Obstruction-induced changes in urinary bladder smooth muscle contractility: a role for Rho kinase

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Submitted 21 October 2002; accepted in final form 30 June 2003

Bing, Wu, Shaohua Chang, Joseph A. Hypolite, Michael E. DiSanto, Stephen A. Zderic, Lester Rolf, Alan J. Wein, and Samuel Chacko. Obstruction-induced changes in urinary bladder smooth muscle contractility: a role for Rho kinase. Am J Physiol Renal Physiol 285: F990–F997, 2003. First published July 8, 2003; 10.1152/ajprenal.00378.2002.—Detrusor smooth muscle (DSM) undergoes hypertrophy after partial bladder outlet obstruction (PBOO) in male rabbits, as it does in men with PBOO induced by benign prostatic hyperplasia. Despite detrusor hypertrophy, some bladders are severely dysfunctional (decompensated). In this study, the rabbit model for PBOO was used to determine the biochemical regulation of the contractile apparatus and force maintenance by the detrusor from decompensated bladders (DB). Bladders from sham-operated rabbits served as a control. On stimulation with 125 mM KCl, the DSM from sham-operated (SB) rabbits showed phasic contractions, whereas the detrusor from DB was tonic, exhibiting slow development of force, a longer duration of force maintenance, and slow relaxation. The Rho kinase (ROK) inhibitor Y-27632 enhanced the relaxation of precontracted DSM strips from DB. The enhancement of relaxation of the KCl-induced contraction of DB by Y-27632 was associated with dephosphorylation of myosin light chain (MLC20). The DSM extract from DB showed low phosphatase activity compared with that from SB. The DB also showed more Ca2+-independent MLC phosphorylation, which was partially inhibited by Y-27632. RT-PCR and Western blotting revealed similar expression levels of MLC kinase and ROK-α in SB and DB, but ROK-β was overexpressed in DB. These results suggest that the ROK-mediated pathway is partly responsible for the high degree of force maintenance and slow relaxation in the detrusor from DB.

The rabbit model of partial bladder outlet obstruction (PBOO) has been used to demonstrate that such an obstruction induces a significant increase in bladder mass (22). PBOO also results in an alteration of DSM contractility and bladder function. However, the degree of bladder remodeling and dysfunction caused by PBOO varies, and a suitable marker for the clinical identification of bladder remodeling is not available.

In some obstructed rabbits, as in humans, detrusor hypertrophy and the associated remodeling are sufficient to maintain bladder function close to normal (compensated), whereas other rabbits show severe bladder dysfunction, i.e., increased void frequency, decreased void volume, and increased residual volume (38). Hypertrophied detrusor muscle from the severely dysfunctional (decompensated) bladder shows altered contractile characteristics (23). The mechanisms that lead to alterations in detrusor contractility are not understood, but hypertrophied detrusor muscle shows tonic characteristics, e.g., slow generation of force and long duration of force maintenance, compared with the phasic contraction of normal detrusor muscle (14, 33). Associated with the change in contractile characteristics, an alteration in the composition of myosin II isoforms has also been reported (11). Myosin II in smooth muscle and nonmuscle cells is activated by actin when the regulatory light chains (myosin light chain (MLC20)) are phosphorylated by a Ca2+-calmodulin-dependent MLC kinase (MLCK) (1, 5, 6, 13). Dephosphorylation of MLC20 by a Ca2+-independent phosphatase (MLCP) lowers the actin-activated ATPase activity of myosin (29). A correlation between myosin phosphorylation-dephosphorylation and contraction-relaxation of the smooth muscle has been demonstrated in several laboratories (3, 9, 16). Myosin phosphorylation and subsequent force can be maintained by inhibiting MLCP (19).

MLCP is regulated by the small GTPase RhoA and Rho-associated kinase (ROK) (17). The inhibition of MLCP by RhoA/ROK is associated with phosphorylation of the MLCP regulatory subunit MYPT-1 and with smooth muscle contraction in the absence of a rise in intracellular Ca2+ concentrations (18, 20). Thus ROK-

PARTIAL OBSTRUCTION of the urinary bladder outlet, one of the major effects of benign prostatic hyperplasia in men, leads to an impaired ability of the bladder to store and empty urine. To generate the increased force required to expel urine against the obstruction, the detrusor smooth muscle (DSM) undergoes hypertrophy.

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mediated regulation of MLC phosphorylation increases the Ca²⁺ sensitivity of smooth muscle contraction (12, 31). There are no data on the expression of ROK or MLCK in detrusor muscle from bladders with obstruction-induced hypertrophy and bladder dysfunction.

The present study was undertaken to determine whether the tonic contractile characteristics exhibited by the hypertrophied detrusor muscle from severely dysfunctional bladders are associated with a change in ROK expression by myocytes and with subsequent inhibition of MLCP activity.

**METHODS**

**PB00.** The protocol for animal surgery was approved by the University of Pennsylvania’s Institutional Animal Care Use Committee. Adult male New Zealand White rabbits weighing an average of 6 pounds were sedated with ketamine-xylazine (25 mg·10 mg⁻¹·kg body wt⁻¹ im). Once sedation was achieved, deep anesthesia was induced with 4–5% isoflurane administered by face mask with monitoring by pulse oximetry. After the animal was shaved and prepared under sterile conditions, an 8-Fr coude-tip catheter was passed into the bladder, and the bladder neck and urethra were exposed via a midline and retroperitoneal incision. Partial outlet obstruction was created by passing a 2-0 silk tie around the urethra. A second 8-Fr catheter was placed outside the urethra, and the silk suture was tied down with a fentanyl patch. Sham surgery was performed in an identical manner, except the silk suture was cut and removed before closure of the abdomen.

**Metabolic monitoring of bladder function.** After 11 days of outlet obstruction, rabbits were transferred to metabolic cages and given 24 h to acclimate to their new surroundings. Thereafter, monitoring was initiated, with food and water given ad libitum. For ≥24 h, urine output was directed to digital scales interfaced to a personal computer, which were monitored at 2-min intervals (Kent Scientific, Torrington, CT). Data transfer from the acquisition software to an Excel spreadsheet allowed for calculation of urinary frequency (voids/24 h) and average voided volume per 24 h. Fourteen-day-obstructed rabbits were grouped as compensated (<30 voids/24 h) or decompensated (>30 voids/24 h). The void volume per void for the compensated and decompensated groups ranged from 18 to 32 ml and from 2 to 4 ml, respectively (Table 1). The urinary frequencies of normal unoperated and sham-operated rabbits were 4–7 and 6–9 voids/24 h, respectively. The volume per void for unoperated normal and sham-operated normal rabbits was similar (31–48 and 28–44 ml, respectively).

**Tissue preparation for analyses.** After completion of the metabolic cage studies, five sham-operated rabbits and five obstructed rabbits that showed decompensated bladder function (>30 voids/24 h and ~2–3 ml/void and 6 ± 3 ml/void in obstructed and sham-operated rabbits, respectively) were selected for the study. The rabbits were sedated with an intramuscular injection of ketamine-xylazine and anesthetized with pentobarbital sodium (25 mg/kg iv). The bladder was removed through an abdominal incision, emptied, and washed in ice-cold phosphate-buffere saline. After the bladder was removed, the animal was euthanized with beuthanasia. As shown in Table 1, the bladder mass for the decompensated bladders (DB) was 9.8 ± 0.7 g compared with 2.3 ± 0.4 g for the sham-operated rabbits (SB). The mucosal and serosal layers were removed, with care taken to not disturb the muscle layer. For physiological studies, the bladder smooth muscle strips (8 × 3 mm) from the midregion of the bladder body were removed and kept in Tyrode solution at 37°C in the presence of 95% O₂-5% CO₂.

For biochemical and molecular biological analyses, the bladder muscle layer from the midbody region was quickly frozen and stored in liquid nitrogen until it was used. Sections (2- to 4-cm long) of the midbladder wall were fixed in 10% buffered (pH 7.4) formalin and processed for paraffin sectioning and used for immunofluorescence studies.

**Phosphatase and kinase assay.** For determination of the phosphatase and kinase activities, the frozen detrusor muscle tissue was pulverized in the presence of liquid nitrogen with use of a porcelain mortar and pestle. The muscle powder was transferred to a centrifuge tube and homogenized in the extraction buffer (60 mM KCl, 20 mM imidazole-HCl, pH 7.1, 4 mM EDTA, 10 mM ATP, and 5 mM DTT) using a Polytron (Brinkman). The muscle homogenate was centrifuged (40,000 g for 15 min), and the supernatant was collected and dialyzed for 4 h against phosphorylation buffer (60 mM KCl, 20 mM imidazole-HCl, pH 7.1, and 10 mM DTT). A protease inhibitor cocktail containing 0.4 mM PMSF, 1 μM antipain, 1 μM pepstatin A, and 5 μM trypsin inhibitor was present in all buffers.

Phosphatase activity in the muscle extract was determined by the release of ³²P from column-purified gizzard smooth muscle myosin previously labeled with [γ-³²P]ATP (4). The concentration of phosphorylated myosin in the phosphatase assay mixture was adjusted to obtain ~15,000 cpm/alloquint (25 μl) removed at the initial time point. The reaction was started by addition of 150 μg of ³²P-labeled myosin (dialyzed against phosphorylation buffer) to the assay mixture (0.15 ml) containing 0.1 ml DSM tissue extract (10 mg/ml) dialyzed against phosphorylation buffer and 5 mM MgCl₂. The assay mixture was incubated at 25°C, and aliquots (25 μl) were taken at 30 s and thereafter at 1-min intervals and added to TCA (10% final concentration) to stop the reaction. The TCA-precipitated protein was heated at

<table>
<thead>
<tr>
<th>Table 1. Urinary bladder function</th>
<th>Unoperated Control</th>
<th>Sham Operated</th>
<th>Compensated</th>
<th>Decompensated</th>
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<tr>
<td></td>
<td>(n = 7)</td>
<td>(n = 8)</td>
<td>(n = 11)</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Bladder mass, g</td>
<td>2 ± 0.1</td>
<td>2.3 ± 0.4</td>
<td>4.4 ± 0.4*</td>
<td>9.8 ± 0.7*</td>
</tr>
<tr>
<td>Frequency, voids/24 h</td>
<td>4 ± 3</td>
<td>6 ± 3</td>
<td>14 ± 9*</td>
<td>43 ± 12*</td>
</tr>
<tr>
<td>Volume/void, ml</td>
<td>31 ± 17</td>
<td>26 ± 16</td>
<td>18 ± 14*</td>
<td>2.5 ± 1*</td>
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Values are means ± SE. We used only obstructed bladders that were decompensated (>30 voids/24 h). Sham-operated rabbits were used as control. *P < 0.05 vs. control or sham control group.

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Fig. 1. Profile of force generation and maintenance by detrusor smooth muscle (DSM) strips from sham (SB) and decompensated bladders (DB). Force profile from a representative muscle strip from SB is compared with DB (n = 5). Force is standardized to muscle strip weight for SB and DB. Muscle strips of similar length and width were stimulated to contract with 125 mM KCl. Force generation in DB DSM is slower but is maintained for a longer period than in SB. Unlike phasic contractile characteristics of SB, DB shows prolonged force maintenance to the point of approaching tonic contraction. Arrow, addition of KCl.

90°C for 45 min and chilled in ice, and 32P release was determined by filtering the protein with a Millipore manifold filter and counting the radioactivity remaining in the protein precipitate, as described elsewhere (4).

Ca2+-independent phosphorylation of MLC was determined by assaying the kinase activity in the DSM extract in the presence of 2 mM EGTA as described by Chacko (4). Briefly, 100 µl of DSM tissue extract (10 mg/ml) was assayed in a total volume of 5 µl containing 5 mM MgCl2, 2 mM EGTA, and 10 mM imidazole-HCl (pH 7.1). The myosin in the extract and exogenously added unphosphorylated myosin (50 µg) purified from chicken gizzard served as substrate. The reaction was started by addition of ATP mixture: 2 mM ATP containing 5 mM MgCl2, 2 mM EGTA, and 10 mM imidazole-HCl (pH 7.1). The myosin in the extract and exogenously added unphosphorylated myosin (50 µg) purified from chicken gizzard served as substrate. The reaction was started by addition of ATP mixture: 2 mM ATP 

Table 2. MLC20 phosphorylation during force maintenance

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<tr>
<th>Time Muscle Strips Were Freeze-Clamped</th>
<th>Phosphorylated MLC20, %</th>
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<tr>
<td>After reaching maximum force</td>
<td>19.8 ± 1.77</td>
</tr>
<tr>
<td>20 min after reaching maximum force</td>
<td>17.83 ± 1.66</td>
</tr>
<tr>
<td>20 min after reaching maximum force</td>
<td>7.76 ± 1.25*</td>
</tr>
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</table>

Values are means ± SE. Muscle strips were freeze-clamped at indicated time, pulverized, and processed for 2-dimensional gel electrophoresis as described elsewhere (14). Percent phosphorylation of myosin light chain (MLC20) was determined by scanning densitometry. *Significant decrease.

Fig. 2. Relaxation of precontracted DSM strips from DB by Rho-associated kinase (ROK) inhibitor Y-27632. DSM strips from DB were stimulated to contract by addition of 125 mM KCl. After the muscle developed maximal force, Y-27632 was added to the muscle bath to obtain a final concentration of 50 µM. Data were collected from 5 experiments using DSM from 5 DB rabbits (P < 0.05, n = 5).

Fig. 3. Phosphatase activity in DSM extracts. Myosin phosphatase activity in extracts derived from DSM of SB and DB was measured using 32P-phosphorylated purified smooth muscle myosin as a substrate. Experiments were repeated 4 times with different animals.
data are expressed as mean optical density (OD) units × area. All PCR products were sequenced to confirm their identities.

**SDS-PAGE and Western blot analysis.** Total extractable protein was isolated from SB and DB that were frozen in liquid nitrogen. Equal amounts of total protein were separated on 7.5% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. After the membranes were blocked with 5% fat-free milk for 1 h, they were incubated at 1:2,000 dilution of anti-MLCK (Calbiochem, San Diego, CA) and anti-ROK-α and anti-ROK-β antibody (Transduction Laboratories, Lexington, KY) for 2 h at room temperature. After the blot was washed, it was incubated with the secondary antibody anti-mouse Ig at 1:4,000 dilution (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. Between incubations, membranes were washed thoroughly with PBS containing 0.05% Tween 20. Antibody reactivity was detected using an enhanced chemiluminescence kit (Amersham Biosciences) and quantitated by scanning densitometry as described above. Amounts of protein loaded on gels for Western blotting were adjusted to the linear portion of the concentration vs. absorption curve.

**Histochemistry.** Paraffin sections (5 μm) were prepared from SB and DB. Briefly, tissue sections were placed in Histoclear solution and washed in descending grades of ethanol and, finally, in PBS. For immunostaining, slides were incubated for 30 min in 1% BSA to block nonspecific binding and then incubated for 1–2 h at room temperature with different primary antibodies [anti-MLCK (1:400), anti-ROK-α (1:200), or anti-ROK-β (1:200)]. Sections were washed three times in PBS and treated with secondary antibody (anti-mouse IgG-Cy3; catalog no. C-2181, Sigma, St. Louis, MO) at 1:400 dilution for 1 h, washed three times with PBS, and mounted with a drop of mounting medium (Aqua-Mount, Lerner Labs, Pittsburgh, PA). Sections were examined under a fluorescence microscope (Leitz) equipped for epifluorescence illumination. Negative control sections were incubated with secondary antibody only.

**Force measurements.** Bladder muscle strips from a total of five rabbits from SB and DB groups (~50 mg) were placed in Tyrode solution and suspended longitudinally in 10 ml of Tyrode buffer at 37°C in the presence of 95% O2-5% CO2. After 30 min of equilibration, the length of optimal force development (L0) was determined by increasing the length of

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**Fig. 4.** Kinase activity in the absence of Ca2+. 32P incorporated into the myosin light chain (MLC20) band from a Coomasie blue-stained gel is plotted as a function of time. A: kinase activity in the absence of Ca2+ (2 mM EGTA) in DSM extracts from SB and DB. B: effect of 5 μM Y-27632 on Ca2+-independent phosphorylation of MLC20. Values are means ± SE of 5 determinations of 4 different rabbits. P < 0.05.

**Fig. 5.** Expression of MLC kinase (MLCK) and ROK isoforms at the mRNA level. Representative ethidium bromide-stained agarose gels are shown from RT-PCR for MLCK (A), ROK-α (B), and ROK-β (C). Results from 2 SB (SB1 and SB2) and 2 DB (DB1 and DB2) rabbits are shown. PCR fragments appear to migrate to the predicted positions of 289 bp for MLCK, 202 bp for ROK-α, and 336 bp for ROK-β. α-Actin served as an internal standard. All PCR products were sequenced to confirm their identity. D: summary of densitometric analysis of PCR gels (n = 6). OD, optical density. *P < 0.05.
each strip in 1.5-mm increments until maximal contractile force to electrical field stimulation (80 V, 32 Hz, 1-ms duration) was achieved. Buffer was replaced with fresh buffer, and tissue was allowed to equilibrate at L_o for 15 min to allow stabilization at resting level. The muscle strips were then rapidly frozen in liquid nitrogen for analysis of phosphorylation levels at resting tone. Longitudinal bladder strips (~2 x 8 mm, 50 mg) were used for depolarization with high-KCl solution (125 mM) to evaluate tonic and phasic properties (32). All strips were prepared according to the protocol described above to reach L_o before stimulation with KCl, and force was measured as described previously (24). To determine the effect of inhibition of ROK on the maintenance of force, muscle strips were stimulated with 125 mM KCl, and the effect of Y-27632 at a constant concentration of 50 μM Y-27632 was determined. The effect of Y-27632 at a constant concentration of 50 μM Y-27632 was determined. The effect of Y-27632 at a constant concentration of 50 μM Y-27632 was determined. The effect of Y-27632 at a constant concentration of 50 μM Y-27632 was determined.

**RESULTS**

Some rabbits obstructed for 14 days revealed severe bladder dysfunction, despite smooth muscle hypertrophy. These bladders were classified as decompensated on the basis of the animal’s voiding pattern. As shown in Table 1, the urinary frequency and void volume for SB rabbits were 6 ± 3 voids/24 h and 26 ± 16 ml/void, respectively. However, the urinary frequency and volume per void for DB rabbits were 43 ± 12 voids/24 h and 2.5 ± 1 ml/void, respectively. SB weight averaged 2.3 ± 0.4 g, whereas DB weight was 9.8 ± 0.7 g (P < 0.05). Rabbits obstructed for 14 days and revealing severe bladder dysfunction, despite DSM hypertrophy, were used for physiological, biochemical, and immunofluorescence studies. SB rabbits served as control.

Figure 1 shows the typical profile of force generated by DSM strips from SB compared with that from DB. Muscle strips were stimulated with 125 mM KCl to circumvent any receptor-mediated changes in the smooth muscle cell membranes caused by hypertrophy. Muscle strips from SB developed a rapid transient force, followed by phasic contraction, whereas force development by strips from DB was slow. DB strips also exhibited a much longer duration of force maintenance and slower relaxation compared with the fast and steady relaxation of the strips from SB. The DSM strips from DB also showed higher amplitude of spontaneous contractile activity (Fig. 1).

To determine whether the long duration of force maintenance and slow relaxation shown by DB are due to ROK-mediated cascade, muscle strips from DB were precontracted with 125 mM KCl, and the effect of the ROK inhibitor Y-27632 on the relaxation was determined. The effect of Y-27632 at a constant concentration of 50 μM on the maximal force produced by DB is shown in Fig. 2. The force remained high at ~80% for 10 min and at 75% even after 20 min in the absence of Y-27632. In the presence of 50 μM Y-27632, the KCl-induced force decreased to ~50% in 10 min, reaching the basal level in 20 min. The level of MLC20 phosphorylation during force maintenance in the presence or absence of Y-27632 is shown in Table 2. The level of phosphorylation in the DB muscle strips was 19.8 ± 1.77% after reaching maximal force. At 20 min after addition of 50 μM Y-27632, the phosphorylation significantly decreased to 7.76 ± 1.25%, compared with 17.83 ± 1.66% in the absence of the inhibitor (P < 0.05, n = 5).

Because ROK has been shown to phosphorylate myosin phosphatase (34) and lower the myosin phosphatase activity, we compared the phosphatase activity in muscle extracts derived from SB and DB. As shown in Fig. 3, 80% of the covalently bound 32P was hydrolyzed in 30 s from phosphorylated myosin incubated with the DSM extract from SB compared with only 50% from DB. A significant difference in the dephosphorylation of phosphorylated myosin was also observed at 1 min between samples incubated with DSM extract from SB and DB (P < 0.05, n = 4), although at 3 min the myosin was completely dephosphorylated in both samples. Thus the dephosphorylation of the myosin incubated with the extract from DB was slower than that from SB, indicating a decrease in phosphatase activity in DB.

On the basis of the finding that ROK phosphorylates MLC20 directly in a Ca2+-independent manner (18), we examined the kinase activity in extracts from SB and DB in the presence of 2 mM EGTA. As expected, the MLCK activity was significantly lower in the extract from SB (P < 0.05, n = 4; Fig. 4A), because MLCK requires Ca2+ and calmodulin for activation. Although...
calmodulin was present in the extract from SB (data not shown), Ca$^{2+}$ was chelated with 2 mM EGTA. Under the same conditions, the extract from DB revealed a significant ($P < 0.05, n = 4$) level of MLC$_{20}$ phosphorylation, and this activity was partially inhibited by 5–10 μM Y-27632 (Fig. 4B).

Analysis of the expression of MLCK, ROK-α, and ROK-β at the mRNA level by RT-PCR revealed similar levels of MLCK transcripts in SB and DB (Fig. 5A), as well as similar ROK-α levels (Fig. 5B); however, the relative expression of mRNA for ROK-β was higher in DB than in SB (Fig. 5C). α-Actin mRNA used as an internal control for RT-PCR analyses was expressed to similar levels in SB and DB. Densitometric scanning of the ethidium bromide-stained bands representing the three molecules revealed a significant quantitative difference ($P < 0.05, n = 6$) only for expression of ROK-β mRNA (Fig. 5D). Western blot analysis using highly specific antibody confirmed the increased expression of ROK-β at the protein level in DB, whereas neither ROK-α nor MLCK differed in SB vs. DB (Fig. 6). A slight increase in the expression of MLCK was observed on Western blotting for some DB; however, the overexpression of MLCK was not significant (data not shown).

Immunofluorescence microscopy of DSM sections showed that antibodies specific to MLCK, ROK-α, and ROK-β localized to the smooth muscle bundles in SB and DB (Fig. 7). Compared with SB, immunofluorescence staining with antibody to ROK-β is more intense in DSM bundles from DB.

**DISCUSSION**

Some obstructed bladders fail to achieve normal bladder function, despite compensatory hypertrophy (21, 38, 39). In these experiments, SB generated and dissipated force rapidly in response to KCl, typical of a phasic smooth muscle (30). However, DB showed tonic characteristics. DB also showed increased amplitude of spontaneous activity (Fig. 1), a property consistent with the overactivity seen in obstructed human bladder in benign prostatic hyperplasia.

The present study focuses on the role of ROK in the regulation and maintenance of force in DSM, exhibiting dysfunction, despite hypertrophy, after PBOO. The RhoA-signaling pathway, through activation of ROK, has been shown to inhibit MLC$_{20}$ dephosphorylation (34) and maintain muscle tone in corpus cavernosum smooth muscle in vivo (7). The relaxant effects of the
ROK inhibitor Y-27632 on the maintenance of force by the DSM strips (Fig. 2) indicate a role for the RhoA/ROK-mediated pathway in detrusor from obstructed dysfunctional bladder.

Extracts from DB reveal very low myosin phosphatase activity compared with extracts from SB. MLC20 phosphorylation levels in the absence of Ca\(^{2+}\) are low for SB, because the MLCK is Ca\(^{2+}\)-calmodulin dependent (20). However, a significantly high MLC20 phosphorylation level was present in the absence of Ca\(^{2+}\) in the extract from DB (Fig. 4). The finding that this MLC20 phosphorylation is independent of Ca\(^{2+}\) and partially inhibited by Y-27632 suggests that it is mediated through the RhoA-ROK pathway. Interestingly, the ROK inhibitor did not completely inhibit the phosphorylation.

The extent of MLC20 phosphorylation in the cell is dependent on the activities of kinase and phosphatase, which catalyze the covalent binding of phosphate to specific amino acid residues (Ser19 and Thr18) and its removal, respectively. The low level of MLC20 phosphorylation by MLCK in the absence of Ca\(^{2+}\) does not lead to significant phosphorylation levels, because the MLCP is active under normal conditions in the cell. Consequently, the incorporation of \(^{32}\)P into MLC20 was very low in the absence of Ca\(^{2+}\) in the extract from SB. The high level of phosphorylation in the absence of Ca\(^{2+}\) in the extract from DB is due to direct phosphorylation of MLC20 (2) by ROK or the presence of other Ca\(^{2+}\)-independent kinases. Direct phosphorylation of MLC20 by a zil kinase (28) and/or integrin-linked kinase (8) may also account for myosin phosphorylation in the absence of Ca\(^{2+}\). Direct phosphorylation of Ser19 and Thr18, the same residues that are phosphorylated by MLCK, has been reported (25); however, the inhibition of phosphorylation by Y-27632 indicates that most of the MLC20 phosphorylation in the absence of Ca\(^{2+}\) is mediated through ROK.

An active RhoA-ROK cascade might inhibit the regulatory subunit of myosin phosphatase (MYPT-1) or act indirectly by phosphorylating another kinase, which in turn affects the phosphatase activity (26). High muscle tone in the presence of low intracellular Ca\(^{2+}\) concentrations in the corpus cavernosum smooth muscle is attributed to a ROK-mediated Ca\(^{2+}\) sensitization (37). ROK is highly expressed in the corpus cavernosum smooth muscle compared with smooth muscle from other sources (37).

The rate of dephosphorylation of phosphorylated gizzard myosin by endogenous phosphatase was significantly lower in the extract of DB than in the extract of SB (Fig. 3), further suggesting an increased level of ROK in DB. RT-PCR and Western blot analyses revealed similar levels of MLCK expression in SB and DB, consistent with the same level of MLCK activity in these bladders. As expected, MLCK activity in both muscles was Ca\(^{2+}\) sensitive. Protein and mRNA analyses also indicated overexpression of ROK-\(\beta\) in the detrusor from DB, a result consistent with the findings from immunofluorescence staining of smooth muscle cells of normal and decompensated bladders. Upregulation of Rho kinase mRNA has also been reported in uterine smooth muscle during pregnancy (35), although the expression of individual ROK isoforms and expression at the protein level remain to be investigated. ROK-\(\alpha\) and ROK-\(\beta\) isolated from smooth muscle and nonmuscle cells are 60% identical overall, and their kinase domains are 86% identified (15, 27, 36). Y-27632 is a competitive inhibitor of both isoforms (15).

The decrease in force and relaxation in the presence of Y-27632 indicates that the longer duration of force maintenance by the detrusor from DB is due to a RhoA/ROK-mediated pathway. The finding that ROK-\(\beta\) is overexpressed in the DB also points to an adaptation of the detrusor to facilitate force maintenance for a long period of time in an attempt to empty the bladder against the obstruction.

In conclusion, our data point to a role for an enhanced RhoA/ROK-mediated signal transduction pathway in the dysfunction of hypertrophied DSM from obstructed bladders. Involvement of this pathway suggests a remodeling in the regulation of myosin phosphorylation, which in turn regulates the actin-myosin interaction and contraction in decompensated detrusor. Ca\(^{2+}\) sensitization, mediated by the RhoA-ROK cascade, enables the DSM from DB to contract at low intracellular Ca\(^{2+}\) concentration. Further studies on the role of the Rho-ROK cascade in detrusor dysfunction are required to determine whether this mechanism is responsible for the pathophysiology of the DB, characterized by detrusor overactivity and incomplete emptying, despite detrusor hypertrophy.

We thank Marina Hoffman and Brian Siano for editorial assistance and photographic reproduction, respectively.

DISCLOSURES

S. Chang is a Research Scholar Fellow from the American Foundation for Urologic Diseases. This research is supported by National Institute of Diabetes and Digestive and Kidney Diseases George M. O’Brien Urology Research Center Grant P50 DK-52620.

REFERENCES


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