it is possible that the greater relaxation induced by ROK inhibitors in CCh-induced contraction reflects a greater contribution of ROK to the constrictor response to M3 stimulation. However, it remains unknown whether adrenergic agonists utilize this signaling pathway more than does muscarinic agonists.

H-1152, Y-27632 and HA-1077 were similarly effective at inhibiting tone raised by CCh and KCl. In the context of smooth muscle contraction, it is well established that membrane depolarization opens voltage-gated Ca^{2+} channels allowing Ca^{2+} entry to the cytosol. The increase in intracellular Ca^{2+} results in binding of Ca^{2+} to calmodulin and subsequent activation of MLC kinase, which catalyzes phosphorylation of myosin, triggering cross-bridge cycling and contraction [1–3]. In this context, CCh, but not KCl-induced depolarization, would be expected to initiate Ca^{2+} sensitization. Raising external K^+ may cause the release of excitatory neurotransmitters from intrinsic nerves within the rat anococcygeus and retractor penis muscle preparations, such that the contraction of the muscle will include a receptor-mediated component produced by these neurotransmitters. However, this is unlikely since the experiments described herein were performed on tissues pretreated with bretylium tosylate, which abolishes noradrenergic contractile responses seen on EFS. In agreement with our results, recent reports demonstrated that the tonic component of K^+-induced contraction involves activation of ROK with resultant inhibition of MLCP in rat tail artery [39]. In addition, Y-27632 has been shown to inhibit non-receptor-mediated contractions in mouse anococcygeus muscle as well as to block Ca^{2+} entry in rat arteries [30,40]. At this point, it is unclear how contractions mediated by receptor-independent mechanisms activate the RhoA/ROK signaling pathway in smooth muscle.

Noradrenergic neurotransmission plays an important role in the regulation of anococcygeus and retractor penis muscle tone. EFS-evoked contractions were fully blocked by tetrodotoxin and phentolamine, but not atropine, at all frequencies of stimulation, indicating that these responses are neural in origin and adrenergic in nature. Transient (phasic) contractile responses elicited by EFS were more resistant to antagonism by either ROK inhibitor than the tonic (sustained) contraction by NE. This is in accordance with previous reports in the guinea-pig ileum, human

trachea and corpus cavernosum in which Y-27632 has been found to be effective against the tonic but not the transient phase of contraction [6,10,12]. Our results further support a role for the RhoA/ROK pathway in regulating the smooth muscle tone in conditions where a tonic contraction or a high basal tone are involved.

In conclusion, the results of the present study demonstrate that RhoA, ROKα and ROKβ are expressed in the rat anococcygeus and retractor penis muscle and that this signaling pathway plays a pivotal role in the regulation of smooth muscle contraction in these tissues. In addition, this work demonstrated the mRNA expression of upstream regulators of RhoA activity, through the identification of RGS domain-containing RhoGEFs. Hence, inhibition of the RhoA/ROK signaling may represent a valuable strategy to counteract an elevated smooth muscle contraction. Also, the manipulation of the expression level of each RhoGEF might provide further understanding of their function in the mechanisms of Ca^{2+} sensitization.

Acknowledgments

This study was supported by grants from the National Institutes of Health (HL-71138, HL-74167). Cleber E. Teixeira is funded by a postdoctoral fellowship (No. 0425437B) from the American Heart Association (Southeast Affiliate). Liming Jin is supported by a fellowship from the American Foundation for Urologic Disease/ American Urological Association.

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