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Comparative pharmacological analysis of Rho-kinase inhibitors and identification of molecular components of Ca^{2+} sensitization in the rat lower urinary tract

Cleber E. Teixeira [a,b,*], *Liming Jin* [b], *Fernanda B.M. Priviero* [b], *Zhekang Ying* [b], *R. Clinton Webb* [b]

[a] *Department of Pharmacology, Faculty of Medical Sciences, UNICAMP, Campinas 13081-970, SP, Brazil*
[b] *Department of Physiology, Medical College of Georgia, Augusta, GA 30912, USA*

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ABSTRACT

We aimed to compare the expression and function of molecular components of the RhoA/Rho-kinase signaling pathway in the contractile responses of detrusor, trigonal and urethral smooth muscle, using selective Rho-kinase inhibitors. Contractility studies and molecular approaches were employed to demonstrate the expression patterns and functional activity of the RhoA/Rho-kinase signaling pathway in the lower urinary tract. Frequency–response curves (1–32 Hz) and concentration–response curves (CRC) to carbachol (CCh, 0.01–30 μM), phenylephrine (PE, 0.01–300 μM) and endothelin-1 (ET-1, 0.01–100 nM) were significantly attenuated ($p < 0.01$) following incubation with the Rho-kinase inhibitors H-1152 (0.1–1 μM), Y-27632 (1–10 μM) or HA-1077 (10 μM). Addition of Rho-kinase inhibitors also markedly reduced ($p < 0.01$) the contractions evoked by either KCl (80 mM) or α,β-methylene ATP (α,β-mATP, 10 μM). Among the Rho-kinase inhibitors tested, H-1152 was approximately 9–16 times more potent than Y-27632 or HA-1077. In addition, basal tone of detrusor and trigonal strips was reduced following addition of Y-27632 (10 μM), H-1152 (1 μM) and HA-1077 (10 μM). The expression of RhoA, RhoGDI, leukemia-associated RhoGEF (LARG) and p115RhoGEF was similar among the detrusor, trigone and urethra, whereas Rho-kinase α, Rho-kinase β and PDZ-RhoGEF protein levels were significantly lower in the urethra. Components of the RhoA/Rho-kinase signaling are expressed in detrusor, trigonal and urethral smooth muscle and dynamically regulate contraction and tone. Manipulation of RhoGEF expression may provide further understanding of mechanisms involving Ca^{2+} sensitization in the lower urinary tract.

* *Corresponding author at*: Department of Pharmacology, Faculty of Medical Sciences, UNICAMP, Campinas 13081-970, SP, Brazil. Tel.: +55 19 35219555; fax: +55 19 32892968.
E-mail address: cet@fcm.unicamp.br (C.E. Teixeira).

Abbreviations: CCh, carbachol; EFS, electrical field stimulation; ET-1, endothelin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEFs, guanine nucleotide exchange factors; HA-1077, (5-isoquinolinesulfonyl)homopiperazine; H-1152, (*S*)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine; LARG, leukemia-associated RhoGEF; α,β-mATP, α,β-methylene ATP; MLC, myosin light chain; 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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1. Introduction

The passive phase of bladder filling accounts for the ability of the bladder to increase in volume at low intravesical pressure to prevent reverse pressure exposure to the upper urinary tract, while the bladder neck and urethra remain in a tonic state to prevent leakage, thus maintaining urinary continence. Bladder emptying is accompanied by a reversal of function in which smooth muscle contraction predominates in the bladder body with a concomitant reduction in outlet resistance of the bladder neck and urethra [1–3]. These regulatory actions are mediated through the interaction of norepinephrine (sympathetic component released by hypogastric nerve stimulation), acetylcholine and ATP (parasympathetic components released by pelvic nerve stimulation) with adrenergic, muscarinic and purinergic receptors, respectively [2]. Stimulation of M_2 and M_3 muscarinic receptors produce detrusor smooth muscle contraction [4–7], whereas sympathetic activation of post-junctional α_1-adrenoceptors in the urethra and bladder trigone plays a key role in the maintenance of urethral closure pressure [4,8–10]. In addition, β_2- and β_3-adrenoceptors mediate relaxation of rat and human detrusor, causing bladder activity inhibition [11–13]. The overactive bladder syndrome is a widespread condition with a significant burden on health resources [14]. This disorder might be associated with involuntary contractions of the detrusor that occur during bladder filling and which are unrelated to the volume of urine in the bladder. These contractions may lead to urgency as well as involuntary loss of urine.

Accumulating evidence reveals the physiological importance of the Ca^{2+}-independent RhoA/Rho-kinase pathway in the regulation of smooth muscle tone by alteration of the sensitivity of contractile proteins for Ca^{2+} [15]. RhoA regulates smooth muscle contraction by cycling between a GDP-bound inactive form (coupled to a guanine dissociation inhibitor, RhoGDI) and a GTP-bound active form [16–18]. Upstream activation of heterotrimeric G proteins lead to the exchange of GDP for GTP, an event carried out by the guanine exchange factors (GEFs) p115RhoGEF [19], PDZ-RhoGEF [20] and LARG (Leukemia-associated RhoGEF) [21], which are able to transduce signals from G protein-coupled receptors to RhoA [22–24]. Rho-kinase is activated by RhoA and inhibits myosin phosphatase through the phosphorylation of its myosin binding subunit, leading to an increase in Ca^{2+} sensitivity. The RhoA/Rho-kinase Ca^{2+} sensitization pathway is involved in the regulation of bladder smooth muscle contraction and tone in humans [25,26] and animals [27–29]. Inhibition of Rho-kinase attenuates bladder hyperactivity in rabbits with partial outlet obstruction [30], diabetic rabbits [31] and hypertensive rats [32].

The report described herein was undertaken to provide further understanding on the contribution of the Rho-kinase-mediated Ca^{2+} sensitization in the contractile properties of detrusor, trigonal and urethral smooth muscles by studying the effects of Rho-kinase inhibitors on the contractions mediated by G protein-coupled and G protein-independent mechanisms. In addition, comparative expression studies were performed to identify molecular components of this pathway in the lower urinary tract, namely RhoA, Rho-kinase, Rho-guanine dissociation inhibitor (RhoGDI) and Rho-guanine nucleotide exchange factors (RhoGEFs).

2. Materials and methods

2.1. Tissue preparation

All experiments were conducted in accordance with institutional guidelines and approved by the local committee on animal experiments. Male Sprague–Dawley rats (weighing 250–300 g) were obtained from Harlan Laboratories (Indianapolis, USA). The animals were anaesthetized with pentobarbital sodium (40 mg/kg, i.p.), killed by decapitation and exsanguinated. The urinary bladder and the urethra were quickly removed and placed 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; $MgSO_4{\cdot}7H_2O$, 1.17; $CaCl_2{\cdot}2H_2O$, 1.6. After the preparations were pinned in a Sylgard Petry dish filled with ice-cold Krebs solution, visible connective tissue and fat were carefully removed by sharp dissection under a stereomicroscope. Four longitudinal full thickness strips (10 mm × 1.5 mm) were excised from the detrusor proximal to the orifices of the two ureters. The urethra was separated from the bladder at the level of the bladder neck and two rings of approximately 2–3 mm in length were prepared. One circular strip (4 mm × 1 mm) of smooth muscle was isolated from the bladder trigone.

2.2. Recording of mechanical activity

For functional studies, detrusor and trigonal strips were horizontally attached between two mounting clamps in 4-ml myograph chambers (Danish Myograph Technology, Aarhus, Denmark) containing Krebs solution at 37 °C continuously aerated with 5% CO_2 in O_2. Urethral rings were transferred to 5-ml chambers and set up between two metal hooks. The preparations were repeatedly stretched so that a stable force of about 20 mN, 3 mN and 2 mN was obtained during the equilibration period for detrusor, trigone and urethra, respectively. When subjected to electrical field stimulation (EFS), the tissues were mounted 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, USA). Square wave pulses (1 ms duration) were delivered at supramaximal voltage and the train duration was 10 s. Changes in isometric force were recorded using a PowerLab 8/SP™ data acquisition system (software Chart 5.0, ADInstruments, Colorado Springs, USA).

All preparations were equilibrated for 1 h and were subsequently challenged with 80 mM KCl (the same composition as Krebs solution with NaCl replaced by equimolar KCl) for 10 min to check for tissue viability. Frequency–response curves (EFS, 1–32 Hz) as well as cumulative concentration–response curves to carbachol (CCh, 0.01–30 μM), phenylephrine (PE, 0.01–300 μM) and endothelin-1 (ET-1, 0.01–100 nM) were obtained in the absence (treated with the appropriate vehicle) and 30 min after the addition of the Rho-kinase inhibitors H-1152 (0.1–1 μM), Y-27632 (1–10 μM) or HA-1077 (1–10 μM). The phasic component of the contractions evoked in the detrusor and trigone is represented by the peak of the responses whereas the tonic component relates to the steady-state contraction that follows.

In some protocols, an initial contractile response to α,β-methylene ATP (α,β-mATP, 10 μM; exposed for 5 min) was

obtained followed by a 60 min washout period, to avoid desensitization of the responses. Tissues were incubated with Rho-kinase inhibitors and re-challenged with α,β-mATP. To evaluate the effect of Rho-kinase inhibitors on the contraction evoked by KCl, atropine (1 μM), phentolamine (1 μM) and α,β-mATP (10 μM) were added to the bathing solution to prevent the effects of acetylcholine, norepinephrine and ATP released from nerve endings, respectively.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Detrusor, trigone and urethra were isolated, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Total RNA (4 μg per reaction) extracted from tissue segments with TRIzol reagent (Invitrogen, Carlsbad, USA) 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, Tokyo, Japan), connected to an IBM AT computer (New York, USA) with a 512 × 512 pixel array imaging board with 256 gray levels. Sequences of forward and reverse primers for PDZ-RhoGEF, LARG, p115RhoGEF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were previously described [33]. The PCR products were quantified by densitometric scanning of gel images using UN-SCAN-IT software (Silk Scientific Corp., USA). Results were then expressed as the densitometric ratio of gene of interest/GAPDH (house-keeping gene).

2.4. Western blot analysis

Rat detrusor, trigone and urethra were removed as described above, snap-frozen in liquid nitrogen and kept at −80 °C until use. The tissue segments 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 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, Rockford, USA). An aliquot of 40 μg protein from each sample was loaded per lane and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins were subsequently transferred onto nitrocellulose membranes (BioRad, Hercules, USA). Membranes were blocked by treatment with 5% milk in Tris-buffered saline containing 0.05% Tween 20, probed with antibodies against RhoA (1:200), Rho-kinase α (1:500), Rho-kinase β (1:200), RhoGDI (1:2000), p115RhoGEF (1:200), PDZ-RhoGEF (1:500) or LARG (1:200) and incubated with a horseradish peroxidase-conjugated second antibody. Immunoreactivity was detected by enhanced chemiluminescence autoradiography. The bands were quantified by densitometric scanning of film images using UN-SCAN-IT software (Silk Scientific Corp., USA). Results were then expressed as the densitometric ratio of protein of interest/β-actin (1:2000).

2.5. Drugs and chemicals

Atropine, carbachol, endothelin-1, α,β-methylene ATP, nifedipine, phentolamine, phenylephrine and tetrodotoxin were purchased from Sigma Chemical Co. (St. Louis, USA). 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 (San Diego, USA). The antibodies used to probe for RhoA, Rho-kinase α and Rho-kinase β were obtained from BD Biosciences (San Diego, CA, USA). The antibodies used to probe for RhoGDI and RhoGEFs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents used were of analytical grade. All drugs were dissolved in deionized water.

2.6. Data analysis

Experimental values were calculated relative to the maximal changes from the contraction produced by KCl (80 mM) in each tissue, which was taken as 100%. The pEC_{50} values were determined as −log of the molar concentration to produce 50% of the maximal response. Data are shown as the percentage of contraction of *n* experiments from different individuals, expressed as the mean ± S.E.M. Analysis of variance (ANOVA) and Student's paired *t*-test were employed to evaluate the results. A *p* value less than 0.05 was considered to indicate significance. A program package was used for the statistical analysis of all data (GraphPAD Software, version 3.00, San Diego, USA).

3. Results

3.1. Agonist concentration–response curves

In all preparations examined, detrusor, trigonal and urethral smooth muscles exhibited spontaneous phasic contractions, with peak amplitudes of 7.2 ± 1.4 mN, 1.5 ± 0.4 mN and 0.4 ± 0.2 mN, respectively. These spontaneous contractions were abolished by nifedipine (0.1 μM; $n = 4$, each), indicating a critical role of Ca^{2+} entry through L-type Ca^{2+} channels in the initiation of phasic contractions.

Increasing concentrations of the muscarinic receptor agonist CCh (0.01–30 μM) evoked contractile responses in detrusor preparations ($n = 24$). Application of CCh caused force development to increase initially to a transient peak (phasic response) and then decline to reach a plateau level (tonic response). This general pattern was superimposed by spontaneous force oscillations of varying intensity. The maximal phasic and tonic contractile responses of the isolated detrusor muscle strips corresponded to 135 ± 3% (at 10 μM) and 87 ± 2% (at 30 μM) of KCl (80 mM)-induced contractions, respectively. The concentration–response curves to CCh obtained from the phasic component of the contractions yielded pEC_{50} values of 5.97 ± 0.08, which were not statistically different when compared to the values obtained from the tonic component

of the responses. CCh caused no significant contractions when applied to trigonal (5 ± 3% maximum response; $n = 6$) or urethral (2 ± 2% maximum response; $n = 5$) preparations.

The α_1-adrenergic agonist PE (0.01–100 μM) acted as a partial agonist in the trigone, eliciting maximal phasic and tonic contractions of 61 ± 2% and 40 ± 1%, respectively ($n = 10$). The pEC_{50} values derived from these curves were 5.30 ± 0.03 (phasic) and 5.25 ± 0.03 (tonic). In urethral preparations, the contractions to PE (0.01–300 μM) were characterized by sustained responses with a pEC_{50} value and maximum response of 5.18 ± 0.03 and 123 ± 3%, respectively. Cumulative addition of ET-1 (0.01–100 nM) also caused sustained contractions of urethral strips with a pEC_{50} value of 8.17 ± 0.03 and maximum response of 98 ± 1%. On the other hand, PE failed to induced significant contractions in the detrusor smooth muscle (6 ± 2% at the highest agonist concentration; $n = 6$).

3.2. *Effects of Rho-kinase inhibitors on basal tone and agonist-evoked contractions*

The contribution of Ca^{2+} influx through voltage-operated Ca^{2+} channels as well as of the Rho-kinase signaling pathway to the baseline bladder tension was assessed using nifedipine and the Rho-kinase inhibitors H-1152, Y-27632 and HA-1077, respectively, during a incubation period of 30 min. Nifedipine (0.1 μM; $n = 6$) was able to decrease the resting tone of detrusor strips by 28 ± 5%. Similarly, H-1152 (1 μM), Y-27632 (10 μM) and HA-1077 (10 μM) reduced the baseline bladder tension by 51 ± 9%, 38 ± 9% and 29 ± 6%, respectively ($n = 6$). In the trigone, baseline tension was reduced by 47 ± 7%, 43 ± 8% and 34 ± 6% in the presence of the above mentioned concentrations of H-1152, Y-27632 and HA-1077, respectively ($n = 4$). These compounds virtually abolished spontaneous phasic contractions. On the other hand, Rho-kinase inhibitors failed to significantly decrease baseline tension in the urethra ($n = 4$).

In order to investigate the role of the RhoA/Rho-kinase signaling pathway in the contractions of detrusor, trigonal and urethral preparations, increasing concentrations of the Rho-kinase inhibitors H-1152 (0.1 μM, 0.3 μM and 1 μM), Y-27632 (1 μM, 3 μM and 10 μM) and HA-1077 (1 μM, 3 μM and 10 μM) were used. Fig. 1 shows that each Rho-kinase inhibitor caused significant rightward shifts in the concentration–response curves to CCh in detrusor smooth muscle ($n = 5$–6).

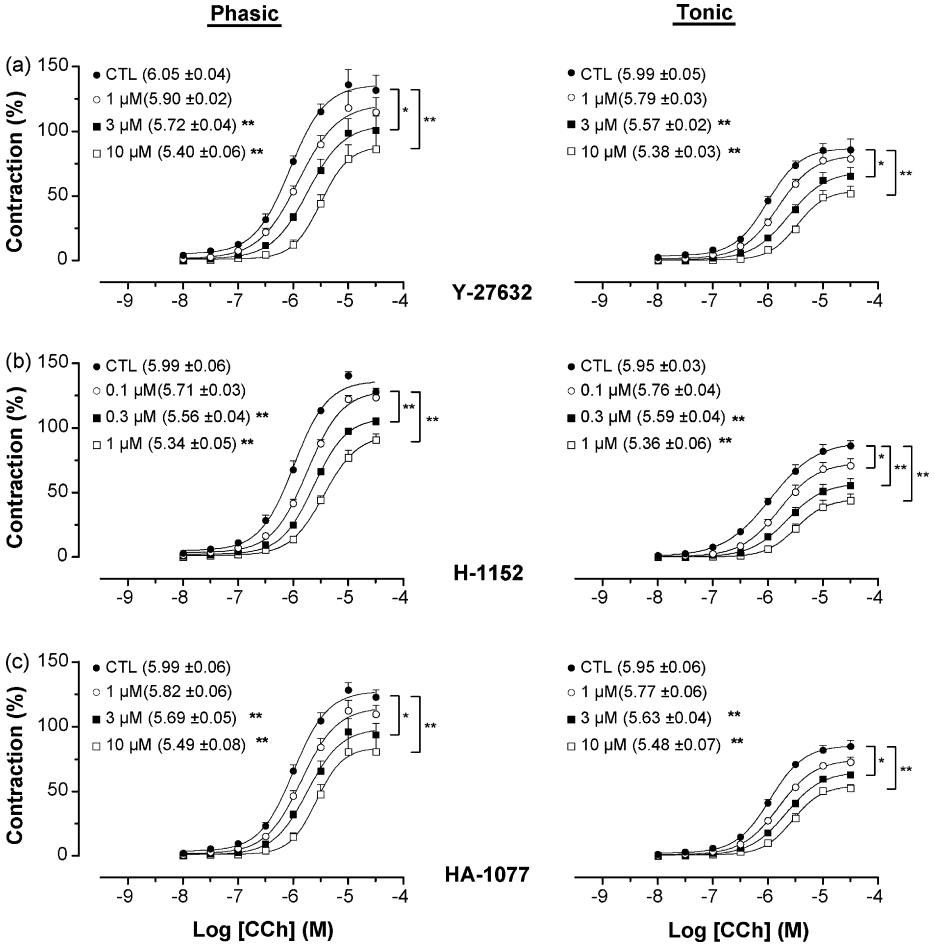

Fig. 1 – Concentration–response curves to carbachol (CCh, 0.01–30 μM) in rat detrusor smooth muscle in control conditions (CTL) and treated with the Rho-kinase inhibitors Y-27632 (1–10 μM; $n = 5$; panel a), H-1152 (0.1–1 μM; $n = 6$; panel b) and HA-1077 (1–10 μM; $n = 5$; panel c). The effects of Rho-kinase inhibitors were evaluated in the phasic (left panels) and tonic (right panels) components of the CCh-induced contractions. Experimental values were calculated relative to the maximal changes from the contraction produced by KCl (80 mM) in each tissue, which was taken as 100%. The corresponding pEC_{50} values are represented in the panel. Data represent the mean ± S.E.M. of n experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to CTL.

In addition to affecting the potency of CCh, H-1152, Y-27632 and HA-1077 significantly reduced maximal contractions to this agonist.

Similar experimental protocols were carried out to evaluate the contribution of Rho-kinase in the contractile mechanism elicited by PE in trigone and urethra. The concentration-response curves evoked by PE were shifted to the right by H-1152, Y-27632 or HA-1077 in both trigonal (Fig. 2; $n = 4$) and urethral (Fig. 3; $n = 4$) smooth muscle preparations, in which the former tissue was less sensitive to Rho-kinase inhibition than the latter ($p < 0.01$). Also, the maximal responses elicited by PE were significantly reduced in trigonal and urethral tissues following treatment with the Rho-kinase inhibitors. Interestingly, the urethral contractions mediated by ET-1 revealed no alteration in pEC_{50} values after incubation with increasing concentrations of H-1152 ($n = 4$). However, maximal responses to ET-1 were reduced by $14 \pm 3\%$, $31 \pm 3\%$ and $52 \pm 1\%$ in the presence of 0.1 μM, 0.3 μM and 1 μM of H-1152.

3.3. *Effects of Rho-kinase inhibitors on contractions mediated by α,β-mATP and EFS*

Addition of α,β-mATP (10 μM) induced phasic contractions in rat detrusor, trigonal and urethral preparations, averaging $36 \pm 1\%$ ($n = 36$), $27 \pm 1\%$ ($n = 18$) and $22 \pm 1\%$ ($n = 18$), respectively. Re-challenge of the tissues with α,β-mATP following incubation with vehicle for 60 min to prevent tissue desensitization, evoked a similar contractile response ($32 \pm 4\%$, $30 \pm 3\%$ and $24 \pm 3\%$ for detrusor, trigone and urethra, respectively). In the presence of nifedipine (0.1 μM; $n = 4$), α,β-mATP-induced contractions were fully blocked. After recording the first set of responses to 10 μM α,β-mATP, the tissues were incubated with either H-1152 (1 μM), Y-27632 (10 μM) or HA-1077 (10 μM) for at least 30 min before a second addition of α,β-mATP ($n = 6$, each). Among all three inhibitors, H-1152 was the most effective ($p < 0.05$) in antagonizing the contractile effect elicited by the purinergic agonist ($45 \pm 7\%$, $42 \pm 5\%$ and $52 \pm 2\%$ inhibition in detrusor, trigone and urethra,

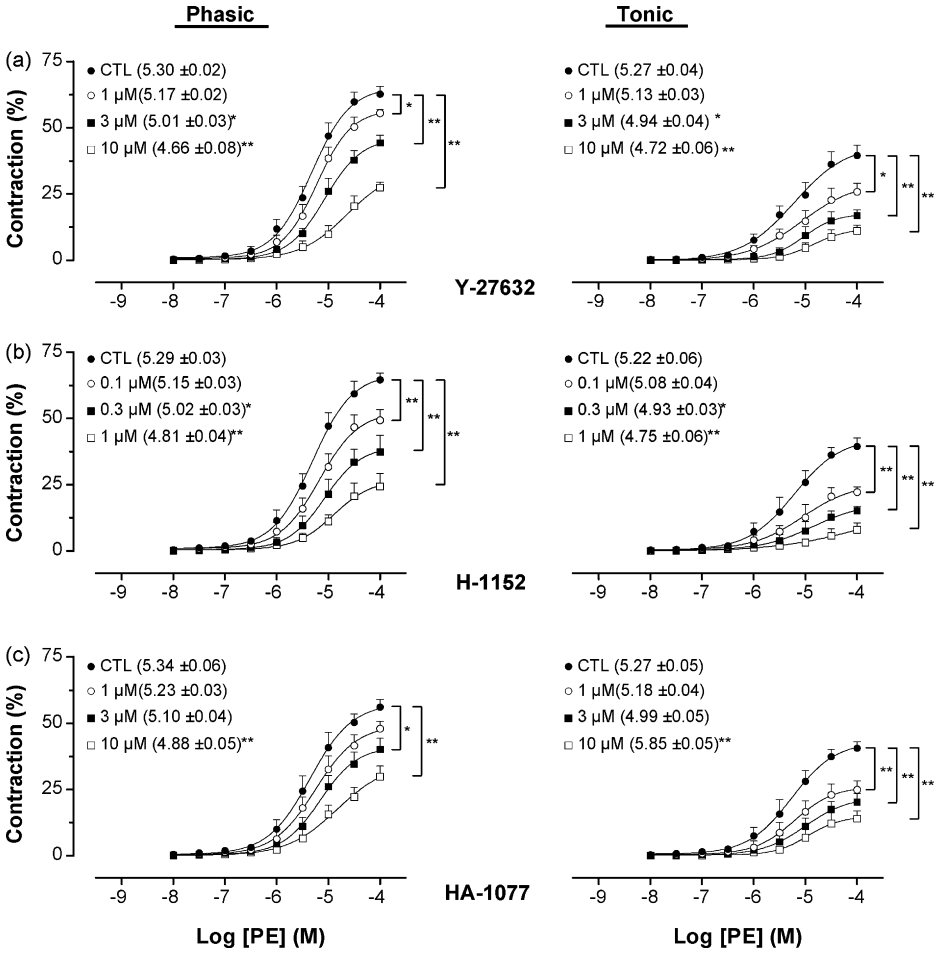

Fig. 2 – Concentration–response curves to phenylephrine (PE, 0.01–100 μM) in rat trigonal smooth muscle in control conditions (CTL) and treated with the Rho-kinase inhibitors Y-27632 (1–10 μM; $n = 4$; panel a), H-1152 (0.1–1 μM; $n = 4$; panel b) and HA-1077 (1–10 μM; $n = 4$; panel c). The effects of Rho-kinase inhibitors were evaluated in the phasic (left panels) and tonic (right panels) components of the PE-induced contractions. Experimental values were calculated relative to the maximal changes from the contraction produced by KCl (80 mM) in each tissue, which was taken as 100%. The corresponding pEC_{50} values are represented in the panel. Data represent the mean ± S.E.M. of n experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to CTL.

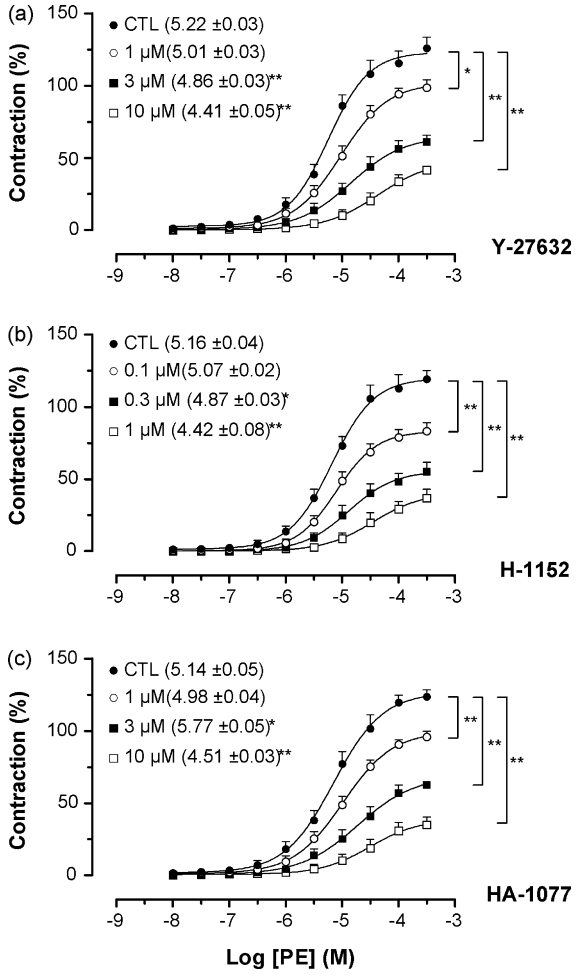

Fig. 3 – Concentration–response curves to phenylephrine (PE, 0.01–300 μM) in rat urethral smooth muscle in control conditions (CTL) and treated with the Rho-kinase inhibitors Y-27632 (1–10 μM; $n = 4$; panel a), H-1152 (0.1–1 μM; $n = 4$; panel b) and HA-1077 (1–10 μM; $n = 4$; panel c). Experimental values were calculated relative to the maximal changes from the contraction produced by KCl (80 mM) in each tissue, which was taken as 100%. The corresponding pEC_{50} values are represented in the panel. Data represent the mean ± S.E.M. of n experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to CTL.

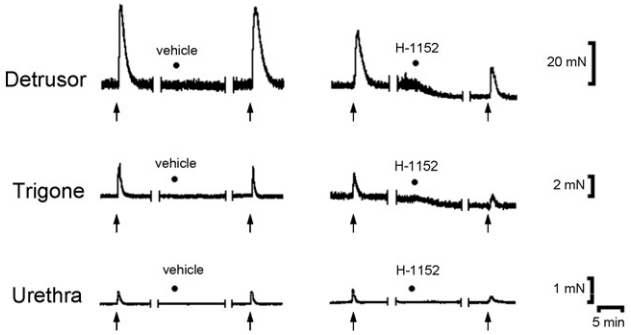

Fig. 4 – Isometric force recordings of contractions evoked by α,β-methylene ATP (α,β-mATP, 10 μM) in the absence (vehicle) and presence of the Rho-kinase inhibitor H-1152 (1 μM) in detrusor, trigonal and urethral smooth muscle. A 60 min washout period was allowed after the first application of α,β-mATP in order to prevent desensitization. Upward arrows indicate α,β-mATP addition to the bathing medium. These are representative tracings of four to six experiments.

respectively) compared to Y-27632 (27 ± 5%, 33 ± 2% and 37 ± 5% inhibition in detrusor, trigone and urethra, respectively) and HA-1077 (23 ± 7%, 31 ± 3% and 34 ± 4% inhibition in detrusor, trigone and urethra, respectively). Fig. 4 shows a representative tracing of the inhibitory effect of H-1152 on α,β-mATP-induced contractions.

The rat detrusor smooth muscle strips developed increasing tension responses with the increase in the frequency of stimulation from 1 to 32 Hz, 10-s trains. These contractions were characterized by a rapid development and consisted of transient responses. The mean maximum peak contraction of 137 ± 4% was developed at 32 Hz, while 13 ± 1%, 31 ± 2%, 62 ± 3%, 99 ± 4% and 124 ± 4% was developed at 1 Hz, 2 Hz, 4 Hz, 8 Hz and 16 Hz, respectively ($n = 14$). Muscle contractions induced by EFS were virtually blocked by the Na^+ channel blocker tetrodotoxin (1 μM; $n = 4$), indicating that these responses are neuronal in origin, and also significantly inhibited by atropine (1 μM; $n = 5$) or purinergic receptor desensitization with α,β-mATP (10 μM; $n = 5$), indicating that these responses are cholinergic/purinergic in nature (not shown). The Rho-kinase inhibitors H-1152 (0.1–1 μM; $n = 6$), Y-27632 (1–10 μM; $n = 5$) and HA-1077 (1–10 μM; $n = 5$) significantly reduced electrically induced contractions in a concentration-dependent manner. Fig. 5 shows an experimental tracing of the inhibitory effects of H-1152 on EFS-induced contractions. The percentage of inhibition of these contractile responses is summarized in Table 1. The inhibition caused by Y-27632 and HA-1077 was similar to that obtained with H-1152.

3.4. *Effects of Rho-kinase inhibitors on contractions induced by KCl*

In the presence of the muscarinic antagonist atropine (1 μM) and α,β-mATP (10 μM) to prevent the effects of acetylcholine and ATP released from nerves, the mean peak (phasic)

Table 1 – Percentage of inhibition of contractile responses induced by electrical field stimulation (EFS, 1–32 Hz) in rat detrusor strips following addition of the Rho-kinase inhibitor H-1152 (0.1–1 μM; $n = 6$)

EFS (Hz)	% Inhibition		
	H-1152 0.1 μM	H-1152 0.3 μM	H-1152 1 μM
1	20 ± 3	40 ± 9	73 ± 5
2	15 ± 4	39 ± 10	60 ± 4
4	15 ± 2	35 ± 3	54 ± 7
8	10 ± 2	21 ± 2	38 ± 3
16	8 ± 2	17 ± 2	30 ± 2
32	8 ± 2	13 ± 2	23 ± 2

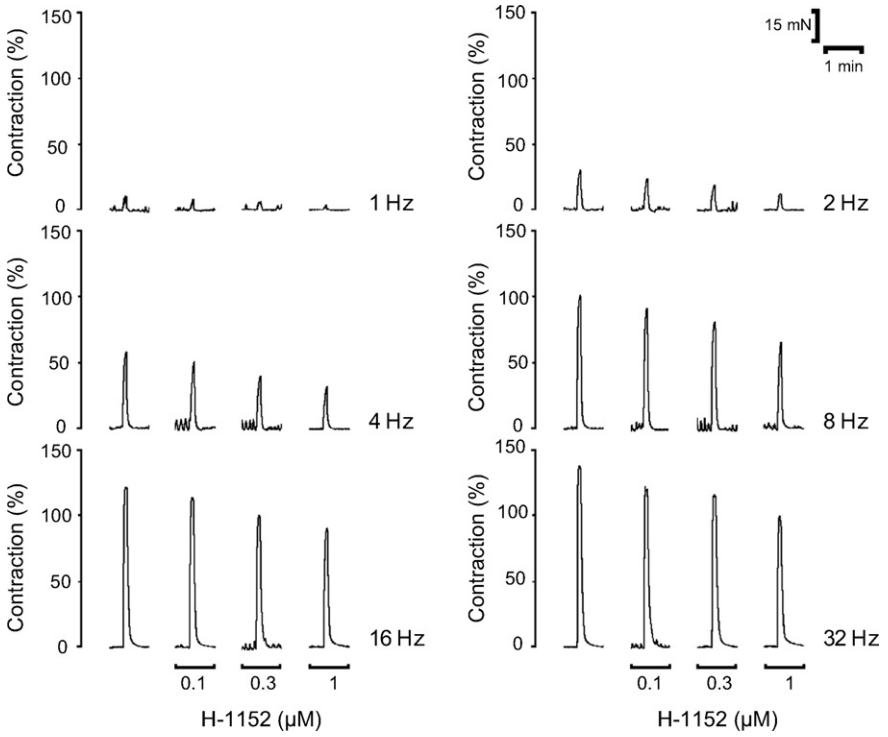

Fig. 5 – Representative tracings showing the inhibitory effects of the Rho-kinase inhibitor H-1152 (0.1–1 μM) on electrically induced contractions (1–32 Hz, 50 V; 10-s trains) of rat detrusor smooth muscle. The strips were treated with each concentration of H-1152 for 30 min prior to electrical field stimulation (see text for details). Vertical bars indicate the percentage of contraction induced by KCl (80 mM). These are experimental tracings of six experiments.

contraction during the exposure to 80 mM KCl was 58.3 ± 8.1 mN ($n = 72$), 4.3 ± 0.8 mN ($n = 40$) and 4.8 ± 1.0 mN ($n = 44$) in detrusor, trigone and urethra, respectively, whereas the plateau (tonic) component of this response achieved 36 ± 3% (detrusor), 81 ± 2% (trigone) and 51 ± 3% (urethra) of the peak. In Ca^{2+}-free medium containing 1 mM EGTA, KCl-induced contractions were completely abolished ($n = 4$). In detrusor and urethral preparations, the addition of H-1152 (1 μM; $n = 4–6$), Y-27632 (10 μM; $n = 5–6$) and HA-1077 (10 μM; $n = 5–6$) caused marked inhibitions ($p < 0.01$) in both components of the KCl-induced contractions, which were significantly greater on the tonic contractile response (Fig. 6). Nevertheless, the degree of inhibition of trigonal smooth muscle contractions evoked by KCl were only marginally affected by each Rho-kinase inhibitor, without significant differences between tonic and phasic components of the response (Fig. 6; $n = 4–6$).

3.5. *Expression of RhoA, RhoGDI, ROKα, ROKβ and RhoGEFs*

Fig. 7a demonstrates the protein expression of RhoA and RhoGDI in detrusor, trigonal and urethral smooth muscle. The antibodies against RhoA and RhoGDI recognized single proteins with an approximate molecular mass of 21 kDa and 27 kDa, respectively, with no significant difference in their expression level among the tissues. Similarly, antibodies against Rho-kinase α and Rho-kinase β revealed the expression of these kinases with approximate molecular weights of 180 kDa and 160 kDa, respectively, as expected (Fig. 7b). Their expression was significantly lower ($p < 0.01$) in the urethra when compared to the levels observed in the trigone and detrusor. A RT-PCR assay was used to identify the mRNA expression of the RGS-containing RhoGEFs in the lower urinary tract. Fig. 8 shows the electrophoretic visualization of the mRNA and protein expression for PDZ-RhoGEF, p115RhoGEF and LARG in detrusor, trigone and urethra. We observed a decreased expression of PDZ-RhoGEF both at the mRNA and protein levels in the urethra. Moreover, PDZ-RhoGEF expression was significantly higher in the trigone, as demonstrated by β-actin-normalized quantification, although such difference was not apparent in the mRNA level.

4. Discussion

The present study was primarily designed to compare the expression levels as well as the functional importance of the RhoA/Rho-kinase Ca^{2+} sensitization pathway in different tissues of the lower urinary tract, namely the bladder body, trigone and urethra. The main findings indicate that the molecular components of this Ca^{2+} sensitization pathway are expressed in the rat lower urinary tract, playing an important role in the maintenance of the contractile activity in detrusor, trigonal and urethral smooth muscle. Furthermore, we have provided the pharmacological basis that points to H-1152 as a more potent Rho-kinase inhibitor compared to the widespread used Y-27632 and HA-1077.

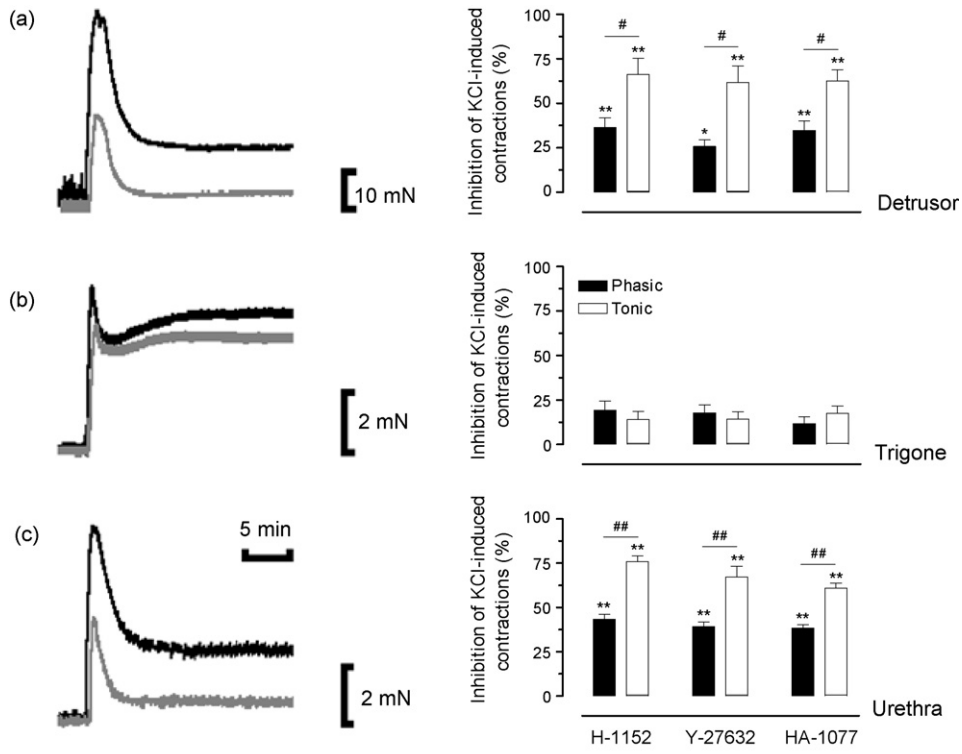

Fig. 6 – Effects of the Rho-kinase inhibitors H-1152, Y-27632 and HA-1077 on KCl (80 mM)-induced contractions of rat detrusor (a), trigonal (b) and urethral (c) preparations. After KCl-evoked contractions were achieved, tissues were relaxed and incubated for 30 min with each Rho-kinase inhibitor prior to a second KCl-induced depolarization. Typical traces (left) show the contractile effect of KCl before (black traces) and after (gray traces) pretreatment with H-1152 in rat detrusor, trigone and urethra. Panels (right) show the inhibitory effects of H-1152 (1 μM), Y-27632 (10 μM) and HA-1077 (10 μM) on the phasic (closed bars) and tonic (open bars) components of the contractile response elicited by KCl in detrusor, trigonal and urethral smooth muscle. The force of the phasic and tonic components was measured as the peak of contraction and 20 min after KCl addition, respectively. Results are calculated as percentage of inhibition of tonic and phasic components of the contractile response and expressed as the mean ± S.E.M. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to pretreatment levels; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the degree of inhibition of phasic contractions.

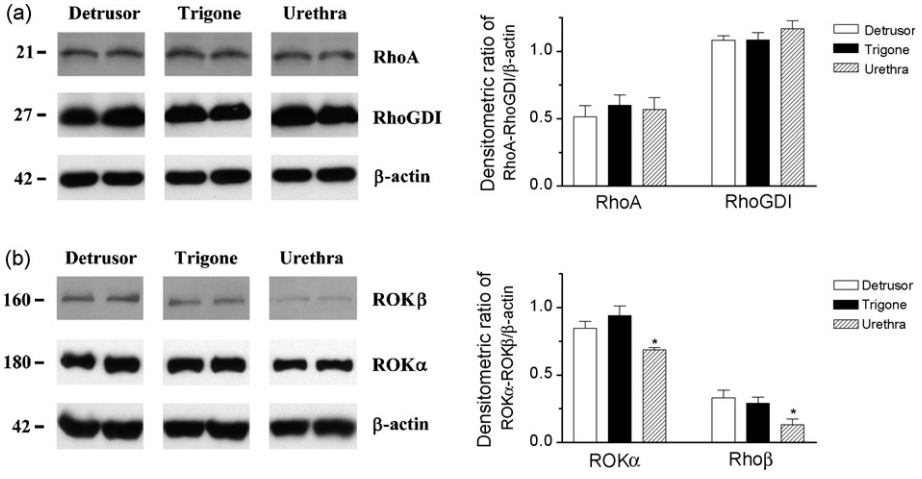

Fig. 7 – Representative Western blot analysis of RhoA (a), RhoGDI (a) and Rho-kinase isoforms (ROKα and ROKβ; b) in detrusor, trigonal and urethral smooth muscle ($n = 6$). Tissue homogenates were subjected to SDS-PAGE and immunoblotted with specific antibodies. Summarized data of densitometrical analyses are represented at the right. Each set of determinations was performed in triplicate.

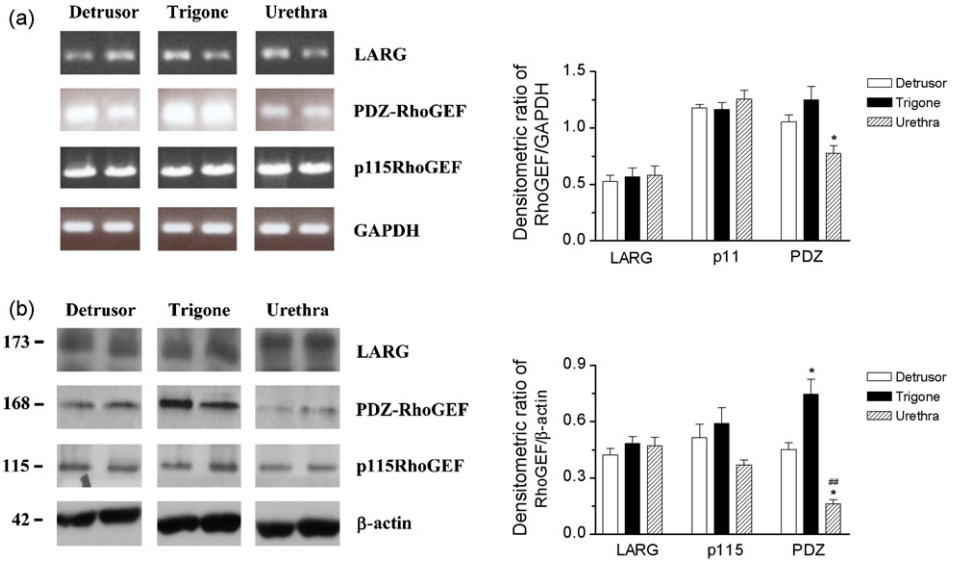

Fig. 8 – Representative mRNA analysis of p115RhoGEF, PDZ-RhoGEF and LARG in rat detrusor, trigone and urethra (a). Total RNA was isolated from crude tissue homogenates and expression of RGS-containing RhoGEFs mRNA was analyzed by semi-quantitative RT-PCR. The electrophoretic visualization of the amplicons represents five sets of separate experiments. Western blot analysis of LARG, p115RhoGEF and PDZ-RhoGEF ($n = 6$; b). Tissue homogenates were subjected to SDS-PAGE and immunoblotted with specific antibodies. The blots were detected with an Advance Chemiluminescence Detection Kit. Summarized data of densitometrical analyses are represented at the right. Each set of determinations were performed in triplicate.

In human, rat, rabbit and guinea pig bladder, the Rho-kinase pathway represents an important regulator of smooth muscle activity and tone [25,27–32]. The prototype Rho-kinase inhibitor Y-27632 has been shown to attenuate contractions of bladder smooth muscle evoked by CCh, through the decrease of Ca^{2+} sensitivity of the contractile apparatus [27–29]. In this work, we confirmed and extended these findings by investigating the effects of three different Rho-kinase inhibitors, namely H-1152, Y-27632 and HA-1077, on the phasic and tonic components of the contractions in response to G protein coupled-receptor agonists, membrane depolarization and nerve stimulation. Each Rho-kinase inhibitor tested caused significant inhibition of both phasic and tonic components of contractions in response to agonists in detrusor, urethra and trigone, indicating that activation of G protein-coupled receptors leads to stimulation of Rho-kinase to cause the force development evoked by distinct agonists in the lower urinary tract. These findings corroborate the above-mentioned studies which demonstrate a role for the RhoA/Rho-kinase pathway in Ca^{2+} sensitization, using both intact and permeabilized strips of detrusor smooth muscle. In addition, we found that H-1152 is approximately 9–16 times more potent than Y-27632 and HA-1077, which is in agreement with its higher potency and selectivity to inhibit Rho-kinase in *in vitro* assays [34] as well as in intact smooth muscle [35].

Detrusor smooth muscle strips taken from the bladder of all species develop spontaneous phasic contractions, although this activity does not contribute to a rise in the intravesical pressure and has been shown to occur locally, without spreading throughout the tissue under physiological conditions [36]. These spontaneous contractions have been recently shown to be suppressed by cyclic nucleotides and Rho-kinase inhibition [28]. Interestingly, Y-27632 suppressed spontaneous contractions without inhibiting either action potentials or associated Ca^{2+} transients, indicating that Rho-kinase in detrusor smooth muscle may be activated in unstimulated conditions. Likewise, all three Rho-kinase inhibitors assayed virtually abolished the spontaneous contractions and decreased the bladder tension in the present investigation, in agreement with previous studies [27,36]. Taken together, these findings point to the occurrence of an intrinsic Rho-kinase-dependent Ca^{2+} sensitization mechanism in detrusor smooth muscle.

Many factors, such as peripheral nerve control and the contribution of other components of the urinary tract, may influence micturition. Both cholinergic (acetylcholine) and purinergic (ATP) components influence different phases of the micturition response, with ATP being largely responsible for the initiation of voiding and cholinergic transmission responsible for the maintenance of voiding [38]. The Rho-kinase inhibitors were found to reduce significantly the contractile effect resulting from nerve depolarization in a concentration-dependent manner, demonstrating the involvement of the Ca^{2+} sensitization mechanism in the nerve-derived endogenous regulation of detrusor smooth muscle tone. We also tested the effects of H-1152, Y-27632 and HA-1077 in the contractions of detrusor, trigone and urethra evoked by α,β-mATP. Stimulation of $P2X_1$ receptors by α,β-mATP mediates the depolarization of the membrane causing Ca^{2+} influx through both voltage-gated Ca^{2+} channels as well as directly through the receptor [39]. Interestingly, the α,β-mATP-induced contractions were blocked by approximately 50% by either Rho-kinase inhibitor regardless of the preparation.

In a recent report supporting a role for a Ca^{2+} sensitization mechanism in urinary bladder, contractile responses evoked by KCl were not affected by Y-27632 [27]. In the context of bladder smooth muscle contraction, it is well established that membrane depolarization opens voltage-gated Ca^{2+} channels allowing Ca^{2+} entry to the cytosol [40]. Nevertheless, H-1152, Y-27632 and HA-1077 were effective at inhibiting contractions of detrusor and urethra evoked by KCl in the presence of receptor blockade to exclude nerve-derived neurotransmitter effects, suggesting that Rho-kinase may be involved in both Ca^{2+}-dependent and independent regulation of contraction. In agreement with our results, recent reports demonstrated that KCl-induced contraction involves activation of Rho-kinase in rat-tail artery [41] and canine tracheal smooth muscle [42]. Y-27632 has also been shown to inhibit non-receptor-mediated contractions in mouse anococcygeus muscle [43] and to block Ca^{2+} entry in rat arteries [44]. Interestingly, KCl-induced contractions of trigone were not significantly affected by either Rho-kinase inhibitor, providing evidence that the Rho-kinase-mediated Ca^{2+} sensitization mechanism is not particularly involved in KCl depolarization-coupled contractile responses of this tissue. It is worth mentioning that raised extracellular KCl as well as $P2X_1$ receptor stimulation depolarize the cell membrane, thus leading to the opening of L-type Ca^{2+} channels. Although Rho-kinase inhibitors affect enzyme activity by competing with ATP for binding at the kinase catalytic site, we speculate that the $P2X_1$ receptor stimulation includes a component of activated Rho-kinase activity.

We identified the expression of RhoA, its endogenous inhibitor Rho-guanine dissociation inhibitor (RhoGDI), as well as the individual Rho-kinase isoforms at the protein level, thus providing support for a RhoA/Rho-kinase pathway in detrusor, trigonal and urethral tissues. This is in agreement with previous work wherein identification of these proteins has also been demonstrated [25,27,30,31,37,45]. In addition, recent reports demonstrate that ROKβ is partly responsible for the high degree of force maintenance in the detrusor smooth muscle in overactive bladders [30–32]. Our experiments revealed that both ROKα and ROKβ are expressed at lower levels in the urethra compared to the detrusor and trigone. Although Rho-kinase has been recently shown to be obligatory for generating urethral tone [46], the Rho-kinase inhibitors used in this study failed to reduce baseline tension in rat urethral smooth muscle, as opposed to their effects on detrusor and trigone. It is noteworthy that responses in the same tissue from different species involve different mechanisms as it is the case of CCh-induced contractions of rat and detrusor smooth muscle [29]. It is possible that our findings are a reflex of a lower Rho-kinase protein expression in the urethra, or else, a constitutive drive of signaling pathways downstream of receptor activation is absent in this tissue. Indeed, low levels of RhoA/ROK may unleash myosin phosphatase, causing rapid dephosphorylation of phosphorylated myosin light chain, preventing the development of any basal tone, and causing rapid fade of stimulated contractions [47]. This observation suggests that the use of Rho-kinase inhibitors in the therapeutic management of overactive bladder could have a more prominent effect on the bladder wall rather than the urethra.

Post-translational modification (geranylgeranylation) enables the necessary membrane binding and subsequent activation of RhoA. Activation also requires a guanine nucleotide exchange factor (GEF), which promotes the exchange of GTP for GDP. Leukemia-associated RhoGEF (LARG) and its homologs PDZ-RhoGEF and p115RhoGEF make up a subgroup of RhoGEFs, known as regulators of G protein signaling (RGS) domain-containing RhoGEFs. To our knowledge, the present report represents the first demonstration of the expression of these RhoGEFs in the lower urinary tract at both gene and protein levels, although another GEF, namely p63RhoGEF, is expressed and binds to RhoA in J82 human bladder carcinoma cells [48]. Because the activation of RhoA comes from an increased activity of RhoGEF or decreased RhoGDI, the lower expression level of PDZ-RhoGEF may explain the lack of effect of Rho-kinase inhibitors to reduce baseline tension in the urethra, given the centrality of RhoGEFs for RhoA activation, notwithstanding that RhoA levels were not different among detrusor, trigonal and urethral strips. The demonstration of a defect at the level of one of the determinant factors for the activation of RhoA (the reduced PDZ-RhoGEF content), makes unlikely a contemporary abnormality for RhoGDI, thus explaining why its level does not differ among the tissues of the urinary system studied. Similarly, downregulation of p115RhoGEF in patients with Bartter's and Gitelman's syndrome was suggested to contribute to reduced vascular tone regulation [49].

In conclusion, given the involvement of the RhoA/Rho-kinase pathway in the maintenance of contractility in bladder and urethra, the data provided by this study could provide further insight on the role of this Ca^{2+} sensitization signaling into the pathophysiology of the lower urinary tract. However, it is important to compare the relative importance of human detrusor and any potential animal models if Ca^{2+} sensitization is to become a target for drug development. A detailed understanding of the mechanisms underlying the regulation of RGS-containing RhoGEFs as well as the pharmacological inhibition of Rho-kinase, should provide insights into the identification of new therapies directed toward alleviating symptoms of lower urinary tract disorders, such as overactive bladder and urge urinary incontinence.

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REFERENCES

[1] Andersson KE, Hedlund P. Pharmacologic perspective on the physiology of the lower urinary tract. Urology 2002;60:13–20.
[2] Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiol Rev 2004;84:935–86.
[3] Peters SLM, Schmidt M, Michel MC. Rho kinase: a target for treating urinary bladder dysfunction? Trends Pharmacol Sci 2006;27:492–7.

[4] Andersson KE. Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. Pharmacol Rev 1993;45:253–308.
[5] Harriss DR, Marsh KA, Birmingham AT, Hill SJ. Expression of muscarinic M3-receptors coupled to inositol phospholipids hydrolysis in human detrusor cultured smooth muscle cells. J Urol 1995;154:1241–5.
[6] Hegde SS, Eglen RM. Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder. Life Sci 1999;64:419–28.
[7] Yamanishi T, Chapple CR, Yasuda K, Chess-Williams R. The role of M_2 muscarinic receptor subtypes in mediating contraction of the pig bladder base after cyclic adenosine monophosphate elevation and/or selective M_3 inactivation. J Urol 2002;167:397–401.
[8] Testa R, Guarneri L, Ibba M, Strada G, Poggesi E, Taddei C, et al. Characterization of α_1-adrenoceptor subtypes in prostate and prostatic urethra of rat, rabbit, dog and man. Eur J Pharmacol 1993;249:307–15.
[9] Chess-Williams R, Aston N, Couldwell C. α_{1A}-Adrenoceptor subtype mediates contraction of the rat urethra. J Auton Pharmacol 1994;14:375–81.
[10] O'Neill AB, Buckner SA, Brune ME, Milicic I, Daza AV, Gauvin DM, et al. Pharmacological properties of A-204176, a novel and selective α_{1A} adrenergic agonist, in *in vitro* and *in vivo* models of urethral function. Life Sci 2001;70:181–97.
[11] Seguchi H, Nishimura J, Zhou Y, Niiro N, Kumazawa J, Kanaide H. Expression of β_3-adrenoceptors in rat detrusor smooth muscle. J Urol 1998;159:2197–201.
[12] Fujimura T, Tamura K, Tsutsumi T, Yamamoto T, Nakamura K, Koibuchi Y, et al. Expression and possible functional role of the β_3-adrenoceptor in human and rat detrusor muscle. J Urol 1999;161:680–5.
[13] Hudman D, Elliott RA, Whitaker P, Terry TR, Sandhu DP, Norman RI. Inhibition of the contractile responses of isolated human and rat bladders by clenbuterol. J Urol 2001;166:1969–73.
[14] Moreland RB, Brioni JD, Sullivan JP. Emerging pharmacologic approaches for the treatment of lower urinary tract disorders. J Pharmacol Exp Ther 2004;308:797–804.
[15] Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin. II. Modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev 2003;83:1325–58.
[16] Wettschureck N, Offermanns S. Rho/Rho-kinase mediated signaling in physiology and pathophysiology. J Mol Med 2002;80:629–38.
[17] Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 2003;4:446–56.
[18] Bhattacharya M, Babwah AV, Ferguson SSG. Small GTP-binding protein-coupled receptors. Biochem Soc Trans 2004;32:1040–4.
[19] Hart MJ, Sharma S, elMasry N, Qiu RG, McCabe P, Polakis P, et al. Identification of a novel guanine nucleotide exchange factor for the Rho GTPase. J Biol Chem 1996;271:25452–8.
[20] Fukuhara S, Murga C, Zohar M, Igishi T, Gutkind JS. A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J Biol Chem 1999;274:5868–79.
[21] Kourlas PJ, Strout MP, Becknell B, Veronese ML, Croce CM, Theil KS, et al. Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukaemia. Proc Natl Acad Sci USA 2000;97:2145–50.
[22] Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem 2000;69:795–827.
[23] Fukuhara S, Chikumi H, Gutkind JS. RGS-containing Rho-GEFs: the missing link between transforming G proteins and Rho? Oncogene 2001;20:1661–8.
[24] Schmidt A, Hall A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. Genes Dev 2002;16:1587–609.
[25] Takahashi R, Nishimura J, Hirano K, Seki N, Naito S, Kanaide H. Ca^{2+} sensitization in contraction of human bladder smooth muscle. J Urol 2004;172:748–52.
[26] Schneider T, Fetscher C, Krege S, Michel MC. Signal transduction underlying carbachol-induced contraction of human urinary bladder. J Pharmacol Exp Ther 2004;309:1148–53.
[27] Wibberley A, Chen Z, Hu E, Hieble JP, Westfall TD. Expression and functional role of Rho-kinase in rat urinary bladder smooth muscle. Br J Pharmacol 2003;138:757–66.
[28] Hashitani H, Brading AF, Suzuki H. Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder. Br J Pharmacol 2004;141:183–93.
[29] Durlu-Kandilci NT, Brading AF. Involvement of Rho kinase and protein kinase C in carbachol-induced calcium sensitization in beta-escin skinned rat and guinea-pig bladders. Br J Pharmacol 2006;148:376–84.
[30] Bing W, Chang S, Hypolite JA, DiSanto ME, Zderic SA, Rolf L, et al. Obstruction-induced changes in urinary bladder smooth muscle contractility: a role for Rho kinase. Am J Physiol Renal Physiol 2003;285:F990–7.
[31] Chang S, Hypolite JA, DiSanto ME, Changolkar A, Wein AJ, Chacko S. Increased basal phosphorylation of detrusor smooth muscle myosin in alloxan-induced diabetic rabbit is mediated by upregulation of Rho-kinase β and CPI-17. Am J Physiol Renal Physiol 2006;290:F650–6.
[32] Rajasekaran M, Wilkes N, Kuntz S, Albo ME. Rho-Kinase inhibition suppresses bladder hyperactivity in spontaneously hypertensive rats. Neurourol Urodyn 2005;24:295–300.
[33] Teixeira CE, Ying Z, Webb RC. Proerectile effects of the Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]homopiperazine (H-1152) in the rat penis. J Pharmacol Exp Ther 2005;315:155–62.
[34] Sasaki Y, Suzuki M, Hidaka H. The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. Pharmacol Ther 2002;93:225–32.
[35] Teixeira CE, Jin L, Ying Z, Palmer T, Webb RC. Ca^{2+} sensitization and the regulation of contractility in rat anococcygeus and retractor penis muscle. Biochem Pharmacol 2005;69:1483–92.
[36] Hashitani H, Bramich NJ, Hirst GD. Mechanisms of excitatory neuromuscular transmission in the guinea-pig urinary bladder. J Physiol 2000;524:565–79.
[37] Hong SK, Yang JH, Kim TB, Kim SW, Paick JS. Effects of ovariectomy and oestrogen replacement on the function and expression of Rho-kinase in rat bladder smooth muscle. BJU Int 2006;98:1114–7.
[38] Streng T, Talo A, Andersson KE. Transmitters contributing to the voiding contraction in female rats. BJU Int 2004;94:910–4.
[39] Schneider P, Hopp HH, Isenberg G. Ca^{2+} influx through ATP-gated channels increments $[Ca^{2+}]_i$ and inactivates I_{Ca} in myocytes from guinea-pig urinary bladder. J Physiol 1991;440:479–96.
[40] Visser AJ, Van Mastrigt R. The role of intracellular and extracellular calcium in mechanical and intracellular electrical activity of human urinary bladder smooth muscle. Urol Res 2000;28:260–8.

[41] Mita M, Yanagihara H, Hishinuma S, Saito M, Walsh MP. Membrane depolarization-induced contraction of rat caudal arterial smooth muscle involves Rho-associated kinase. Biochem J 2002;364:431–40.
[42] Gosens R, Schaafsma D, Meurs H, Zaagsma J, Nelemans SA. Role of Rho-kinase in maintaining airway smooth muscle contractile phenotype. Eur J Pharmacol 2004;483:71–8.
[43] Ayman S, Wallace P, Wayman CP, Gibson A, McFadzean I. Receptor-independent activation of Rho-kinase-mediated calcium sensitisation in smooth muscle. Br J Pharmacol 2003;139:1532–8.
[44] Ghisdal P, Vandenberg G, Morel N. Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries. J Physiol 2003;551:855–67.
[45] Lin AD, Levin RM, Kogan BA, Whitbeck C, Leggett RE, Kearns C, et al. Alteration of contractile and regulatory proteins in estrogen-induced hypertrophy of female rabbit bladder. Urology 2006;68:1139–43.
[46] Malmqvist U, Hedlund P, Sward K, Andersson KE. Female pig urethral tone is dependent on Rho guanosine triphosphatases and Rho-associated kinase. J Urol 2004;171:1955–8.
[47] Patel CA, Rattan S. Spontaneously tonic smooth muscle has characteristically higher levels of RhoA/ROK compared with the phasic smooth muscle. Am J Physiol Gastrointest Liver Physiol 2006;291:G830–7.
[48] Lutz S, Freichel-Blomquist A, Rümenapp U, Schmidt M, Jakobs KH, Wieland T. p63RhoGEF and GEFT are Rho-specific guanine nucleotide exchange factors encoded by the same gene. Naunyn-Schmiedeberg's Arch Pharmacol 2004;369:540–6.
[49] Pagnin E, Semplicini A, Sartori M, Pessina AC, Calo LA. Reduced mRNA and protein content of rho guanine nucleotide exchange factor (RhoGEF) in Bartter's and Gitelman's syndromes: relevance for the pathophysiology of hypertension. Am J Hypertens 2005;18:1200–5.