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

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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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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*