Rho-associated kinase plays a role in rabbit urethral smooth muscle contraction, but not via enhanced myosin light chain phosphorylation

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1Smooth Muscle Research Group and Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada; 2Smooth Muscle Research Centre, Dundalk Institute of Technology, Dundalk, Ireland; and 3Smooth Muscle Research Group and Department of Physiology and Pharmacology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

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Walsh MP, Thornbury K, Cole WC, Sergeant G, Hollywood M, McHale N. Rho-associated kinase plays a role in rabbit urethral smooth muscle contraction, but not via enhanced myosin light chain phosphorylation. Am J Physiol Renal Physiol 300: F73–F85, 2011. First published September 22, 2010; doi:10.1152/ajprenal.00011.2010.—The involvement of Rho-associated kinase (ROK) in activation of rabbit urethral smooth muscle contraction was investigated by examining the effects of two structurally distinct inhibitors of ROK, Y27632 and H1152, on the contractile response to electric field stimulation, membrane depolarization with KCl, and α1-adrenoceptor stimulation with phenylephrine. Both compounds inhibited contractions elicited by all three stimuli. The protein kinase C inhibitor GF109203X, on the other hand, had no effect. Urethral smooth muscle strips were analyzed for phosphorylation of three potential direct or indirect substrates of ROK: 1) myosin regulatory light chains (LC20) at S19, 2) the myosin-targeting subunit of myosin light chain phosphatase (MYPT1) at T697 and T855, and 3) cofilin at S3. The following results were obtained: 1) under resting tension, LC20 was phosphorylated to 0.65 ± 0.02 mol P/mol LC20 (n = 21); 2) LC20 phosphorylation did not change in response to KCl or phenylephrine; 3) ROK inhibition had no effect on LC20 phosphorylation in the absence or presence of contractile stimuli; 4) under resting conditions, MYPT1 was partially phosphorylated at T697 and T855 and cofilin at S3; 5) phosphorylation of MYPT1 and cofilin was unaffected by KCl or phenylephrine; and 6) KCl- and phenylephrine-induced contraction-relaxation cycles did not correlate with actin polymerization-depolymerization. We conclude that ROK plays an important role in urethral smooth muscle contraction, but not via inhibition of MLCP or polymerization of actin.

urethra; urinary incontinence; myosin light chain phosphatase; cofilin

LOWER URINARY TRACT FUNCTION is dependent on the concerted action of the smooth and striated muscles of the urinary bladder, urethra, and periurethral region. Failure to store urine can lead to various forms of incontinence, which is a major health concern (26), but current therapies for incontinence have severe limitations (19). Further therapeutic development will depend on the identification of novel targets. The bladder and urethra work as a functional unit with a reciprocal relationship under normal conditions; i.e., during the storage phase, the detrusor muscle of the bladder is relaxed while the urethra is contracted to allow gradual filling of the bladder with urine and prevent leakage. On the other hand, during voiding, the urethra relaxes and the detrusor contracts to facilitate emptying of the bladder (36). An isolated rat urethral preparation is “continent” in the absence of external neural input, but flow ensues when the smooth muscle is relaxed (29). Furthermore, stimulation of the skeletal muscle makes remarkably little difference to the ability of the contracted urethra to retain fluid. Conte et al. (12) also found that paralyzing the striated muscle encircling the urethra of anesthetized rats with D-tubocurarine did not result in urine leakage. It would appear, therefore, that skeletal muscle is more important for resisting rapid pressure rises caused by coughing or laughing, for example, than for maintaining a constant urethral tone, which makes sense from an energetic standpoint. The smooth muscle cells of the urethra, therefore, play a critical role in continence by remaining in a contracted state most of the time, thereby retaining urine within the bladder. This smooth muscle tone can be modified by adrenergic and cholinergic nerve stimulation (4). Norepinephrine, released by adrenergic neurons, is the major excitatory transmitter in the rabbit urethra (3, 13). At the appropriate time, the smooth muscle cells relax in response to inhibitory nerves, and detrusor smooth muscle contraction voids the bladder through a relaxed urethra. Nitric oxide, released by nonadrenergic, noncholinergic neurons, is an important mediator of urethral smooth muscle relaxation (5, 11).

Deficiencies in urethral closure can result in stress urinary incontinence. Treatment of this condition, which is based mainly on α1-adrenoceptor agonists, has been disappointing (6). To improve strategies for treatment of incontinence, it is essential to understand urethral function and regulation in greater detail.

Smooth muscle contraction and relaxation are regulated primarily by the phosphorylation of myosin light chain 2 (LC20), phosphorylated by myosin light chain kinase (MLCK). Activated MLCK phosphorylates LC20 at S19, which is followed by calcium-induced Ca2+-calmodulin (CaM) binding, leading to formation of Ca2+-MLCK and LC20 complexes, which increases adenylyl cyclase activity and results in smooth muscle relaxation (31). Calcium-induced contractile response elicited by contraction of smooth muscle cells is mediated by cross-bridge cycling of actin and myosin (40). The mechanism by which calcium induces smooth muscle contraction is not well understood; however, calcium is known to be a key regulator of smooth muscle contraction. Calcium is also known to induce smooth muscle contraction by activating the actin-myosin system, which results in the formation of cross-bridge cycling. Calcium is known to activate the actin-myosin system by increasing the number of cross-bridges, which results in smooth muscle contraction. Calcium is also known to activate the actin-myosin system by increasing the number of cross-bridges, which results in smooth muscle contraction. Calcium is known to activate the actin-myosin system by increasing the number of cross-bridges, which results in smooth muscle contraction.

An important aspect of the regulation of smooth muscle contraction that has emerged in recent years concerns the phenomenon of Ca2+-sensitization, i.e., the ability of a variety
of agonists to elicit a contractile response without an increase in [Ca$^{2+}$], (33). Ca$^{2+}$ sensitization involves agonist-induced activation of signaling pathways, primarily the RhoA/Rho-associated kinase (ROK) pathway, that terminate in the inhibition of MLCP. This shifts the balance between kinase and phosphatase in favor of MLCK so that a higher level of LC$_{20}$ phosphorylation (and force) is achieved at a given [Ca$^{2+}$]. ROK mediates MLCP inhibition via phosphorylation of the myosin-targeting subunit of the phosphatase (MYPT1) at T697 and/or T855 (rat numbering; NCBI accession no. EDM16761) (16, 31, 39) or of the 17-kDa cytosolic protein CPI-17, which becomes a potent inhibitor of MLCP when phosphorylated at T38 (27).

Studies regarding the role of LC$_{20}$ phosphorylation in urethral contractile physiology have been rather limited. Hypolite et al. (21) reported that the rabbit urethra exhibits a low level of basal LC$_{20}$ phosphorylation (12.8%). Modest increases in LC$_{20}$ phosphorylation, from 16% at rest to 28% at half-maximal bethanecol-induced force, 29% at 80% maximal force, and 27% at maximal force, were measured (21). A role for RhoA and ROK in urethral tone was indicated by the demonstration that inhibition of RhoA with Clostridium difficile toxin B or of ROK with Y27632 abolished porcine urethral tone without affecting [Ca$^{2+}$], (28). ROK inhibition also inhibited the contractile response of rat urethral smooth muscle to phenylephrine, endothelin-1, α,β-methylene ATP, and membrane depolarization but had no significant effect on baseline tension (37).

The overall aim of this work, therefore, was to gain further insights into the molecular mechanisms involved in the regulation of urethral smooth muscle contraction. The following specific questions were addressed: 1) Is the contraction of urethral smooth muscle in response to electric field stimulation, membrane depolarization by KCl or α1-adrenoceptor stimulation with phenylephrine attenuated by inhibition of ROK? 2) Does the sustained contraction of urethral smooth muscle involve phosphorylation of LC$_{20}$? 3) Does contraction of urethral smooth muscle correlate with the phosphorylation of MYPT1 at T697 and/or T855? 4) Does the contraction-relaxation cycle of urethral smooth muscle correlate with actin polymerization-depolymerization?

**MATERIALS AND METHODS**

**Materials.** Rabbit polyclonal anti-calponin antibody was raised in-house against purified full-length chicken gizzard calponin (42). Commercial antibodies were purchased from the following sources: rabbit polyclonal anti-LC$_{20}$ (Santa Cruz Biotechnology), raised against the full-length human protein; rabbit polyclonal anti-pS19-LC$_{20}$ (Rockland), raised against a synthetic phosphopeptide corresponding to the region around pT855 of the human protein; rabbit polyclonal anti-pT855-MYPT1 (Upstate), raised against a synthetic phosphopeptide corresponding to the region around pT855 of the human protein; rabbit polyclonal anti-pS3-cofilin (Cell Signaling Technology), raised against a synthetic phosphopeptide corresponding to human cofilin containing phosphoserine at position 3; and rabbit polyclonal anticofilin (Cell Signaling Technology), raised against a synthetic peptide corresponding to human cofilin containing serine at position 3, N$^\omega$-nitro-$\omega$-arginine, arginine, phenylephrine, and phorbol 12,13-dibutyrate (PdBu) were purchased from Sigma; Y27632 from BioMol International; H1152, GF109203X, wortmannin, and calyculin-A from Calbiochem; and microcystin-LR from Alexis Biochemicals. Molecular weight markers were purchased from Fermentas.

**Isolation of urethral tissue for tension measurements.** Male New Zealand White rabbits (3–4 kg) were maintained and killed with a lethal injection of pentobarbital according to the standards of the Canadian Council on Animal Care and a protocol approved by the Animal Care Committee of the Faculty of Medicine, University of Calgary, and in accordance with the European Union legislation and ethical standards. Male Sprague-Dawley rats (250–275 g) were maintained and killed by halothane inhalation and decapitation according to the standards of the Canadian Council on Animal Care and a protocol approved by the Animal Care Committee of the Faculty of Medicine, University of Calgary. The proximal 1 cm of the urethra was removed and placed in Krebs solution (120 mM NaCl, 5.9 mM KCl, 25 mM NaHCO$_3$, 5.5 mM glucose, 1.2 mM NaH$_2$PO$_4$, 1.2 mM MgCl$_2$, and 2.5 mM CaCl$_2$) plus 100 μM N$^\omega$-nitro-$\omega$-arginine and 1 μM atropine with pH adjusted to 7.4 with 95% O$_2$-5% CO$_2$. The muscarinic antagonist atropine was included to block the effects of acetylcholine released from nerves, and N$^\omega$-nitro-$\omega$-arginine to block nitric oxide effects. In separate experiments, we found that omission of these inhibitors had no statistically significant effect on LC$_{20}$ phosphorylation levels, as determined by paired Student’s t-test (0.62 ± 0.11 mol P/mol LC$_{20}$ in their presence and 0.52 ± 0.10 mol P/mol LC$_{20}$ in their absence, P > 0.05, n = 3). Circularly oriented strips (8 x 1 x 1 mm) of smooth muscle were dissected. For investigation of the effects of protein kinase inhibitors, muscle strips were placed in a water-jacketed organ bath maintained at 37°C and perfused with warmed Krebs solution bubbled with 95% O$_2$-5% CO$_2$. Strips were adjusted to a tension of 0.5 g, the optimal tension for KCl-induced force development, and allowed to equilibrate for 60 min before experimentation began. During the period of equilibration of the tissue after mounting, it was necessary to stretch the tissue periodically to maintain resting tension at 0.5 g. Stable tension was always achieved within the 60-min equilibration period. Prior experiments indicated that a resting tension of 0.5 g for tissue strips of the dimensions used in this study gave a maximal contractile response to KCl. Contractions in response to electric field stimulation (EFS), KCl, and phenylephrine were measured using Statham UC3 and Dynamometer UF1 transducers, with the outputs recorded on a Grass 7400 chart recorder. Tissues did not exhibit significant loss of maximal tension in response to repetitive stimuli (EFS, KCl, or phenylephrine) over several hours. Field stimulation was applied via platinum ring electrodes mounted at either end of the tissue strip. Pulses of 0.3 ms in duration were delivered in trains at constant frequencies of 4 Hz from a Grass S48 stimulator at a nominal voltage of 50 V. Responses were blocked with 1 μM tetrodotoxin, confirming that they were nerve mediated (38).

**Demembranation (skinning) of urethral smooth muscle strips.** Urethral smooth muscle strips mounted on a force transducer at resting tension were incubated at 21°C with 1% (vol/vol) Triton X-100 in 30 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 50 mM NaCl, 5 mM K$_2$EGTA, 150 mM sucrose, 0.5 mM diithioerythritol, pH 7.4, for 2 h. In establishing the optimal conditions for Triton skinning of urethral smooth muscle strips, the Triton X-100 concentration and time of treatment were varied. The contractile response of the Triton-skinned tissue to Ca$^{2+}$ was comparable to that of the KCl-induced contraction of the intact tissue before Triton treatment when 1% Triton X-100 was used for 2 h. Skinned tissues were then equilibrated with 3.2 mM MgATP, 2 mM free MgCl$_2$, 0.5 mM NaN$_3$, 30 mM TES, 12 mM phosphocreatine, 15 U/ml creatine kinase, and the contractile response of tissue strips to 0.1 and 0.2 mM KCl was similar to that of the intact tissue before Triton treatment.

**Staining for actin filaments.** Muscle strips were mounted immediately after demembranation, covered with 250 mM sucrose, 10 mM Na$_2$EGTA, 10 mM HEPES, 1 mM CaCl$_2$, pH 7.4, and fixed in 3% paraformaldehyde, 2% sucrose with 0.1% Triton-X100 for 30 min at 4°C. Muscle strips were rinsed at least three times in PBS, fixed again in 1% paraformaldehyde and 2% sucrose for 30 min, rinsed again in PBS, and incubated for 60 min with fluorescein isothiocyanate (FITC)-phalloidin (1:200, Molecular Probes) diluted in PBS containing 1% Triton-X100. Coverslips were mounted with DAPI 60 anti-fade solution (25°C for 1 h) and visualized using a Nikon Eclipse 80i microscope equipped with an attached DS-5M digital camera.
Protein extraction. For investigation of protein phosphorylation, muscle strips were immersed in 10% trichloroacetic acid (TCA)/10 mM diithiothreitol (DTT) in acetone that had been precooled on dry ice or wet ice, washed (3 × 5 min) with DTT/acetone, and lyophilized overnight, and the dried tissues were cut into small pieces. SDS-PAGE sample buffer containing 0.1 M DTT (1 ml) was added. Tissue strips of comparable dimensions were extracted with identical volumes of SDS-gel sample buffer, and identical volumes of extract were loaded on gels for Western blot analysis. The amount of extract loaded depended on the sensitivity of the individual antibodies utilized. The samples were heated at 95°C for 10 min, rotated overnight in the cold room, and stored at −20°C until SDS-PAGE was performed.

**RESULTS**

Effects of ROK inhibition on urethral smooth muscle contraction. The effects of ROK inhibition on the contractile response of rabbit urethral smooth muscle to EFS, membrane depolarization with KCl, and α1-adrenoceptor stimulation by phenylephrine were investigated at 37°C. After dissection, mounting, and equilibration of the tissue, several control contraction-relaxation cycles were recorded. The tissue was then incubated with ROK inhibitor (Y27632 or H1152) for 15 min before two additional contraction-relaxation cycles in the continued presence of inhibitor. The ROK inhibitor was then washed out, and two control contraction-relaxation cycles were recorded again. Representative data showing the effects of H1152 on these contractile responses are shown in Fig. 2A with cumulative quantitative data for both inhibitors in Fig. 1B. The contractile responses to EFS, KCl, or phenylephrine were all markedly inhibited by both ROK inhibitors (Fig. 1, A and B). Furthermore, inhibition of ROK during steady-state force maintenance in the presence of phenylephrine elicited relaxation (Fig. 1C). The general protein kinase C (PKC) inhibitor, GF109203X, on the other hand, had no significant effect on the contractile response of the urethra to any of the stimuli (Supplemental Fig. 1). (Supplemental data for this article is available online at the *American Journal of Physiology-Renal Physiology* website.) The efficacy of the PKC inhibitor was verified by its ability to block the contractile response of the rabbit urethra to 0.5 μM PdBu: steady-state force in response to 0.5 μM PdBu (0.47 ± 0.13 g) was reduced to 0.20 ± 0.09 g (n = 6; P < 0.01) following preincubation with 2 μM GF109203X. It is noteworthy that the contractile response to PdBu was very slow and reached a steady-state level of force that was much less than that induced by KCl or phenylephrine.

To pursue the mechanism underlying the contractile responses and the effects of ROK inhibition shown in Fig. 1, it was necessary to use an experimental system that is amenable to rapid quenching for biochemical analysis. For this purpose, urethral muscle strips were mounted in a 1-ml cuvette at room temperature, and the effects of ROK inhibition on phenylephrine- and KCl-induced contraction were examined. Figure 2A shows the inhibition of phenylephrine-induced contraction by H1152 (1 μM): the mean maximal tension in response to phenylephrine in the presence of H1152 was 56.8% of control, compared with 118% of control following washout of the ROK inhibitor. The concentration dependence of H1152-induced relaxation of tissue precontracted with phenylephrine is shown in Fig. 2B. Additional experiments in which longer time intervals were used between successive additions of H1152 indicated that the concentration required for half-maximal relaxation was 0.3 μM H1152 (Fig. 2C). H1152 had a similar
inhibitory effect on KCl-induced contraction under these conditions (data not shown).

These results indicate that contraction of rabbit urethral smooth muscle evoked by EFS, membrane depolarization, or \(\alpha_1\)-adrenoceptor activation involves activation of ROK, but not PKC, and suggest that contraction of the urethra may involve a significant Ca\(^{2+}\) sensitization. Activation of ROK has been implicated in the inhibition of MLCP through the phosphory-

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**Fig. 1. Effects of ROK inhibition on urethral smooth muscle contraction.** Rabbit urethral smooth muscle strips were dissected, mounted on a force transducer at resting tension and perfused with Krebs solution at 37°C for at least 1 h. Two control contraction-relaxation cycles were recorded prior to incubation of the tissue with either H1152 (1 \(\mu\)M) or Y27632 (10 \(\mu\)M) for 15 min. Two contraction-relaxation cycles were recorded in the continued presence of inhibitor. The inhibitor was then washed out and two control contraction-relaxation cycles recorded again. A, representative traces depicting the effects of H1152 on contractions elicited by EFS (upper panel), KCl (middle panel) and phenylephrine (lower panel). B, cumulative data showing the effects of ROK inhibitors on contractions elicited by: electric field stimulation (EFS) at 4 Hz in the presence of H1152 (\(n = 6\)) or Y27632 (\(n = 8\) with Y27632 and \(n = 6\) following washout), KCl (80 mM) stimulation in the presence of H1152 (\(n = 5\) with H1152 and \(n = 7\) following washout) or Y27632 (\(n = 4\)), or phenylephrine (PHE; 10 \(\mu\)M) stimulation in the presence of H1152 (\(n = 6\)) or Y27632 (\(n = 9\) with Y27632 and \(n = 8\) following washout). Values indicate maximal tension as a percentage of the average tension of the initial control responses. Recovery (grey bars) indicates the average tension response following washout of the inhibitor (not significantly different from control). Statistically significant differences from control (absence of H1152), detected by one-way ANOVA with Dunnett’s post-hoc test, are indicated by asterisks (\(**P < 0.01\), \(n = 5\)). C, Effect of H1152 on sustained phenylephrine-induced contraction of urethral smooth muscle. Rabbit urethral smooth muscle strips equilibrated in Krebs solution at 37°C were contracted with phenylephrine (10 \(\mu\)M). Following washout and relaxation, a sustained contractile response was elicited with phenylephrine. H1152 (1 \(\mu\)M) was added in the continued presence of phenylephrine, following which both phenylephrine and H1152 were washed out and a final control sustained contraction elicited with phenylephrine.
lation of MYPT1 at T697 and/or T855 (16, 31, 39). ROK is also known to phosphorylate CPI-17 at T38, which converts it into a potent inhibitor of MLCP (27). Inhibition of MLCP activity would shift the kinase-phosphatase activity balance in favor of MLCK and therefore increase LC20 phosphorylation.

To initiate the investigation of the mechanism whereby ROK regulates urethral smooth muscle contraction, we measured LC20 phosphorylation levels in extracts of tissues quenched at rest and at the peak of contraction induced by phenylephrine or KCl. Phosphorylated and non-phosphorylated LC20 were separated by Phos-tag SDS-PAGE and detected by Western blotting with an antibody that recognizes both forms of the light chain (Fig. 3A): LC20 was phosphorylated to \( \sim 0.6 \text{ mol Pi/mol LC20} \) at resting tension (lanes 5 and 6). Western blotting with a phosphospecific antibody that recognizes only LC20 phosphorylated at S19, the MLCK site (Fig. 3B), and Phos-tag SDS-PAGE in the presence of EDTA to chelate Mn\(^{2+}\) ions (Fig. 3C), confirmed the identities of the bands as labeled. Thus only the slower migrating band was recognized by the phosphospecific antibody (Fig. 3B), and when Mn\(^{2+}\) ions were chelated with EDTA, LC20 migrated as a single band with the mobility of nonphosphorylated LC20 observed in the presence of Mn\(^{2+}\) (Fig. 3C).

Surprisingly, treatment with phenylephrine (Fig. 3A, lanes 1 and 2) or KCl (Fig. 3A, lanes 8 and 9) did not increase the level of LC20 phosphorylation. The cumulative quantitative data in Table 1 confirm this conclusion. Also shown in Fig. 3A, lanes 3 and 4, is LC20 mono- and diphosphorylation (S19 and T18) in rabbit urethral smooth muscle strips contracted in response to the phosphatase inhibitor calyculin-A. No LC20 diphosphorylation was detected in response to phenylephrine or KCl (Fig. 2).
strips treated with KCl (80 mM) or phenylephrine (10 μM) were investigated. Figure 4 shows that there was no change in LC20 phosphorylation throughout the time course of KCl- or phenylephrine-induced contraction. The position of the 26 kDa marker is indicated at the right.

3B). Preincubation with H1152 had no effect on LC20 phosphorylation in the presence of KCl or phenylephrine (Fig. 3D and Table 1).

The possibility arose that because LC20 phosphorylation was quantified after steady-state force was achieved, a transient increase in LC20 phosphorylation correlating with force development might have been missed. Therefore, the time courses of LC20 phosphorylation in response to treatment with KCl and phenylephrine were investigated. Figure 4 shows that there was, in fact, no change in LC20 phosphorylation throughout the time course of KCl- or phenylephrine-induced contraction.

MYPT1 phosphorylation. Since MYPT1 is a well-known substrate of ROK in various smooth muscles, we examined the phosphorylation of MYPT1 at the two ROK sites by performing Western blotting with phosphospecific antibodies. Both sites were phosphorylated at resting tension, and neither KCl nor phenylephrine changed the level of phosphorylation at either site under the peak of the contractile response (Fig. 5 and Table 2). Analysis of the time course of phosphorylation revealed that MYPT1 phosphorylation at T697 and T855 did not change during the contraction elicited by either KCl (Fig. 6, A and C) or phenylephrine (Fig. 6, B and C). To determine whether T697 and T855 were stoichiometrically or only partially phosphorylated, MYPT1 phosphorylation in intact rabbit urethral strips treated with KCl was compared with that in Triton-skinned tissues treated with the phosphatase inhibitor microcystin. Resting levels of MYPT1 phosphorylation at T697 and T855 were unaffected by KCl treatment of intact tissues, whereas substantial increases in phosphorylation at both sites occurred on treatment of the demembranated tissue with microcystin (Fig. 7). Densitometric analysis of the Western blots gave a ratio of KCl to control signals (normalized to calponin) of 1.03 ± 0.13 and 0.79 ± 0.21 (means ± SE, n = 3) for T697 and T855, respectively, and a ratio of microcystin to control of 2.03 ± 0.08 and 8.78 ± 1.00 (means ± SE, n = 3) for T697 and T855, respectively.

CPI-17. Although CPI-17 is well established as a PKC substrate (24, 27), it also has been implicated as a ROK substrate, at least in vitro (15, 25). Phosphorylation by both kinases occurs at T38 and renders CPI-17 a potent MLCP inhibitor. Therefore, we investigated the expression of CPI-17 in the rabbit urethera. Consistent with previous findings with nonvascular smooth muscles (43), the level of CPI-17 in the urethera is considerably less than in vascular smooth muscles and comparable to the level in bladder (Supplemental Fig. 2). Given the low tissue content of CPI-17, the fact that LC20 phosphorylation was unaffected by ROK inhibition (Fig. 3D and Table 1), and the lack of effect of PKC inhibition on urethral contractility (Supplemental Fig. 1), we decided not to pursue the analysis of CPI-17 phosphorylation.

Cofilin phosphorylation. LIM kinase (10) has also been shown to be a ROK substrate, and phosphorylation at T508 within the activation loop activates this kinase (32). Activated LIM kinase in turn phosphorylates the actin-capping and -severing protein cofilin at S3 (7, 44). In the nonphosphorylated state, cofilin binds to and severs actin filaments, and this effect is alleviated on phosphorylation at S3 (1). We examined the time course of cofilin phosphorylation in rabbit urethral smooth muscle in response to KCl and phenylephrine by performing

Table 1. LC20 phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine, and the effect of ROK inhibition

<table>
<thead>
<tr>
<th>Conditions</th>
<th>LC20 Phosphorylation, mol Pi/mol LC20</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.65 ± 0.02 (21)</td>
</tr>
<tr>
<td>KCl</td>
<td>0.63 ± 0.03 (22)</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0.62 ± 0.03 (19)</td>
</tr>
<tr>
<td>H1152</td>
<td>0.55 ± 0.05 (7)</td>
</tr>
<tr>
<td>KCl + H1152</td>
<td>0.56 ± 0.03 (6)</td>
</tr>
<tr>
<td>Phenylephrine + H1152</td>
<td>0.57 ± 0.06 (7)</td>
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Values are means ± SE indicating phosphorylation stoichiometry; the no. of tissue strips analyzed (n) is shown in parentheses. Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine, or vehicle with or without preincubation with the Rho-associated kinase (ROK) inhibitor H1152 (see legend to Fig. 2). Tissues were immersed in trichloroacetic acid (TCA)-acetone-dithiothreitol (DTT) on wet ice at the peak of contraction, and phosphorylation of 20-kDa regulatory light chains of myosin (LC20) was analysed by Phos-tag SDS-PAGE. No significant differences from control were indicated by 1-way ANOVA with Dunnett’s post hoc test (P > 0.05).
Western blotting with a phosphospecific antibody that recognizes cofilin only when phosphorylated at S3. As shown in Fig. 8, cofilin was phosphorylated at S3 in the unstimulated tissue, and its phosphorylation level did not change significantly during the time course of contraction in response to either KCl or phenylephrine.

Actin polymerization. We also measured actin polymerization during the time course of the contractile response to KCl and phenylephrine. Urethral tissue strips were homogenized in F-actin stabilization solution at the times indicated in Fig. 4, and F- and G-actin were separated by high-speed centrifugation. Figure 9A demonstrates that SM-22, a 22-kDa smooth muscle-specific protein (30), was recovered exclusively in the high-speed supernatant and therefore provides a suitable protein for normalization of loading levels. The high-speed supernatant, containing all the G-actin, was then analyzed by Western blotting with anti-actin, and loading levels were normalized to SM-22 (Fig. 9B). If KCl and phenylephrine were to induce actin polymerization that is required for force production in the urethra, we would anticipate that the G-actin content would decline rapidly in response to both stimuli and would increase again during relaxation on washout. This was not found to be the case (Fig. 9B).

Comparison of LC20 phosphorylation in rabbit urethra, bladder, and aorta. The very high level of basal phosphorylation of LC20 in the rabbit urethra at resting tension (0.65 mol Pi/mol LC20) was unexpected, as was the absence of an increase in response to membrane depolarization or /β2-adrenoceptor activation (Table 1). Therefore, we investigated whether the same was true for another phasic urogenital muscle system.

Table 2. MYPT1 phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pT697</th>
<th>pT855</th>
</tr>
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<tbody>
<tr>
<td>KCl</td>
<td>0.86 ± 0.09 (7)</td>
<td>0.88 ± 0.118 (7)</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>1.25 ± 0.21 (9)</td>
<td>1.38 ± 0.14 (9)</td>
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Values indicate means ± SE of signal intensities relative to control after normalization of loading levels; n is shown in parentheses. Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2). Tissues were immersed in TCA-acetone-DTT on wet ice at the peak of contraction, and phosphorylation of the myosin-regenerating subunit of myosin light chain phosphatase (MYPT1) at T697 and T855 was analyzed by Western blotting with phosphospecific antibodies. No significant differences from control (set at a value of 1) were indicated by Student’s t-test (P > 0.05).
smooth muscle (bladder) and a tonic vascular smooth muscle (aorta). As for the urethra, the basal level of LC20 phosphorylation in bladder smooth muscle was high and did not significantly change in response to KCl or phenylephrine (Table 3). On the other hand, the basal level of LC20 phosphorylation in the aorta was lower and did increase in response to both KCl and phenylephrine (Table 3).

LC20 phosphorylation in rat urethra and bladder. The role of LC20 phosphorylation in contraction of urethra and bladder smooth muscles was also investigated in rat tissues. In contrast to the situation with rabbit tissues, LC20 phosphorylation in rat urethra and bladder was relatively low at resting tension and increased in response to both KCl and phenylephrine (Table 4).

Comparison of methods for quenching of tissues before LC20 phosphorylation analysis. During the course of this work, we discovered that the method for quenching of rabbit urethral smooth muscle strips for biochemical analysis was crucial. This is often achieved by immersing the tissue in TCA-acetone-DTT on dry ice or by clamping the tissue between liquid nitrogen-cooled tongs, immersing it in TCA-acetone-DTT on dry ice, and slowly warming the tissue to room temperature in TCA-acetone-DTT. However, we found that these procedures resulted in very low levels of LC20 phosphorylation (Table 5). On the other hand, immersing the tissue in TCA-acetone-DTT on wet ice gave much higher and reproducible phosphorylation stoichiometry (Table 1). We also quenched the tissues by immersion in liquid nitrogen, followed by transfer directly to boiling SDS-gel sample buffer. After incubation at 95°C for 10 min, samples were rotated overnight at 4°C before Phos-tag SDS-PAGE. As shown in Supplemental Figure 3 and Supplemental Table 1, very low levels of LC20 phosphorylation were measured under these conditions. In addition, rabbit urethral smooth muscle strips were clamped between liquid nitrogen-cooled tongs at rest and at the plateau of KCl- or phenylephrine-induced contractions and then treated in one of the following ways: 1) tissues were immersed in TCA-acetone-DTT on wet ice, 2) tissues were immersed in TCA-acetone-DTT on dry ice, or 3) tissues were immersed in liquid nitrogen. Tissues from treatments 1 and 2 were then lyophilized overnight after washing with acetone-DTT. All three sets of tissues were then immersed in boiling SDS-gel sample buffer, maintained at 95°C for 10 min, and rotated at 4°C overnight before Phos-tag SDS-PAGE to quantify LC20 phosphorylation levels. The results confirmed a high level of resting LC20 phosphorylation when tissues freeze-clamped.

Fig. 6. Time courses of MYPT1 phosphorylation in rabbit urethral smooth muscle treated with KCl or phenylephrine. Rabbit urethral smooth muscle strips were treated with 80 mM KCl (A) or 10 μM phenylephrine (B) and the phosphorylation of MYPT1 was analysed in triplicate at selected times during the contractile response by western blotting with phosphospecific antibodies that recognize MYPT1 phosphorylated at T697 or T855. Loading levels were normalized to actin. Numbers beneath gel lanes indicate the times during contractions at which tissues were quenched for western analysis of MYPT1 phosphorylation (see Fig. 4A and B). C, Cumulative data (values indicate the mean ± SEM, n = 4 for KCl and n = 3 for phenylephrine).

Fig. 7. Phosphatase inhibition with microcystin markedly increases MYPT1 phosphorylation at T697 and T855. Rabbit urethral smooth muscle strips (intact or Triton-skinned) were treated with KCl (80 mM, intact strips), microcystin (10 μM, Triton-skinned strips) or vehicle (control, intact strips) and the phosphorylation of MYPT1 (130 kDa) analysed in triplicate by western blotting with phosphospecific antibodies. Loading levels were normalized to calponin (32 kDa). “M” denotes the molecular weight marker lane.
with liquid nitrogen-cooled tongs were immersed in TCA-acetone-DTT on dry ice. Even lower levels of LC20 phosphorylation were measured when tissues freeze-clamped with liquid nitrogen-cooled tongs were immersed in liquid nitrogen.

*Effects of removal of extracellular Ca2+ and preincubation with wortmannin on rabbit urethral smooth muscle contraction and LC20 phosphorylation.* Finally, to identify the kinase responsible for the high level of LC20 phosphorylation in the rabbit urethra under resting conditions, we investigated the effect on LC20 phosphorylation of removal of extracellular Ca2+ and preincubation with wortmannin (10 μM), which at this concentration is a MLCK inhibitor. Incubation of urethral tissue strips in Ca2+-free Krebs solution containing 10 mM EGTA for 30 min resulted in a marked decrease in LC20 phosphorylation and resting tension (Table 6). Treatment with KCl (80 mM) or phenylephrine (10 μM) after preincubation of tissue strips in Ca2+-free Krebs solution containing 10 mM EGTA for 30 min failed to elicit a contractile response or an increase in LC20 phosphorylation (Table 6). Preincubation of tissue strips with wortmannin (10 μM) in Krebs solution also reduced the resting level of LC20 phosphorylation and reduced resting tension by over 50% (Table 6). Addition of KCl (80 mM) after incubation with wortmannin for 30 min failed to elicit a contractile response or an increase in LC20 phosphorylation (Table 6).

**DISCUSSION**

The most interesting findings from this study were that the urethra and bladder of the rabbit had a high level of LC20 phosphorylation at rest (Figs. 3 and 4 and Table 1), despite inducing robust contractile responses (Figs. 1 and 2). Vascular smooth muscle of the rabbit (aorta), on the other hand, behaved as expected; i.e., LC20 phosphorylation increased in response to membrane depolarization and α1-adrenoceptor stimulation (Table 3). Rat urethra and bladder, however, had a low level of LC20 phosphorylation at rest, which increased in response to membrane depolarization and α1-adrenoceptor stimulation, indicating species specificity (Table 4). It will be important in the future to determine whether human urethral and bladder smooth muscles behave like the rabbit or rat counterparts.

Another key finding from this study was that the contractile responses to electric field stimulation, KCl, and phenylephrine were all potently inhibited by Y27632 and H1152, two structurally unrelated inhibitors of ROK (Figs. 1 and 2). It is important to note that, although highly selective, these compounds can inhibit other kinases such as PKC-related kinase-2 (PRK2), AMP-activated protein kinase (AMPK), and, to a lesser degree, pyruvate dehydrogenase kinase-11 (PDK11) (9). PRK2 and PDK11 have not been implicated in the regulation of smooth muscle contraction, and AMPK has been shown to phosphorylate and inactivate smooth muscle MLCK (20). Given our observation that Y27632 and H1152 had no effect on LC20 phosphorylation (Fig. 3D and Table 1), it is unlikely that the inhibitory effects of Y27632 and H1152 on contraction involve inhibition of AMPK.

The RhoA/ROK pathway has previously been implicated in agonist-induced contraction of the rat urethra (37) and spontaneous tone in female porcine urethral smooth muscle (28). Extensive studies of various smooth muscles have implicated the RhoA/ROK pathway in Ca2+ sensitization, i.e., an increase in force at a given [Ca2+]. This pathway results in inhibition of MLCP via phosphorylation of the myosin-targeting subunit of MLCP (MYPT1) and/or CPI-17 by ROK, which results in increased LC20 phosphorylation and contraction (33).
Tissues were immersed in TCA-acetone-DTT on wet ice at the peak of contraction, and LC20 phosphorylation was analyzed by Phos-tag SDS-PAGE. No statistically significant differences from control were indicated by 1-way ANOVA with Dunnett’s post hoc test. Another ROK substrate that may play a role in regulation of contractility is LIM kinase, which contains two LIM domains, zinc finger domains originally identified in the proteins Lin11, Isl-1, and Mec-3 (8). This kinase is activated by ROK-catalyzed phosphorylation within the activation loop. The activated LIM kinase phosphorylates the actin-capping and -severing protein cofilin at S3, whereupon it loses the ability to bind to actin and sever actin filaments (10). Actin polymerization has been implicated in smooth muscle contraction, and dephosphorylation of cofilin at S3 favors actin polymerization by increasing the availability of barbed ends of actin filaments.

Table 3. LC20 phosphorylation in rabbit urethral, bladder, and aortic smooth muscles treated with KCl or phenylephrine

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Urethra</th>
<th>Bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.67 ± 0.07</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>KCl</td>
<td>0.70 ± 0.03</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0.70 ± 0.02</td>
<td>0.59 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE indicating phosphorylation stoichiometry; n = 6 for urethra, 3 for bladder, and 5 for aorta. Rabbit urethral, bladder, and aortic smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2). Tissues were immersed in TCA-acetone-DTT on wet ice at the peak of contraction, and LC20 phosphorylation was analyzed by Phos-tag SDS-PAGE. *P < 0.05, significantly different from control as indicated by 1-way ANOVA with Dunnett’s post hoc test.

Table 4. LC20 phosphorylation in rat urethral and bladder smooth muscles treated with KCl or phenylephrine

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Urethra</th>
<th>Bladder</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.13 ± 0.05</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>KCl</td>
<td>0.37 ± 0.07*</td>
<td>0.43 ± 0.06*</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0.41 ± 0.04†</td>
<td>0.45 ± 0.06*</td>
</tr>
</tbody>
</table>

Values are means ± SE indicating phosphorylation stoichiometry; n = 4. Rat urethral and bladder smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2). Tissues were immersed in TCA-acetone-DTT on wet ice at the peak of contraction, and LC20 phosphorylation was analyzed by Phos-tag SDS-PAGE. *P < 0.5; †P < 0.01, significantly different from control as indicated by 1-way ANOVA with Dunnett’s post hoc test.

Table 5. LC20 phosphorylation in rabbit urethral smooth muscle treated with KCI or phenylephrine and quenched in TCA-acetone-DTT on dry ice or clamped between liquid nitrogen-cooled tongs

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Treatment 1</th>
<th>Treatment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05 ± 0.01 (14)</td>
<td>0.04 ± 0.01 (3)</td>
</tr>
<tr>
<td>KCl</td>
<td>0.12 ± 0.03 (10)</td>
<td>0.07 ± 0.02 (3)</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0.07 ± 0.02 (11)</td>
<td>0.04 ± 0.01 (3)</td>
</tr>
</tbody>
</table>

Values are means ± SE indicating phosphorylation stoichiometry; n is shown in parentheses. Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2). In treatment 1, tissues at the peak of contraction were immersed in TCA-acetone-DTT on dry ice and then washed (3 × 1 ml) in acetonate-DTT before the liquid was poured off and the tissue was lyophilized. In treatment 2, tissues at the peak of contraction were quickly frozen by being clamped between liquid nitrogen cooled tongs, immersed in TCA-acetone-DTT on dry ice, slowly (1 h) warmed to room temperature, and washed (3 × 1 ml) in acetonate-DTT, and then the liquid was poured off and the tissue was frozen on dry ice and lyophilized. LC20 phosphorylation was analyzed by Phos-tag SDS-PAGE. No statistically significant differences from control were indicated by 1-way ANOVA with Dunnett’s post hoc test (P > 0.05).
in the rabbit urethra at rest and in the presence of KCl and phenylephrine.

The method used to quench the urethral tissue strips for biochemical analysis proved crucial. If tissues were immersed in TCA-acetone-DTT on dry ice or clamped between liquid nitrogen-cooled tongs before immersion in TCA-acetone-DTT on dry ice and gradually warmed to room temperature, the levels of LC20 phosphorylation were consistently very low at rest and did not change in response to KCl or phenylephrine (Table 5). On the other hand, if tissues were immersed in TCA-acetone-DTT on wet ice, the levels of LC20 phosphorylation were consistently much higher at rest and again did not change in response to KCl or phenylephrine (Table 1). These results suggest that the use of TCA-acetone-DTT on dry ice does not rapidly quench cellular biochemical reactions so that LC20 is dephosphorylated by MLCP that remains active under these conditions. If the tissue was freeze-clamped between liquid nitrogen-cooled tongs and subsequently immersed in TCA-acetone-DTT on wet or dry ice, or in liquid nitrogen, differences in LC20 phosphorylation levels were again observed (Table 5 and Supplemental Table 2). In the case of immersion in wet ice-cooled TCA-acetone-DTT, resting LC20 stoichiometry was 0.36 mol P/mol LC20, which did not change in response to contractile stimuli (Supplemental Table 2). On the other hand, very low levels of LC20 phosphorylation were measured in the cases of tissues that had been freeze-clamped between liquid nitrogen-cooled tongs and then immersed in dry-ice-cooled TCA-acetone-DTT or liquid nitrogen. Furthermore, for tissues that were immersed directly in liquid nitrogen and then transferred to boiling SDS-gel sample buffer, the measured stoichiometry of LC20 phosphorylation was again very low (Supplemental Fig. 3 and Supplemental Table 1), consistent with a very high tissue phosphatase activity. We interpret these results as follows: freeze-clamping the tissue between liquid nitrogen-cooled tongs or plunging the tissue in liquid nitrogen or dry ice-cooled TCA-acetone-DTT dramatically reduces the phosphatase activity, but as the tissue warms up in SDS-gel sample buffer or TCA-acetone-DTT, the phosphatase becomes active again and dephosphorylates LC20 before a sufficiently high temperature is reached to denature the phosphatase. It appears unlikely that immersion of tissue in wet ice-cooled TCA-acetone-DTT would lead to activation of MLCK, since the kinase requires Ca2+, Mg2+, and ATP for activity and none are present in the quench solution. Our observation that immersion of unstimulated rat tissues in wet ice-cooled TCA-acetone-DTT gave the expected low basal levels of LC20 phosphorylation also argues against activation of MLCK under these quenching conditions. It is also clear from the results presented that any manipulation involving rapid freezing of the rabbit urethra results in low levels of LC20 phosphorylation under both resting and stimulated conditions. In particular, it is apparent from Supplemental Table 2 that freeze-clamping the tissue with liquid nitrogen-cooled tongs followed by immersion in wet ice-cooled TCA-acetone-DTT yields lower and more variable LC20 phosphorylation stoichiometry (0.36 ± 0.07 mol P/mol LC20) than direct immersion in wet ice-cooled TCA-acetone-DTT (0.65 ± 0.02 mol P/mol LC20), consistent with phosphatase activity dephosphorylating LC20 during sample workup. Furthermore, we compared the basal level of LC20 phosphorylation at 0.5 g of resting tension with that when no tension was applied to the tissue and found

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Table 6. Effects of removal of extracellular Ca2+ and preincubation with wortmannin on LC20 phosphorylation and contraction in rabbit urethral smooth muscle

<table>
<thead>
<tr>
<th>Conditions</th>
<th>LC20 Phosphorylation, mol P/mol LC20</th>
<th>Contractile Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.70 ± 0.02</td>
<td>None</td>
</tr>
<tr>
<td>0 Ca2+</td>
<td>0.13 ± 0.08†</td>
<td>Relaxation (43.8 ± 5.8%)</td>
</tr>
<tr>
<td>KCl</td>
<td>0.66 ± 0.26</td>
<td>Contraction</td>
</tr>
<tr>
<td>0 Ca2+ + KCl</td>
<td>0.16 ± 0.03†</td>
<td>None</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0.65 ± 0.05</td>
<td>Contraction</td>
</tr>
<tr>
<td>0 Ca2+ + phenylephrine</td>
<td>0.06 ± 0.04†</td>
<td>None</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>0.16 ± 0.07†</td>
<td>Relaxation (52.8 ± 9.0%)</td>
</tr>
<tr>
<td>Wortmannin + KCl</td>
<td>0.06 ± 0.02†</td>
<td>None</td>
</tr>
</tbody>
</table>

Values are means ± SE indicating phosphorylation stoichiometry; n = 2 tissue strips from each of 2 animals in each case. Rabbit urethral smooth muscle strips were treated with KCl, phenylephrine, or vehicle (see legend to Fig. 2), and the contractile responses were recorded. Tissues were immersed in TCA-acetone-DTT on wet ice, and LC20 phosphorylation was analyzed by Phos-tag SDS-PAGE. †P < 0.01, significantly different from control as indicated by 1-way ANOVA with Dunnett’s post hoc test.

(17). This would predict that dephosphorylation of cofilin, and not ROK/LIM kinase-mediated phosphorylation, at S3 would occur in response to contractile stimulation, and a decrease in phosphocofilin has been demonstrated in canine tracheal smooth muscle treated with acetylcholine or KCl, which is associated with actin polymerization and contraction (45). Nevertheless, we investigated cofilin phosphorylation in rabbit urethral smooth muscle. Although cofilin exhibited a basal level of S3 phosphorylation under resting tension, there was no change in cofilin phosphorylation in response to membrane depolarization or α1-adrenoceptor stimulation (Fig. 8). Furthermore, there was no change in G-actin content in response to depolarization or 1-adrenoceptor stimulation (Fig. 9). Furthermore, for tissues that were immersed directly in liquid nitrogen and then transferred to boiling SDS-gel sample buffer, the measured stoichiometry of LC20 phosphorylation was again very low (Supplemental Fig. 3 and Supplemental Table 1), consistent with a very high tissue phosphatase activity.
no statistically significant difference indicated by Student’s t-test (P > 0.05): 0.66 ± 0.03 (n = 18) and 0.71 ± 0.11 (n = 10) mol P/mol LC20, respectively. Also, if tissue at zero tension was quenched with dry ice-cooled TCA-acetone-DTT, low levels of LC20 phosphorylation were measured (0.11 ± 0.05 mol P/mol LC20; n = 6). Caution must therefore be exercised when choosing a method of tissue quenching for quantification of LC20 phosphorylation in rabbit tissues.

Future studies need to be directed toward identification of ROK substrates in rabbit urethra with a view to defining the mechanism of activation of contraction without an increase in LC20 phosphorylation.

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REFERENCES


