Proerectile Effects of the Rho-Kinase Inhibitor (S)-(+)2-Methyl-1-[(4-methyl-5-isooquinolinyl)sulfonyl]homopiperazine (H-1152) in the Rat Penis

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Received March 8, 2005; accepted June 14, 2005

ABSTRACT

The Rho-kinase pathway mediates Ca$^{2+}$ sensitization in the penile circulation, which maintains the penis in the flaccid state. We aimed to investigate the functional effect of a novel Rho-kinase inhibitor, H-1152 [(S)-(+)2-methyl-1-[(4-methyl-5-isooquinolinyl)sulfonyl]homopiperazine], both in vitro and in vivo as well as to demonstrate the expression of Rho guanine nucleotide exchange factors (RhoGEFs) in the rat corpus cavernosum (CC), by using a semiquantitative reverse transcription-polymerase chain reaction assay to measure their mRNA expression. Cumulative addition of H-1152 (0.001–3 μM) or Y-27632 (0.01–30 μM; (R)-(+)trans-N-(4-pyridyl)-4-(1-aminomethyl)-cylohexanecarboxamide) caused sustained relaxations of precontracted CC strips, which were not affected by inhibition of the nitric oxide signaling pathway. Addition of H-1152 (0.1 μM), Y-27632 (1 μM), or sodium nitroprusside (SNP; 0.1 μM) caused rightward shifts in the curves to phenylephrine (PE), but it had little effect on the contractions mediated by electrical field stimulation (EFS). It is noteworthy that when H-1152 or Y-27632 was combined with SNP, a marked synergistic inhibition was noted both on PE- and EFS-induced contractions. Intraperitoneal administration of H-1152 (100 nmol/kg) had a discrete effect on mean arterial pressure and significantly enhanced erectile responses evoked by stimulation of the cavernous nerve. The mRNA expression for PDZ-RhoGEF, p115RhoGEF, and leukemia-associated RhoGEF in cavernosal segments was visualized by electrophoresis on agarose gel. The results indicate that H-1152 is a powerful Rho-kinase inhibitor, giving rise to its therapeutic potential in the treatment of erectile dysfunction. The regulator of G-protein signaling-containing RhoGEFs may represent key components of the molecular mechanisms associated with the abnormal function of the cavernosal smooth muscle.

The contraction of the cavernosal smooth muscle has an important role in maintaining the penis in the flaccid state and is considered to be mediated by the release of several substances, such as noradrenaline, angiotensin II, prostanoids, and endothelin-1 (Andersson, 2001, 2003). The main contractile factor is thought to be noradrenaline released from sympathetic nerve endings, acting on postjunctional $\alpha_1$-adrenoceptors (Andersson, 2001, 2003). The mechanism of noradrenergic contraction is based on the activation of subcellular signaling systems that mobilize Ca$^{2+}$ from extracellular and intracellular stores, resulting in an increased intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]i) (Himpens et al., 1995). The elevation of [Ca$^{2+}$]i causes calmodulin-mediated activation of myosin light chain (MLC) kinase, MLC phosphorylation, and cross-bridge cycling, which results in contraction. On the other hand, a decrease in [Ca$^{2+}$]i, permits dephosphorylation of MLC by MLC phosphatase, thus, causing relaxation (Somlyo and Somlyo, 2003).

In addition, distinct regulatory mechanisms brought about by activation of heterotrimeric G protein-coupled receptors are known to elevate the degree of contractile force without further increases in [Ca$^{2+}$]i, a process termed Ca$^{2+}$ sensitization. One of these mechanisms involves RhoA, a small, monomeric G protein that activates Rho-kinase, which in turn phosphorylates the regulatory subunit of MLC phospha...
tase and leads to sensitization of myofilaments to Ca$^{2+}$ (Riento and Ridley, 2003; Somlyo and Somlyo, 2003). Rho-kinase is expressed in human, rabbit, and rat cavernosal smooth muscle (Mills et al., 2001; Rees et al., 2002; Wang et al., 2002). Using an in vivo rat model, we have demonstrated that a Rho-kinase inhibitor, Y-27632, caused a dramatic increase in intracavernosal pressure by a mechanism independent of nitric oxide (NO), with minimal effects on systemic blood pressure (Chitaley et al., 2001). In addition, this inhibitor has been shown to antagonize noradrenergic contractions in human and rabbit penile tissue (Rees et al., 2002; Wang et al., 2002; Takahashi et al., 2003). These findings collectively indicate that the RhoA/Rho-kinase pathway is present and active in the resting (contracted) state of the corpus cavernosum.

A pivotal role for NO in the relaxation of the corpus cavernosum and vasculature is widely accepted (Andersson and Wagner, 1995). The endothelium and nerve fibers innervating the cavernosal tissue may be the source of NO, which is released after stimulation of neuronal and endothelial NO synthase (eNOS), respectively. Chitaley et al. (2001) have reported that cavernosal relaxations induced by Y-27632 were not affected by treatment with inhibitors of either NO synthase or soluble guanylyl cyclase. In contrast, recent studies reported that stimulation of the eGMP-dependent protein kinase leads to phosphorylation of RhoA and its inhibition. Therefore, inhibition of RhoA and its downstream target Rho-kinase by NO would contribute to the vasodilator action of NO (Sauzeau et al., 2000; Sandu et al., 2001; Chitaley and Webb, 2002).

Because cavernosal smooth muscle is kept contracted by basal noradrenergic activity in the detumescent state, we were interested in investigating the in vitro and in vivo effects of the novel Rho-kinase inhibitor H-1152 (Sasaki et al., 2002) in the penile tissue. Moreover, multiple mechanisms were recently described to link the Rho-kinase pathway and NO-generating system; therefore, we decided to reexamine the role of endogenous NO in the modulation of the mechanisms underlying Ca$^{2+}$ sensitization in the corpus cavernosum. Furthermore, previous studies demonstrated that the regulator of G protein signaling (RGS) domain is responsible for the physical association with the Go subunit of GTPase and acts to regulate G protein-coupled receptor signaling (Ross and Wilkie, 2000; Sah et al., 2000; Schmidt and Hall, 2002). Therefore, to further explore the signal transduction pathway for the increases in Ca$^{2+}$ sensitivity, we investigated the mRNA expression of the RGS-containing Rho guanine nucleotide exchange factors (RhoGEFs) in the corpus cavernosum.

**Materials and Methods**

**Animals.** Adult male Sprague-Dawley rats (250–300 g; Harlan, Indianapolis, IN) were used in these studies. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education. The animals were housed two per cage on a 12-h light/dark cycle and fed a standard chow diet with water ad libitum.

**Drugs and Solutions.** Krebs’ solution of the following composition was used: 130 mM NaCl, 14.9 mM NaHCO$_3$, 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH$_2$PO$_4$, 1.17 mM MgSO$_4$·7H$_2$O, and 1.6 mM CaCl$_2$·2H$_2$O. Acetylcholine, bretlyium tosylate, N$^\bullet$-nitro-l-arginine methyl ester (l-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), phenylephrine, prazosin, sodium nitroprusside, and tetrodotoxin were purchased from Sigma Chemical Co. (St. Louis, MO). Pentobarbital sodium was obtained from the university pharmacy. H-1152 and Y-27632 were acquired from Calbiochem (San Diego, CA). TRizol and superscript II kit were purchased from Invitrogen (Carlsbad, CA). 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. Controls were prepared in methyl sulfoxide. Solutions were stored at −20°C and further diluted in deionized water just before use.

**Functional Studies in Cavernosal Strips.** The animals were anesthetized with pentobarbital sodium (40 mg/kg i.p.), killed by decapitation, and exsanguinated. The penis was excised, transferred into ice-cold Krebs’ solution, and surgically dissected to remove the tunica albuginea. One crural strip preparation (1 × 10 mm) was obtained from each corpus cavernosum. Cavernosal strips were mounted in 4-ml myograph chambers (Danish Myo Technology, Aarhus, Denmark) containing Krebs’ solution at 37°C continuously bubbled with a mixture of 95% O$_2$ and 5% CO$_2$. The tissues were stretched to a resting force of 2 mN and allowed to equilibrate for 60 min. Changes in isometric force were recorded using a PowerLab/8SP data acquisition system (Chart software, version 5.0; ADInstruments, Colorado Springs, CO). To verify the contractile ability of the preparations, a high 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) and Y-27632 (0.01–30 μM) were obtained in cavernosal strips contracted with phenylephrine (PE; 10 μM). 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 West Warwick, RI). EFS was conducted at 50 V, 1-ms pulse width and trains of stimuli lasting 10 s at varying frequencies (1–32 Hz).

**Measurement of Intracavernosal Pressure.** Rats were anesthetized with intramuscular ketamine (87 mg/kg) plus xylazine (13 mg/kg) and maintained on supplemental ketamine as needed. The right carotid artery was cannulated for continuous monitoring of mean arterial pressure (MAP), and a 30-gauge needle was inserted into the right corpus cavernosum to record intracavernosal pressure (ICP) simultaneously via Grass pressure transducers (Astro-Med). The abdominal cavity was opened, exposing the right cavernous nerve at the dorsolateral aspect of the prostate. Stainless steel bipolar electrodes connected to a Grass SD9 stimulator (Astro-Med) were positioned on the cavernosal nerve. Electrical stimulation of the cavernous nerve (5-ms pulse width; 60 s; 5 V) at different frequencies (1, 2, 4, 8, and 16 Hz) was performed. All pressure data were collected for analysis using PowerLab/8SP data acquisition system.

**Primer Design and 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. The PDZ-RhoGEF primers were as follows: forward, 5′-GGGACCCCTTTCTGAGACGCAA-3′; reverse, 5′-GGCCAGCCAC-TTGCTCTGTGAG-3′; LARG primers were forward, 5′-AGCCATG-CGGCGTGGAGTAAAC-3′; and reverse, 5′-GTCCAGGGAAATGGAGGTGTC-3′. p115RhoGEF primers were forward, 5′-TCCGGCAACAGATGTGGGACAGA-3′; and reverse, 5′-TACCGCGTCACGGTGAGAAGTG-3′. Glyceraldehyde-3-phosphate dehydrogenase primers were forward, TGATCT-CTGACACGACACTGCT; and reverse, ACAGCCTTGGGAG-ACCC-AGTGAT.

Penile tissue was isolated, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent analysis. Total RNA (4 μg/reaction) extracted from tissue segments with TRizol reagent (Invitrogen) was used for the first-strand cDNA synthesis with superscript II kit.

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(Invitrogen), according to manufacturer’s specifications. cDNA equal to 0.04 μg of total RNA was used for each PCR reaction under the following conditions: 94°C for 2 min and 22 (for glyceraldehyde-3-phosphate dehydrogenase) 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 videocamera (Sony Video Camera Module Charge-Coupled Device; Sony, Tokyo, Japan), connected to an IBM AT computer (IBM, White Plains, NY) with a 512 × 512-pixel array imaging board with 256 gray levels.

Statistical Analysis. Experimental values of relaxation were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Calculations were based on the number of experiments performed in each individual. In vivo erectile responses were expressed as the ratio of ΔICP (mm Hg)/MAP (mm Hg) × 100, with ΔICP, the difference between ICP in the flaccid state and ICP during the plateau phase of the erectile response and MAP, the mean arterial pressure during the plateau phase of the erectile response. During detumescence, the time (D20) and the rate (ΔD20) for ICP to decrease to 20% of maximal increase were determined (Mizusawa et al., 2001). Data are shown as the percentage of relaxation of n experiments, expressed as the mean ± S.E.M. Analysis of variance and Student’s paired t test were used 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 (Instat, ver. 3.0; GraphPad Software Inc., San Diego, CA).

Results

Relaxing Activity of Rho-Kinase Inhibitors. The addition of PE (10 μM) to the bathing medium caused a submaximal contraction of rat cavernosal segments and generated active force of 9.3 ± 0.8 mN (n = 18), which consisted of a rapid rise in force followed by a slower rise to a sustained level within 10 min (Fig. 1). The cumulative addition of H-1152 (0.001–3 μM) and Y-27632 (0.01–30 μM) produced long-lasting and concentration-dependent relaxations in contracted tissues (Fig. 1), with pEC50 values of 7.04 ± 0.04 and 6.02 ± 0.07, respectively (p < 0.01; n = 9). Maximal responses elicited by these compounds were not statistically different (94 ± 2% for H-1152 and 90 ± 2% for Y-27632; Fig. 1).

Effect of L-NAME and ODQ on Relaxations Induced by H-1152 and Y-27632. Under our experimental conditions, the incubation of rat cavernosal strips with either L-NAME (100 μM), a nonselective inhibitor of NO synthase, or ODQ (10 μM), an inhibitor of soluble guanylyl cyclase, virtually abolished the endothelium-dependent relaxations mediated by acetylcholine (10 μM). It is interesting that acetylcholine relaxed precontracted rat corporeal tissue at the above-mentioned concentration by only 10 ± 2%, unlike in corpus cavernous isolated from humans, rabbits, or mice, where this concentration has been shown to cause 40 to 70% relaxation. The addition of L-NAME (n = 5) or ODQ (n = 4) caused a discrete contractile effect (∼5% increase) on the tone induced by PE (10 μM). As shown in Table 1, treatment of cavernosal strips with either L-NAME or ODQ did not significantly affect the relaxations evoked by H-1152 (0.001–3 μM) or Y-27632 (0.01–30 μM).

Effect of Rho-Kinase Inhibition on PE- and EFS-Induced Contractions. Addition of PE (0.01–100 μM) to the bathing medium concentration dependently contracted the

![Fig. 1. Effects of the Rho-kinase inhibitors H-1152 and Y-27632 in cavernosal smooth muscle strips contracted with PE (10 μM). a, typical tracing showing the sustained and concentration-dependent relaxant response of H-1152 (0.001–3 μM). This is a representative tracing of nine experiments. b, experimental values of the relaxations induced by H-1152 (open symbols; n = 9) and Y-27632 (closed symbols; n = 9) were calculated relative to the maximal changes from the contraction produced by PE in each tissue, which was taken as 100%. Data represent the mean ± S.E.M. of n experiments.](image-url)
cavernosal smooth muscle strips with a pEC\textsubscript{50} value of 6.27 ± 0.06 (n = 18), reaching a peak (166 ± 4% of KCl-induced contraction) at 30 μM. Addition of the Rho-kinase inhibitors H-1152 (0.1–1 μM) or Y-27632 (1–10 μM) significantly attenuated the contractions evoked by PE in a concentration-dependent manner. At their highest concentration, H-1152 and Y-27632 caused 9.7- and 11.5-fold rightward shifts in the curves to PE, respectively (n = 4; p < 0.01). The NO donor SNP (0.1–1 μM; n = 4) also inhibited the contractile activity evoked by PE in a concentration-dependent manner, culminating with a 6.7-fold shift to the right after treatment of the preparations with 1 μM SNP. As shown in Fig. 2, H-1152 (0.1 μM), Y-27632 (1 μM), and SNP (0.1 μM) caused similar rightward shifts in the curves to PE of 2.8-, 2.9- and 2.4-fold, respectively (n = 4). However, the coincubation of H-1152 (0.1 μM) or Y-27632 (1 μM) with SNP (0.1 μM) resulted in significantly higher shifts in the curves to PE, compared with their actions individually, as represented by the 9.9- (H-1152 plus SNP) and 7.5-fold (Y-27632 plus SNP) displacement to the right (Fig. 2; p < 0.01).

In cavernosal smooth muscle strips under resting conditions, EFS (1–32 Hz) produced frequency-dependent contractions that were virtually abolished by the Na\textsuperscript{+} channel blocker tetrodotoxin (1 μM; n = 6) and by the α\textsubscript{1}-adrenergic antagonist prazosin (1 μM; n = 5). In addition, electrically induced contractions were fully blocked by the noradrenergic nerve blocking agent bretylium tosylate (30 μM; n = 6), confirming that these responses are neuronal in origin and adrenergic in nature. Addition of H-1152 (0.1–1 μM), Y-27632 (1–10 μM), or SNP (0.1–1 μM) caused concentration-dependent reductions of EFS-induced contractions, with maximal inhibitions at their highest concentration of 42 ± 4, 44 ± 3, and 16 ± 3%, respectively, when the strips were stimulated at 32 Hz (n = 4, each). As shown in Fig. 3, each agent had little effect on the contractile activity elicited by EFS over the full range of the frequency-response curve when their lowest concentration was used (H-1152, 0.1 μM; Y-27632, 1 μM; and SNP, 0.1 μM). However, the combination of either Rho-kinase inhibitor with SNP produced a significant inhibition of the electrically evoked contractions compared with the controls (Fig. 3; p < 0.01; n = 4).

**Effect of H-1152 on Penile ICP.** ICP and MAP under resting conditions in anesthetized rats averaged 8 ± 2 and 116 ± 5 mm Hg, respectively (n = 8). Electrical stimulation of the cavernous nerve induced frequency-dependent (1–16 Hz) and sustained increases in ICP, accompanied by transient decreases in the MAP (n = 8). The ratio of ICP over MAP was used to provide an index of erectile activity that controls for changes in MAP. The highest frequency used (16 Hz) produced a mean ICP of 77 ± 4 mm Hg, which corresponded to a mean of 0.74 ± 0.05 ICP/MAP.

Intraperitoneal administration of H-1152 (100 nmol/kg; n = 4) did not considerably affect the ICP/MAP ratio in the absence of electrical stimulation of the cavernous nerve.
Indeed, AUC was increased by 42\% (AUC) parameter in vehicle- and H-1152-treated animals. The demonstration of the area under the curve (AUC) parameter in vehicle- and H-1152-treated animals. The enhancement by H-1152 was evinced previously in vascular smooth muscle tone because of the inhibition of MLC phosphatase activity, resulting in increased MLC phosphorylation and force in the penile tissue. First, we compared the relaxant responses of two Rho-kinase inhibitors, H-1152 and Y-27632, in the isolated rat corpus cavernosum, by applying either compound to strips precontracted with the $\alpha_1$-agonist phenylephrine. The contractions were concentration dependently inhibited by H-1152 and Y-27632, indicating that the Rho-kinase pathway is involved in the adrenergic contraction of the cavernosal tissue, supporting previous studies (Chitaley et al., 2001; Rees et al., 2002; Wang et al., 2002; Takahashi et al., 2003). The tonic force produced by phenylephrine in rat corpus cavernosum was more sensitive to H-1152 than Y-27632, because the former compound was approximately 10 times more potent than the latter, rendering H-1152 as a more effective inhibitor of Rho-kinase.

Previous studies demonstrated that in vascular smooth muscle RhoA activation is accompanied by the membrane translocation of GTP-bound RhoA (Fujihara et al., 1997; Scholtz et al., 1999; Gong et al., 2001). On the other hand, the NO signaling pathway exerts an inhibitory action on RhoA function via cGMP-dependent protein kinases, which phosphorylate RhoA, thus inhibiting its translocation to the membrane (Gohla et al., 2000; Sauzeau et al., 2000; Sandu et al., 2001; Sawada et al., 2001; Chitaley and Webb, 2002). In rat cavernosal strips, we found that neither L-NAME nor ODQ affected the relaxant responses evoked by H-1152 or Y-27632, corroborating previous observations that inhibition of NOS or soluble guanylyl cyclase does not influence relaxations mediated by Rho-kinase inhibitors (Chitaley et al., 2001). Nevertheless, although pharmacological inhibition of the NO/cGMP pathway failed to affect both H-1152 and Y-27632-evoked relaxations, the lines of evidence presented in this study suggest that attenuation of RhoA/Rho-kinase signaling in rat cavernosal smooth muscle represents part of the NO-induced inhibitory effects on contractile activity, as evidenced previously in vascular smooth muscle. First, relaxations evoked by acetylcholine at a concentration that markedly relaxes both human and rabbit cavernosal preparations (10 $\mu$M), caused only a small relaxant response in precontracted rat corporeal tissue, in agreement with previous studies (Hedlund et al., 1999; Claudino et al., 2004). Second, addition of either L-NAME or ODQ had little effect on the tone of the preparations, whereas these inhibitors have been shown to induce marked contractions in the corpus cavernosum from other species (Teixeira et al., 2004a,b). Although a
positive NADPH diaphorase has been described in some parts of the sinusoidal endothelium of the rat penis (Keast, 1992), this has not been found in later investigations (Schirar et al., 1994; Dail et al., 1995; Hedlund et al., 1999). Bivalacqua et al. (2004) have demonstrated that the RhoA/Rho-kinase pathway down-regulates rat penile eNOS in diabetes and that inhibition of Rho-kinase led to a restoration of the erectile function, which was accompanied by an improvement of eNOS expression. Based on these observations, one could argue in favor of endothelium-derived NO as a key mediator of erectile function. Nevertheless, the erectile process is initiated by stimulation of NO release from nitrergic nerves, with a subsequent dilation of the penile arteries to permit an increase in blood flow to the penis (Andersson and Wagner, 1995). In response to stretch and possibly shear stress, the sinusoidal endothelium may release NO to help maintain the erectile state. In this previous study (Bivalacqua et al., 2004), the functional data presented were obtained in the intact animal by measuring intracavernosal pressure, thus representing an action of nerve-derived NO on the arteries and the sinuses (stretch). The authors did not test the responsiveness to acetylcholine in the isolated cavernosal tissue; therefore, a confusing issue remains with regard to the physiological basis for erection and the means to establish this basis ex-
kinase pathway plays an important role in the maintenance of the vasoconstrictive state of the cavernosal vasculature.

The activity of Rho itself is regulated by distinct groups of proteins, such as RhoGEPs, which facilitate the activation of Rho by promoting the dissociation of GDP and subsequent binding of GTP (Ross and Wilkie, 2000; Sah et al., 2000; Schmidt and Hall, 2002). A group of RhoGEPs has recently been identified and characterized by a moiety resembling a regulator of GDS domain that binds the activated α subunit of GTPase. This group of RhoGEP proteins currently comprises three members: LARG, p115RhoGEP, and PDZ-RhoGEP (Ross and Wilkie, 2000; Schmidt and Hall, 2002; Somlyo and Somlyo, 2003). Although p115RhoGEP is found predominantly in hematopoietic cells (Girkontaite et al., 2001), PDZ-RhoGEP and LARG seem to be more widely expressed (Fukuhara et al., 1999, 2000; Kourlas et al., 2000). We have developed a method for semiquantification of mRNA expression of the GDS domain-containing RhoGEPs in biological samples (Ying et al., 2004). To our knowledge, this is the first study that demonstrates the presence of GDS domain-containing RhoGEPs in corpus cavernosum, suggesting their potential role in the regulation of cavernosal smooth muscle tone.

In conclusion, our results showed that the compound H-1152 can completely and more potently reverse the Ca\(^{2+}\) sensitization process in the cavernosal tissue, both in vitro and in vivo. On this basis, Rho-kinase antagonism represents another potential line of therapy in the treatment of erectile dysfunction, especially in cases where either noradrenergic tone is elevated or nitricergic/endothelial-dependent responses are blunted. Most importantly, the design and synthesis of selective inhibitors of GDS domain-containing RhoGEPs should provide the framework for defining new physiological roles for these factors, not to mention the possibility of identifying novel agents to treat erectile disorders.

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