Rhoa-mediated Ca\textsuperscript{2+} Sensitization in Erectile Function*

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A Rho-kinase inhibitor increases corpus cavernosum (CC) pressure in an in vivo rat model (Chitaley, K., Wingard, C. J., Webb, R. C., Branam, H., Stopper, V. S., Lewis, R. W., and Mills, T. M. (2001) Nat. Med. 7, 119–122) suggesting that Rho-mediated Ca\textsuperscript{2+} sensitization of CC smooth muscle maintains the flaccid (contracted) state. We directly demonstrate Ca\textsuperscript{2+} sensitization of permeabilized rabbit and human CC and identify a highly expressed molecular component of this pathway. Ca\textsuperscript{2+} sensitization of force induced by endothelin or GTP\textsuperscript{S} was significantly greater in CC than in rabbit ileum smooth muscle and was accompanied by a 17-fold higher RhoA content. Pull-down assays with the Rhoa binding domain of mDia showed the high RhoA content of CC to be available for activation by GTP\textsuperscript{S}. Ca\textsuperscript{2+} sensitization induced by endothelin, phenylephrine, or GTP\textsuperscript{S} was completely relaxed by the Rho kinase inhibitor Y-27632. Human and rabbit CC both express the phosophatase inhibitor CPI-17, the myosin phosphatase regulatory (MYPT-1) and catalytic (PP1\textsuperscript{c}) subunits, and two isoforms of Rho kinase. We suggest that high expression of Rhoa contributes, through Rhoa-mediated Ca\textsuperscript{2+} sensitization, to the flaccid state of CC that can be reversed by a water-soluble, orally active Rho kinase inhibitor suitable for therapy of erectile dysfunction.

Relaxation of the smooth muscle trabeculae of the corpus cavernosum (CC) and of the helicine arteries leads to blood filling of the sinuses, occlusion of the venous outflow (reviewed in Ref. 1), and penile erection. Nitric oxide (NO), generated by both nerves and the endothelial cells that cover the trabeculae of the CC, through stimulation of soluble guanylate cyclase and the generation of cyclic GMP (for review see Ref. 2), is thought to play a dominant role in relaxation of smooth muscle in this tissue. The pathophysiological relevance of the cyclic GMP-protein kinase G (PKG) pathway is evidenced by the successful use of cyclic GMP phosphodiesterase inhibitors (PDE-5) such as sildenafil (Viagra) in the treatment of erectile dysfunction (for review see Ref. 3). Other signaling pathways involving vasoactive intestinal polypeptide/cAMP may also be operative in relaxation of the CC. Severe erectile dysfunction in cGMP-dependent kinase 1-deficient mice, with normal cAMP signaling, also demonstrated the importance of PKG and the inability of the cAMP pathway to compensate for the absence of the cGMP signaling cascade in vivo (4, 5).

The contracted (resting) state of the CC smooth muscle is considered to be mediated by release of norepinephrine, endothelin-1, neuropeptide Y, prostanoids, and angiotensin II (1, 6–11). Inhibition of these agonists at the receptor level or in their downstream signaling pathways should, like NO stimulation, also lead to a decrease in myosin regulatory light chain (RLC\textsubscript{200}) phosphorylation and consequent relaxation through decreased cytoplasmic [Ca\textsuperscript{2+}] and/or inhibition of Ca\textsuperscript{2+} sensitization (for review see Refs. 12 and 13). Calcium sensitization is brought about by agonist activation of heterotrimeric G-protein-coupled receptors, the exchange of GTP for GDP on the small GTPase Rhoa, its activation and dissociation from its partner Rho-GDI (guanine nucleotide dissociation inhibitor). GTPrhoa activates Rho-kinase, which inhibits myosin light chain phosphatase, resulting in an increase in RLC\textsubscript{200} phosphorylation and force at constant [Ca\textsuperscript{2+}] (13–17). Ca\textsuperscript{2+} sensitization has been shown to make a significant contribution to agonist-induced contraction under physiological conditions in vascular smooth muscle (12, 18). A recent study of an in vivo rat model demonstrated that a Rho-kinase inhibitor caused a dramatic increase in CC pressure and erection independent of NO (19) while exerting a minimal effect on systemic arterial pressure. This finding suggests that the Rhoa-Ca\textsuperscript{2+} sensitization pathway is present and active in the “resting state” of CC smooth muscle. These authors proposed that antagonism of Rho-kinase introduces a potential alternate avenue for the treatment of erectile dysfunction. The Rho-kinase inhibitor Y-27632 (20, 21) also relaxed intact phenylephrine-contracted cavernosal strips of smooth muscle in a dose-dependent fashion (22, 23) similar to other smooth muscles (20, 24).

In this study we directly demonstrate very potent Ca\textsuperscript{2+} sensitization of force in permeabilized rabbit and human CC in which Ca\textsuperscript{2+} concentrations are clamped and show that a component of this pathway, Rhoa, is very highly expressed in CC compared with its content in other smooth muscles; the Rhoa binding partner, guanine nucleotide dissociation inhibitor (GDI), is also present at high concentrations. Using a Rho binding domain pull-down assay, we also show that, during Ca\textsuperscript{2+} sensitization with GTP\textsuperscript{S}, a large fraction of the endogenous Rhoa is in the active, GTP\textsuperscript{S}-bound form, and that the Rho-kinase inhibitor inhibits calcium sensitization induced by GTP\textsuperscript{S}, phenylephrine, and endothelin-1. Furthermore, the magnitude of relaxation of maximal GTP\textsuperscript{S}-induced calcium-sensitized force by Y-27632 is 2- to 3-fold greater than by 8-Br-cGMP in rabbit CC. We also find that CC expresses telokin, a target of PKG (25), myosin phosphatase regulatory MYPT-1 and catalytic PP1\textsuperscript{c} subunits (12, 13, 17), both Rho-
kinase isoforms ROKα and ROKβ (26–29), and the myosin phosphatase inhibitor protein, CPI-17 (30).

**EXPERIMENTAL PROCEDURES**

**Tissue Preparation and Force Measurements**—New Zealand rabbits (2–3 kg) were anesthetized with halothane and killed by exsanguination according to protocols approved by the Center for Comparative Medicine at the University of Virginia. The penis was excised, and the CC was dissected free from the tunica albuginea. Human CC specimens were obtained at the time of implantation of a penile implant from four patients, one diagnosed with vasculogenic erectile dysfunction due to atherosclerotic disease and the other three with type II diabetes mellitus. These three patients had failed to achieve an adequate erection for intercourse following administration of the PDE-5 inhibitor sildenafil and intracavernous prostaglandin E1, which raises cAMP. Small strips (0.15–0.2 mm wide and 2 mm long) were cut with razor knives from the regions where longitudinal bundles could be well resolved under a dissecting microscope. The ends of the muscle bundles were tied with monofilament silk to a force transducer and a stationary hook and mounted on a bubble plate for force measurements. After recording the contractile response to high potassium, the muscle was transferred to relaxing solution (G1) containing 1 mM EGTA and permeabilized with 0.1 mDia (33). The following dilutions of primary antibodies were used: anti-RhoA (monoclonal antibody, Transduction Laboratories) at 1:1,000, anti-ROKα (rabbit polyclonal) at 1:2,000 and anti-ROKβ antibodies (Santa Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-ROKα (rabbit polyclonal) at 1:2,000 and anti-ROKβ antibodies (Santa Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROK (1:5,000, anti-Rho-kinase ROK (Cruz Biotechnology) at 1:5,000. All solutions and the use of α-toxin and A23187 have been described previously (15, 31, 32).

**mDia Pull-down Assays**—Affinity precipitation of active Rho was performed using the Rho binding domain (RBD) of the fusion protein, mDia (33). pEGX-IT-1 mouse mDia RBD (amino acids 2–204) was introduced in Escherichia coli BL21(DE3) pTrx, and the GST fusion protein was expressed, conjugated to glutathione beads, and purified. Small strips of CC (0.15–0.2 mm wide and 2 mm long) were permeabilized with α-toxin for 2 h at room temperature, washed in G1, and contracted with pCa6.5. After 10 min, 10 μM GTP-γS was added for a predetermined amount of time, and tissues were snap frozen and pulverized at liquid nitrogen temperature. The powder was rapidly homogenized in the homogenization buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 0.5% Nonidet P-40, and a protease inhibitor mixture (P-8340, Sigma Chemical Co., St. Louis, MO). Homogenized samples were centrifuged at 14,000 rpm at 4°C for 10 min, and a small quantity of supernatant was saved to be analyzed later for total RhoA content. The remaining supernatant was incubated with 30 μg of GST-RBD fusion protein conjugated with glutathione beads at 4°C for 2 h. The beads were washed twice with homogenization buffer and then resuspended in 48 μl of non-reducing sample buffer with 2 μl of 1 M dithiothreitol and heated to 100°C for 5 min. Positive and negative control samples, into which GTP-γS or GDP was exchanged into the endogenous RhoA of rabbit tissues, were homogenized in homogenization buffer and centrifuged at 14,000 rpm at 4°C for 10 min, and the supernatant was collected. For nucleotide exchange, 10 mM EDTA was added to each sample, 1 mM GDP was added to the negative control and 50 μM GTP-γS to the positive controls. Samples were incubated at 30°C for 15 min, and then the reaction was stopped by addition of 60 mM MgCl2. These controls samples were then incubated with glutathione-mDia beads and run in parallel with the tissue samples. After binding to mDia RBD, the samples were subjected to SDS-polyacrylamide gel electrophoresis on a 4–20% gradient gel. Bound RhoA was detected by Western blot analysis using a monoclonal antibody against RhoA (Santa Cruz Biotechnology).

**Western Blot Analysis**—Strips of rabbit and human CC, strips of rabbit femoral artery and sheets of the longitudinal muscle of rabbit ileum at rest or following stimulation were homogenized in ice-cold buffer (250 mM sucrose, 25 mM Tris, 5 mM MgCl2, 5 mM EDTA, 1 mM dithiothreitol, and protease inhibitor mixture, see above), pH 7.4. Tissue homogenates were submitted to SDS-PAGE with 8% polyacrylamide for MYPT-1, PP16, ROKα, and ROKβ and 12% for all other proteins followed by transfer to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk and phosphate-buffered saline containing 0.05% Tween-20 (PBST) for 1 h and then incubated with primary antibody for 2 h at room temperature. The following dilutions of primary antibodies were used: anti-RhoA (monoclonal antibody SC-418, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:10,000, rabbit polyclonal anti-telokin antibody prepared against re-combinant full-length rabbit telokin at 1:1,000, anti-MLC20 (monoclonal antibody, Sigma) at 1:200, anti-GDI (rabbit polyclonal antibody, Santa Cruz Biotechnology) at 1:5,000, anti-Rho-kinase ROKα (monoclonal antibody, Transduction Laboratories) at 1:1,000, anti-ROKβ (monoclonal antibody, Transduction Laboratories) at 1:500, anti-MYPT-1 (sheep polyclonal antibody (Upstate, Waltham, MA) at 1:1,000, anti-PP16 (Upstate) at 1:5,000, anti-CPT-17 (rabbit polyclonal) at 1:2,000 (34). The blots were washed in PBST, incubated in horseradish peroxidase-conjugated secondary antibodies to mouse, rabbit, or sheep for 1 h at room temperature and detected with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The protein signals were quantitated by densitometry using an Eppendorf Expression 1600 scanner (Long Beach, CA) and the IMAGE Gel Quantitation Program (National Institutes of Health). Protein was determined with the Bio-Rad Protein Assay kit. Quantitation of RhoA, GDI, telokin, and MLC20 in tissues was carried out in the linear range of the antibody reactivity.

For electron microscopy, strips of CC smooth muscle were fixed in 2% glutaraldehyde with 0.1% tannic acid in cacodylate buffer followed by osmium tetroxide, stained with uranyl acetate en bloc, dehydrated, and embedded in Spurr’s resin. Sections were viewed in a Phillips CM12 electron microscope.

**RESULTS**

The respective activities of the Rho-kinase inhibitor Y-27632 and of 8-BrcGMP to relax permeabilized Ca2+-sensitized CC muscle strips were compared (Fig. 1). Muscles were maximally...
sensitized with GTPγS or endothelin plus GTP with Ca^{2+} concentrations clamped at pCa 6.9–6.7. GTPγS (10 μM) and endothelin (10 nM) were highly effective in eliciting Ca^{2+} sensitization. 10 μM GTPγS induced Ca^{2+} sensitization as follows: 64% ± 11.6 S.D. of maximal Ca-induced force (pCa 4.5) (n = 42) in rabbit CC and 52% ± 4.9 S.D. (n = 7) in several strips from three human CC specimens. Endothelin induced Ca^{2+} sensitization as follows: 35% ± 5.2 S.D. of pCa 4.5 (n = 11) in rabbit CC and 46% ± 4.1 S.D. (n = 6) in human CC. Phenylephrine-induced Ca^{2+} sensitization in rabbit CC was significantly smaller, −10–20% of the CaG response. Y-27632 (10 μM) completely reversed the GTPγS-, endothelin-, and phenylephrine-induced Ca^{2+} sensitization force. Saturating concentrations of 8-Br-cGMP (50 μM) produced only a small relaxation of GTPγS-induced Ca^{2+} sensitization in rabbit CC, whereas subsequent addition of 10 μM Y-27632 induced complete relaxation of endothelin-, phenylephrine-, or GTPγS-mediated Ca^{2+} sensitization. The results showing inhibition of maximal GTPγS-induced Ca^{2+} sensitization are summarized in Fig. 2 where 50 μM 8-Br-cGMP induced 35% ± 11.4 S.D. (n = 13) relaxation compared with 97% ± 4.3 S.D. (n = 10) relaxation induced by 10 μM Y-27632. The IC_{50} = 0.9 μM for Y-27632-induced relaxation of GTPγS-mediated Ca^{2+} sensitization is shown in Fig. 3. 8-Br-cGMP was more effective in relaxing endothelin than GTPγS-mediated Ca^{2+} sensitization, 96% ± 4.4 S.D. (n = 7) in rabbit CC. In several strips of human CC obtained from three of the patients, Y-27632 completely relaxed GTPγS as well as endothelin plus GTP-induced calcium sensitization (n = 3) with a similar sensitivity as found in rabbit CC (Fig. 4); 50 μM 8-Br-cGMP was more effective than in the rabbit CC (compare Figs. 1 and 4). As in the rabbit CC (Fig. 1C), even when 8-Br-cGMP induced a large reversal of sensitized force, subsequent additions of Y-27632 induced a further relaxation (Fig. 4, A and D).

The concentrations of the small GTPase RhoA, its partner Rho-GDI, and the protein kinase G (PKG) target, telokin, were estimated from Western blots of tissues, and known amounts of purified standards of recombinant proteins were loaded in the linear range of the antibody reactivity (Fig. 5). Rabbit ileum smooth muscle is included for comparison with rabbit and human CC. Because the CC has an exceedingly high content of connective tissue relative to smooth muscle, to obtain comparable estimates of the concentrations of RhoA, Rho-GDI, and telokin in smooth muscle, the concentrations were normalized to tissue myosin content estimated from known concentrations of purified turkey gizzard myosin loaded on the same gel (Fig. 5D). The myosin in the standards and tissue specimens was detected with an antibody to MLC_{20}. Comparison of myosin contents detected by Western blotting with a MLC_{20} antibody (Fig. 5D) clearly shows the lower myosin content of equal protein loads in the cavernosum samples compared with rabbit ileum as well as the significantly higher RhoA content (Fig. 5A) and Rho-GDI (Fig. 5B) in cavernosum compared with ileum at equal protein loads, which is obvious even without correcting for the lower cellular content of the CC samples. The relative amounts of myosin, telokin, and RhoA in the CC and rabbit ileum, estimated by comparison with known concentrations of those proteins, are shown in Table I. The myosin content per microgram of total protein in the ileum is 3.4 times that in the rabbit CC. The RhoA content per microgram of total protein was 5.3 ng in the corpus compared with 1.1 ng in the ileum sample. Correcting for the large contribution of connective tissue to total protein in the CC by normalizing the RhoA measurements to the myosin content results in a 17-fold higher RhoA concentration in the rabbit CC than in rabbit ileum; the relative expression of telokin was not significantly different. A similar 16-fold greater RhoA content was measured in human CC samples compared with rabbit ileum (Fig. 5). Normalization of Rho-GDI to myosin content in the same sample (not shown) also gave a content that was ∼17-fold higher in CC than in ileum smooth muscle.

The density of myosin filaments seen in electron micrographs of rabbit CC smooth muscle (Fig. 6) was typical of other
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smooth muscles such as ileum, showing normal arrays of myosin and actin filaments with a high actin to myosin ratio, justifying our above normalization of RhoA content to myosin content. Incidentally, a well-developed sarcoplasmic reticulum forming surface couplings with the plasma membrane as well as the presence of a basement membrane were also observed.

To determine whether the very high concentrations of CC RhoA were accessible to activation during Ca\textsuperscript{2+} sensitization with GTP\textsuperscript{S}, we used a pull-down assay for GTP\textsuperscript{S} RhoA. The mDia Rho binding domain (RBD) conjugated with glutathione beads extracted a larger fraction of RhoA\textsuperscript{GTP\textsuperscript{S}} per total RhoA in the homogenate from rabbit CC compared with rabbit ileum under identical assay conditions (Fig. 7). The RhoA remaining in the supernatant may have also included some GTP\textsuperscript{S}-bound but not GDP-bound RhoA from tissue homogenates treated to load the given nucleotide onto the endogenous RhoA. These results indicate that the highly expressed RhoA in CC is correctly folded and available to be activated by Ca\textsuperscript{2+} sensitization.

Other proteins, thought to participate in Ca\textsuperscript{2+} sensitization signaling pathways such as Rho-kinase, myosin phosphatase regulatory subunit MYPT-1, PP1\textsuperscript{δ}, and the myosin phosphatase inhibitor protein CPI-17 were also detected in human and rabbit CC (Figs. 8 and 9). ROK\textsuperscript{α} content at identical protein loading was greater in both human and rabbit CC compared with ileum, even without correcting for the lower myosin content in the CC (Fig. 8A). The reverse relationship occurred with ROK\textsuperscript{β} content. Rabbit femoral artery contained both isoforms. The absolute amounts of ROK\textsuperscript{α} and \textsuperscript{β} could not be quantitated due to the lack of recombinant ROK proteins and the unknown differences in sensitivities of the two antibodies used for detection. The MYPT-1 and PP1\textsuperscript{δ} contents in both human and rabbit CC, scaling to myosin contents, were similar to the phasic rabbit ileum and greater than in the tonic femoral artery (Fig. 8B). CPI-17 content in the rabbit CC (Fig. 9A), scaled to myosin

![Fig. 4. 10 μM Y-27632 and 50 μM 8-Br-cGMP-induced reversal of GTP\textsuperscript{S} calcium-sensitized force in α-toxin-permeabilized human cavernosal smooth muscle at constant Ca\textsuperscript{2+}. Strips of CC were contracted with pCa 6.9 plus 10 μM GTP\textsuperscript{S} (A and B), pCa 6.7 plus 100 nm endothelin in the presence of 10 μM GTP (C and D). Maximal force was elicited by CaG (pCa 4.5).](http://www.jbc.org/)

![Fig. 5. Western blots for RhoA, RhoGDI, telokin, and MLC\textsubscript{20} in human and rabbit tissues. Protein loads are indicated below each lane. A, Western blots for RhoA in human and rabbit tissues. Lanes 1 and 2, recombinant His-RhoA. (His-tagged RhoA runs higher than the tissue RhoA.) Lanes 3 and 4, human CC; lanes 5 and 6, rabbit CC; lanes 7 and 8, rabbit ileum. B, Rho-GDI. Lane 1, recombinant GDI; lanes 2 and 3, human CC; lanes 4 and 5, rabbit CC; lanes 6 and 7, rabbit ileum. C, telokin in rabbit tissues. Lanes 1–3, rabbit ileum smooth muscle; lanes 4–6, rabbit CC; lanes 7–9, recombinant mouse telokin (FLAG-tagged telokin runs higher than tissue telokin). D, MLC\textsubscript{20}. Lanes 1 and 2, purified chicken gizzard MLC\textsubscript{20}; lanes 3 and 4, human CC; lanes 5 and 6, rabbit CC; lanes 7 and 8, rabbit ileum smooth muscle. The MLC\textsubscript{20} samples are from the same tissue specimens as used for A and C. The paired MLC\textsubscript{20} Western blot from the same tissue, used for quantitating the amount of RhoGDI in smooth muscle excluding connective tissue, is not shown but was similar to D.](http://www.jbc.org/)

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The magnitudes of Ca\(^{2+}\) sensitivity (38). Yet in intact tissue endothelin induces a maintained contraction suggesting that, if [Ca\(^{2+}\)], is low as in cultured cells, are likely due to GTP\(\gamma\)S being able to fully activate the entire RhoA pathway, whereas activation by agonists may be limited by availability of receptors and their coupling to RhoA and RhoA guanine nucleotide exchange factors. Although the human sample size is small (and included because of its relevance, despite the difficulty in obtaining such specimens), its somewhat smaller magnitude of calcium sensitization, compared with rabbit CC, does not reflect a deficiency in contractile proteins, because maximal Ca\(^{2+}\)-induced forces were similar to rabbit CC. It could reflect the rundown of sensitized force of the human tissues from an unknown cause, or it could be related to disease. Further support for a physiological role of Ca\(^{2+}\) sensitization arises from the report that endothelin-1 induces an intracellular Ca\(^{2+}\) transient, which peaks at 250–400 nM before returning to near resting levels of 60–80 nM in about 2 min (38). Yet in intact tissue endothelin induces a maintained contraction suggesting that, if [Ca\(^{2+}\)], is low as in cultured cells,

**DISCUSSION**

The RhoA signaling pathway that, through activation of Rho-kinase, inhibits myosin phosphatase resulting in an increase in MLC\(_{20}\) phosphorylation and force in smooth muscle has been implicated in the regulation of tone in CC smooth muscle in studies in the rat in vivo and on isolated intact strips of cavernosal tissue from rat, rabbit, and human (19, 23, 35). The NO-cGMP pathway also plays a significant role in CC relaxation of cavernosal tissue from rat, rabbit, and human (19, 23, 35). The NO-cGMP pathway also plays a significant role in CC relaxation of cavernosal tissue from rat, rabbit, and human (19, 23, 35).

**TABLE I**

| Protein contents in rabbit corpus cavernosum |
|---------------------------------------------|
| 1 \(\mu\)g of total rabbit corpus cavernosum protein | 1 \(\mu\)g of total ileum protein |
| Myosin | 19.0 | 65.0 |
| Telokin | 4.0 | 19.0 |
| RhoA | 5.3 | 1.1 |

**FIG. 6.** Electron micrograph of a transversely sectioned portion of a smooth muscle cell from rabbit CC showing a normal distribution of myosin filaments (arrowheads) surrounded by actin filaments. A well-developed sarcoplasmic reticulum (SR) forming a surface coupling with the plasma membrane as well as typical caveolae (C) are present.

**Fig. 6.** Electron micrograph of a transversely sectioned portion of a smooth muscle cell from rabbit CC showing a normal distribution of myosin filaments (arrowheads) surrounded by actin filaments. A well-developed sarcoplasmic reticulum (SR) forming a surface coupling with the plasma membrane as well as typical caveolae (C) are present.

**FIG. 7.** Affinity precipitation of active RhoA using the Rho binding domain of mDia conjugated to glutathione beads. \(\alpha\)-Toxin-permeabilized CC or ileum samples run in parallel were stimulated with 10 \(\mu\)M GTP\(\gamma\)S in pCa 6.5 with unstimulated controls incubated in G1 solution (A and B). 10 \(\mu\)l of whole tissue extract (WE) was loaded in lane 1, the remaining extract was incubated with mDia beads and centrifuged, and the pellet (RB) and supernatant (SN) were separated. GTP\(\gamma\)S-stimulated tissues had significantly greater amounts of active RhoA compared with tissues in G1 solution, lanes 2 and 5 in A and B. The mDia precipitated RhoA (RB), as a fraction of WE, was greater in the CC than in ileal tissues (A and B). Starting tissue sample sizes were slightly different for CC and ileum as indicated in the WE lanes. C and D, control assays showing that, when the endogenous RhoA in tissue homogenates was loaded with GTP\(\gamma\)S or GDP, mDia beads extracted GTP\(\gamma\)S but not GDP RhoA.
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been well characterized (31), but it may also reflect inhibition of an initially high myosin phosphatase activity in this tissue. Rabbit portal vein, femoral, and bladder have similar RhoA contents as ileum. The MYPT-1 and PP1 contents of rabbit and human CC, when scaled to myosin content, are similar to that of the phasic ileum rather than the tonic femoral artery, in keeping with the higher phosphatase activity in phasic compared with the tonic smooth muscles (39). Current evidence strongly suggests that Ca$^{2+}$-sensitized force and MLC$_{20}$ phosphorylation reflect inhibition of myosin phosphatase. GTP-RhoA activates Rho-kinase (16), and both translocate to the membrane with a time course compatible with the onset of Ca$^{2+}$ sensitization (40). Active Rho-kinase either directly phosphorylates and inhibits the regulatory subunit of myosin phosphatase (MYPT-1) or acts indirectly by phosphorylating another kinase, which in turn modulates the phosphatase activity (41). Our finding of a 17-fold greater RhoA content in CC than in ileum smooth muscle suggests that it may contribute to the flaccid state of CC. Mills and colleagues (42) have speculated that penile erection occurs as a result of two distinct processes. First, NO-initiated relaxation is mediated by a GMP-dependent increase in PKG activity with subsequent fall in [Ca$^{2+}$]$^i$, decreased MLC kinase activity, and MLC$_{20}$ phosphorylation. In addition to mediating NO-induced relaxation, PKG may inhibit or prevent activation of RhoA and indirectly relax CC through dis inhibition of MYPT-1 (43). The molecular basis and mechanism(s) of GMP-induced relaxation of sensitized force remain to be determined. It is also tempting to speculate that dysregulation of the RhoA pathway may occur with prolonged, non-sexually mediated erection termed priapism. In this case the treatments of choice are α-adrenergic agonists (44). Lastly, recent data suggest that the Rho/Rho-kinase pathway is activated in the CC following castration and contributes to erectile dysfunction due to loss of testosterone (45). Thus the Rho kinase pathway may be involved in the physiology of erection and alterations in signaling contribute to erectile dysfunction.

A landmark paper by Narumiya and colleagues (20) described a series of compounds that potently relaxed agonist-induced smooth muscle contraction through inhibition of Rho-kinase. One of these compounds, Y-27632, has been widely used to characterize Rho/Rho-kinase signaling pathways in many cell types. The IC$_{50}$ value for Y-27632-induced inhibition of GTP$_{S}$-induced Ca$^{2+}$-sensitized force in rabbit CC was 0.9 μM, somewhat lower than the IC$_{50}$ of 2.8 μM found by Rees (23), using Y-27632 in intact rabbit CC contracted with electric field stimulation. Y-27632 completely relaxed the GTP$_{S}$Ca$^{2+}$-sensitized force in both rabbit and human CC, whereas supramaximal concentrations of 8-Br-cGMP were much less effective in the rabbit (Figs. 1 and 2). In the three human CC tissue samples, 8-Br-cGMP induced a greater inhibition of GTP$_{S}$-sensitized force than in the rabbit. In keeping with observations in cGMP-dependent kinase I-deficient mice in which PKA could not compensate for the lack of PKG (4, 5), the human samples in the present study showed a robust response to 8-Br-cGMP despite the patients’ poor response to intercavernous prostaglandin E1 that raises cAMP. However, they also did not respond to the PDE-5 inhibitor sildenafil, which should lead to increased cGMP, perhaps indicating altered PDE-5 activity as these isolated permeabilized CC samples responded well to 8-Br-cGMP. In support of a significant role for the RhoA/Rho-kinase contribution, Chitalay et al. (19) found major increases in CC pressure with injection of Y-27632 in vivo, with

this tonic phase of force maintenance is due to the activation of the Ca$^{2+}$ sensitization pathway. The observed magnitude of Ca$^{2+}$ sensitization may be related to the very high RhoA content of human and rabbit CC smooth muscle (Fig. 5), which is 16- to 17-fold greater, respectively, than in rabbit ileum smooth muscle in which the Ca$^{2+}$ sensitization signaling pathway has

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or without electrical stimulation, as well as in the presence of nitric-oxide synthase inhibitors or cGMP inhibitors. This is consistent with the capabilities of the NO pathway and the Rho-kinase pathway to function independently. Thus, one would expect that the erectile dysfunction and low reproductive ability of cGMP-dependent kinase I-deficient mice (4) should be alleviated by inhibition of Rho-kinase. However, there may also be cross-talk between these pathways (46–49), and their balance may be altered in different types of erectile dysfunction.

Both Rho-kinase isoforms ROKα (ROCKII) and ROKβ (p160ROCK) (reviewed in Ref. 50) were present in CC where their ratio differed from that in ileal muscle. The two isoforms have similar functional domains and have no known functional differences or different targets, and their Kα values for Y-27632 are very similar (21, 50). In the absence of recombinant ROK proteins, total ROK content and the ratios of ROKα and ROKβ could not be accurately determined. A rough estimate of total ROK from Western blots (e.g., Fig. 8A), scaled to the different myosin contents, suggests a higher ROK content of CC than ileal muscle. Thus, the large Ca2+ sensitization observed in CC compared with other smooth muscles may reflect the 17-fold greater RhoA content that would favor formation of the RhoA-GTP-ROK complex and may also be related to a high RhoA effector, ROK.

Stimulation of intact or permeabilized smooth muscle with 8-Br-cGMP or forskolin leads to phosphorylation of telokin, a 17-kDa protein whose sequence is identical to the C terminus of RhoA effector, ROK. Proteins, total ROK content and the ratios of ROK/H18528 complex and may also be related to a high RhoA content that would favor formation of the RhoA signally and Ca2+ sensitivity as well as previous 

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J. Biol. Chem. 2002, 277:30614-30621.
doi: 10.1074/jbc.M204262200 originally published online June 11, 2002

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