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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

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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.

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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