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Modulation of smooth muscle tonus in the lower urinary tract: Interplay of MLCK and MLCP

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Abstract

OBJECTIVE—Smooth muscle tonus in the bladder and urethra plays a critical role in the normal function of the lower urinary tract. The phosphorylation status of the myosin light chain (MLC) is a key regulator of smooth muscle tonus. MLC phosphorylation is modulated by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). We investigated expression and activity of MLCK and MLCP in the rat bladder and urethra.

MATERIALS AND METHODS—Bladder and urethral smooth muscles were obtained from 2-month-old female Sprague Dawley rats. Real-time PCR and Western blotting were used to assess expression of MLCK and myosin phosphatase-targeting subunit of protein phosphatase type 1 (MYPT1), an inhibitor of MLCP. A two-step enzymatic activity assay using phosphorylated and dephosphorylated smooth muscle myosin was used to assess MLCK and MLCP activity.

RESULTS—MLCK mRNA expression was higher in the bladder than the urethra but this difference was not statistically significant (0.26 ± 0.17 vs. 0.14 ± 0.12, p = 0.09). MYPT1 mRNA expression was significantly higher in the bladder than the urethra (2.31 ± 1.04 vs. 0.56 ± 0.36, p = 0.001). Expression of both MLCK and MYPT1 protein was significantly higher in the bladder compared to the urethra (1.63 ± 0.25 vs. 0.91 ± 0.29 and 0.97 ± 0.10 vs. 0.37 ± 0.29, respectively, p <0.001 for both). Functional enzymatic assay identified significantly greater MLCK activity in the bladder compared to the urethra. MLCP activity was lower in the bladder compared to the urethra but the difference was statistically significant only at 1 minute after initiation of assay.

CONCLUSION—In healthy young female rats, MLCK activity is higher and MLCP activity lower in the bladder relative to the urethra. These differences likely play a role in modulating the functional differences between bladder and urethral smooth muscle tone.

Keywords

bladder; urethra; myosin light chain kinase; myosin light chain phosphatase

INTRODUCTION

Coordinated modulation of smooth muscle components in the urinary bladder and urethra is a prerequisite for effective storage and voiding function of the lower urinary tract [1]. During normal bladder filling, the detrusor muscle is relaxed to accommodate increasing urine volumes. Meanwhile, the urethral sphincter remains tonically contracted to prevent urine leakage. Activity of both muscle groups rapidly changes when voiding is initiated.

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During normal micturition the detrusor muscle contracts and the urethra relaxes to permit urination [2]. Thus, the tonic state of the urethra progressively increases during filling-phase to prevent urine leakage and is rapidly reversed in a coordinated fashion with bladder contraction to permit voiding [3].

Upon stimulation, smooth muscle cells release Ca^{2+} from the sarcoplasmic reticulum. Ca^{2+} binds to calmodulin and the Ca^{2+}/calmodulin complex activates myosin light chain kinase (MLCK). MLCK phosphorylates the myosin light chain (MLC), which activates myosin ATPase. Activity of myosin ATPase permits ratcheting of myosin and actin myofilaments and muscular contraction [4–6]. The phosphorylation status of MLC is therefore the final determinant of smooth muscle contraction state [7]. The importance of MLCK and its antagonist, myosin light chain phosphatase (MLCP), which dephosphorylates MLC, is thus readily apparent [8].

Although smooth muscles in the bladder and urethra play important roles in continence, to our knowledge there has been no systematic analysis of their MLCK and MLCP expression and activity. In the present study, we assessed the expression of MLCK and myosin phosphatase-targeting subunit of protein phosphatase type 1 (MYPT1), a negative inhibitor of MLCP, in the bladder and urethra. Furthermore, we conducted enzymatic activity assay of MLCK and MLCP in these tissues.

MATERIALS AND METHODS

Eighteen healthy female Sprague-Dawley rats (2 months old) were obtained from Charles River Laboratories (Wilmington, MA), and randomly divided into three equal groups. Bladder and urethra tissue was harvested for real-time PCR, Western blot, and enzyme activity analysis (n=6 each). All experiments were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Surgery was performed under deep Nembutal anesthesia (25 mg/kg IP). Bladder and urethra were exposed through a lower abdomen midline incision and transection of the pubic symphysis. The bladder was separated from the urethra below the bladder neck. Both tissues were removed as a whole and were placed in ice cold phosphate buffered saline (PBS). The mucosal and serosal layers of these tissues were mechanically separated from the smooth muscle layer. Smooth muscle samples were kept in RNAlater (Ambion, Austin, TX) for RNA isolation or PBS for protein and enzyme isolation. Animals were euthanized at the end of the procedure.

Total ribonucleic acid (RNA) from urethra and bladder (n=6, each) was isolated utilizing the RNAeasy Isolation Kit (Qiagen, Valencia, CA). All RNA was of high quality as indicated by a 2:1 ratio for 28S/18S ribosomal RNA and an optical density (OD) ratio of >1.9 for OD_{260}/OD_{280}. The isolated RNA (2.5 μg) was annealed to 0.4 μg of oligo-dT primer in a volume of 12 μl. The final volume was brought to 20 μl by adding 4 μl of 5X buffer, 2 μl of 0.1 M dithiothreitol (DTT), 1 μl of 10 mM deoxyribonucleotide triphosphate (dNTP), and 1 μl of SuperScript reverse transcriptase (Invitrogen, La Jolla, CA). After one hour of incubation at 42°C, the RT mixture was incubated at 70°C for 10 min to inactivate the reverse transcriptase. The cDNA library was diluted fivefold for real-time PCR by adding 80 μl of TE buffer. All reagents for SYBR Green real-time PCR, including the primers for rat MLCK, MYPT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Applied Biosystems (Foster City, CA). Primer sequences for real-time PCR are presented in Table 1. The reactions were conducted in the Prism 7300HT sequence detection system (Applied Biosystems) using a 96-well plate format. Cycling conditions included an initial phase at 95°C for 3 min, 40 cycles at 95°C for 15 s, and 55°C for 60 s

followed by a melting curve analysis at 95°C for 15 s, 55°C for 15 s, and 95°C for 15 s. Real-time PCR results were analyzed by SDS 7000 software (Applied Biosystems) to determine the expression levels for genes of interest relative to that of GAPDH. Each measurement was performed in triplicate.

Protein from bladder and urethral smooth muscle was extracted in isolation buffer consisting of 60 mM KCl, 40 mM imidazole-HCl (pH 7.1), 2 mM EDTA, 2 mM DTT, 10 mM ATP, and 1% protease inhibitor cocktail (PIC). The extract was centrifuged at 20,000 ×g for 20 min and the supernatant was used for enzyme extraction by protein dialysis as follows: the dialysis tube was pre-wetted with H_2O for 10 min. After drying, 200 μl of the total protein sample was added with resin in phosphorylation buffer of 10 mM imidazole-HCl (pH 7.1), 5 mM DTT, and 1% PIC. The dialyzed samples were transferred to a new tube and used for activity assay. The Bradford assay (Pierce Biotechnology, Rockford, IL) was performed in triplicate and the protein concentration of the extracts was determined.

The protein dialysate from urethral and bladder smooth muscle tissue was assessed by Western blot analysis. Cell lysates containing 20 μg of protein were electrophoresed in SDS–PAGE and then transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was stained with Ponceau S to verify the integrity of the transferred proteins and to monitor the unbiased transfer of all protein samples. Detection of protein on the membrane was performed with the ECL kit (Amersham Life Sciences, Arlington Heights, IL) using anti-MLCK (1:300; Santa Cruz Biotechnology, CA) and anti-MYPT1 antibody (1:500; BD Biosciences, San Jose, CA), followed by exposure of the membrane to X-ray film. The resulting images were analyzed with ChemiImager 4000 (Alpha Innotech, San Leandro, CA) to determine the integrated density value (IDV) of each protein band normalized to the IDV of β-Actin. Before re-probing with a different antibody, the membrane was stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, 10 mM 2-mercaptoethanol at 55°C for 30 min and then washed 4 times in 1× TBS.

MLCK activities of urinary bladder and urethral smooth muscle extracts were determined as previously described [9]. Briefly, kinase was extracted and dialyzed as described above. Seventy μl of cell extract were used for kinase activity in a total volume of 150 μl reaction buffer containing 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM imidazole-HCl (pH 7.1), and 2 mM of ATP at 25°C. Fifty μg of exogenous purified chicken gizzard smooth muscle myosin were added to each assay and the reaction was started by adding [^{32}P]ATP. Individual reactions were stopped at 1, 3 and 5 minutes by transferring 50 μl of the reaction mix to 1 ml of 10% trichloroacetic acid (TCA). The mixture was centrifuged at 14,000 ×g for 20 minutes. Precipitates were then dissolved directly in SDS-PAGE sample buffer. The pH was adjusted to neutrality by adding Tris and the samples were separated in a 14% SDS-PAGE gel. The gel was stained with Coomassie blue and exposed to X-Ray film. The radio-labeled band representing the regulatory 20 kDa light chain (MLC20) was excised from each lane, added to a separate scintillation vial, crushed with a spatula, mixed with scintillation fluid and counted in a scintillation counter. The specific activity of the kinase was calculated as picomoles (pMole) of phosphate bound to the MLC20 per g of cell extract protein.

^{32}P-labled MLC20 (300 cpm/pMol) was prepared by phosphorylation of muscle MLC (1 mg/ml) with endogenous MLCK from chicken gizzard in a 0.5 ml incubation mixture containing 50 mM Tris-HCl, 5 mM magnesium acetate, 0.1 mM $CaCl_2$, 0.35 M NaCl, and 50 μM [^{32}P]ATP. After 1 hour of incubation at room temperature, the reaction mixture was passed through 2×2 ml spin columns with Sephadex G-25 (Sigma-Aldrich, St. Louis, MO). The ^{32}P-labeled substrates were stored at 4°C prior to the following experiments.

MLCP activity was determined by the release of ^{32}P from ^{32}P-labled MLC20. The cell extracts prepared as described above were assayed for phosphatase activity in phosphorylation buffer containing 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 10 mM imidazole-HCl (pH 7.1) at 25°C. Exogenous purified gizzard myosin phosphorylated with [^{32}P]ATP was used as the substrate. The reaction was started by adding 50 μg of the exogenous myosin with covalently bound [^{32}P] phosphate. The reaction was stopped at 1, 3, and 5 minutes by transferring 50 μl of the reaction mixture to TCA to a final concentration of 10%. The TCA precipitated protein was heated to 90°C for 45 min and then chilled on ice. The amount of phosphate (pMole) released per g protein was determined by standard filter binding. The protein was trapped on the filter, and the radioactivity remaining bound to it was determined by counting the filter in a Beckman 2000LS liquid scintillation spectrometer using Ready-Solve HP scintillation cocktail (Beckman, Fullerton, CA).

Statistical analysis was performed with Prism 4 (GraphPad Software, San Diego, CA). Analysis of variance was used to determine differences between groups, followed by pair-wise comparison to determine the significance of differences observed. Statistical significance was set at $P<0.05$.

RESULTS

We first determined the mRNA expression of the contraction agonist MLCK and the antagonist of muscle relaxation MYPT1 in bladder and urethral tissue (Fig. 1). MLCK mRNA expression was two-fold higher in the bladder than in urethral tissue; however, this difference did not reach statistical significance ($P=0.09$). MYPT1 mRNA expression was four-fold higher in the bladder relative to the urethra ($P=0.001$). In both tissues, the contraction agonist (MLCK) was expressed at lower levels than the antagonist of muscle relaxation (MYPT1). Compared to MYPT1 expression, MLCK expression was nine-fold lower in the bladder and four-fold lower in urethral tissue.

MLCK and MYPT1 protein expression were elevated in the bladder as compared to urethral specimens (Fig. 2). This finding is reflected by a two-fold increased MLCK content and a 2.5-fold increased MYPT1 content in the bladder ($p<0.001$ for both). In contrast to real-time PCR results, the contraction agonist MLCK was expressed at higher levels in the bladder and urethra than the antagonist of muscle relaxation (MYPT1). At the protein level, MLCK expression was 1.5-fold higher in the bladder and 2.5-fold higher in urethral tissue.

The activity of MLCK in bladder and urethral tissue is presented in Fig. 3. The activity of the contraction agonist was higher in the bladder than in urethral tissue at all time-points measured. The ratio of bladder to urethral MLCK activity was 2, 1.75, and 1.5 at one, three, and five minutes, respectively. Although the ratio of activity declined over time, differences in MLCK activity between bladder and urethra were statistically significant at each time-point ($p<0.01$).

The activity of MLCP in bladder and urethral specimens is presented in Fig. 4. Activity of this relaxation agonist was higher in urethral tissue as compared to the bladder. The ratio of urethral to bladder MLCP was 2, 1.4, and 1.4 at one, three, and five minutes, respectively. The MLCP ratio declined over time, and differences in activity between bladder and urethra were statistically significant only at the one-minute time-point ($p<0.05$).

DISCUSSION

Smooth muscle is a heterogeneous tissue type. In broad terms smooth muscle may be classified into a “tonic” type that tends to remain in a contracted state and is slow to change its’ contraction state and a “phasic” type that is often in a relaxed state and is relatively

quick to change its' contraction state [10]. Differences in the contractile apparatus of actin and myosin are key factors differentiating the tonic and phasic types of smooth muscle. Actin is relatively conserved among different cell types [11]; therefore, variations in myosin isoform type and other local factors account for a great deal of the variation in smooth muscle function.

Myosin molecules consist of two heavy chains and two pairs of light chains. Each light chain pair consists of a regulatory chain and an essential chain. The myosin heavy chain (MHC) is derived from a single gene but alternative translation of mRNA transcripts leads to the expression of different MHC isoforms. Modification to the carboxy terminal of the protein transcript leads to production of the SM1 or SM2 MHC isoform, whereas modification of the amine terminal leads to production of the SM-A or SM-B isoforms. SM-B and SM2 are associated with higher maximal shortening velocity in a variety of species and therefore imply a more phasic phenotype [1].

SM-B is much more common in the healthy urinary bladder, consistent with a phasic phenotype for this organ [1, 12]. In contrast, the expression of SM-A and SM-1 are higher in the healthy urethra relative to the healthy bladder, which is evidence towards a tonic phenotype for the urethra [12]. Interestingly, when compared to healthy controls, decompensated bladders from rabbits and mice demonstrate increased expression of SM-A and SM-1 relative to SM-B and SM-2 [13, 14]. These changes in protein expression are associated with decreased velocity of contraction and more tonic type activity of the decompensated bladder.

The 20 kDa regulatory chain of the MLC is associated with the amino-terminal portion of the MHC and plays a crucial role in modulating contraction. Phosphorylation and dephosphorylation of MLC lead to smooth muscle contraction and relaxation, respectively and are mediated by MLCK and MLCP, respectively. Gong et al. demonstrated that phasic smooth muscle has greater MLCK activity compared to tonic smooth muscle. This in turn leads to higher levels of MLC phosphorylation in phasic smooth muscle [15]. Hypolite *et al*. demonstrated that phosphorylation of MLC is progressively lower in smooth muscle obtained from the bladder dome, mid-bladder, bladder base, and the urethra. This differential MLC phosphorylation is important for bladder and urethral functions [12], and is now supported by findings in the present study. Specifically, our findings lend support to conceptualization of the bladder as having a phasic type of smooth muscle, at least relative to the urethra.

Treatment of smooth muscle cells with a Rho kinase inhibitor has been demonstrated to decrease smooth muscle contraction force without changing MLCK activity [8]. One possible explanation for this finding is that MLCP may be inhibited by the action of numerous intracellular molecules, including Rho kinase [6]. Rho kinase appears to exert its' effect on MLCP via phosphorylation and activation of MYPT1 [16]. In rat urethra the protein expression of Rho kinase is significantly lower relative to the bladder [17]. Lower expression of Rho kinase is expected to lead to a commensurate reduction in MYPT1 activity and hence a greater activity of MLCP. Thus, our finding of greater MLCP activity in the urethra is consistent with the Rho kinase data of Teixeira et al. Interestingly, the difference in activity of MLCP between urethra and bladder was significant only at the 1 minute time point. Thus, it is conceivable that rapid dephosphorylation of MLC by MLCP in the urethra might permit rapid relaxation of the urethra and thus enable synergic voiding. Further research is required to assess this possibility.

We believe our data represents an important contribution to the understanding of the normal physiological balance between MLCK and MLCP in the lower urinary tract. Specifically, it

provides evidence at the molecular level for the concept of the bladder being phasic while the urethra being tonic at their respective default states.

Acknowledgments

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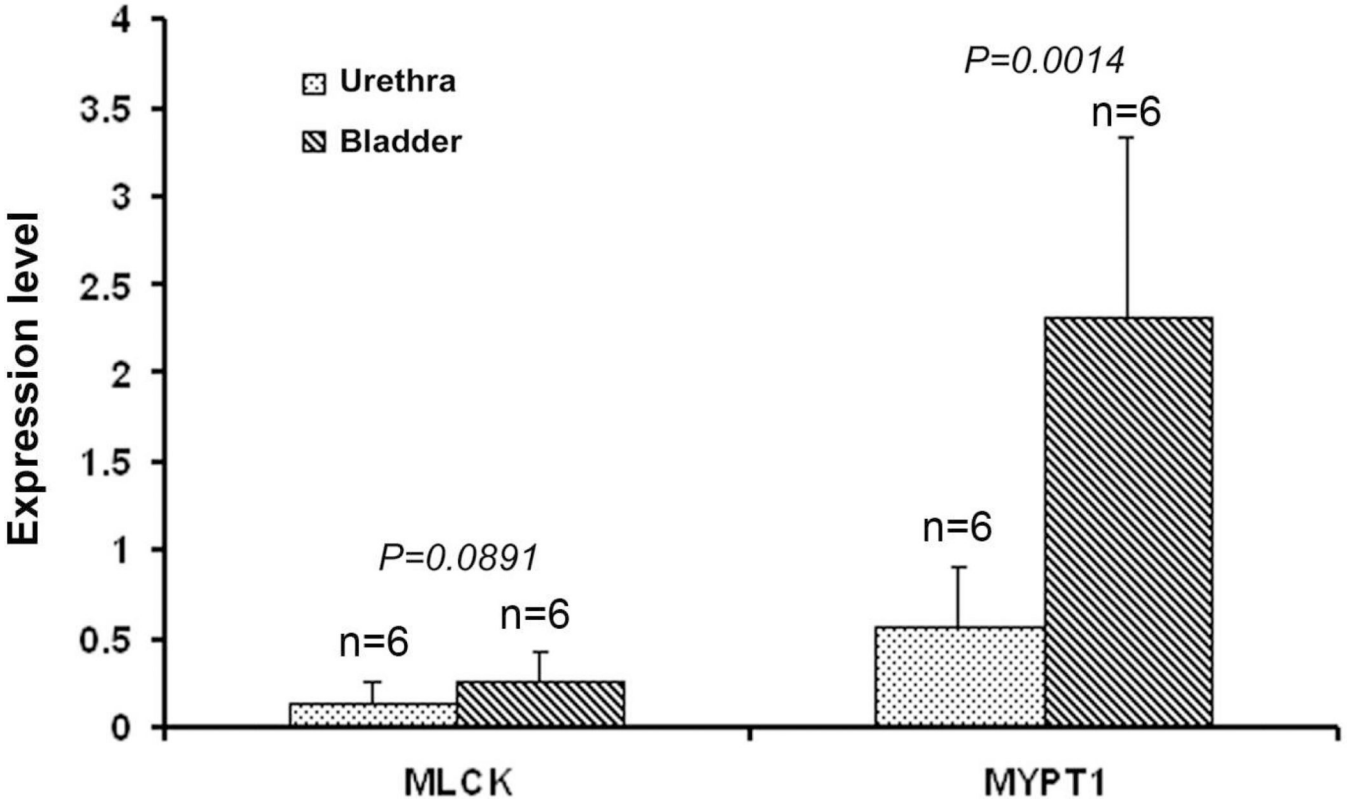

FIG. 1.

Rat bladder and urethra (n=6 each) were analyzed by real-time PCR for MLCK and MYPT1 mRNA expression. Expression level is relative to GAPDH expression and based on 3 independent experiments.

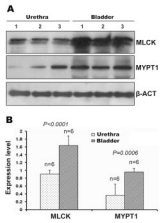

FIG. 2.

Rat bladder and urethra (n=6 each) were analyzed by western blot for MLCK and MYPT1 expression. **A**. Representative gel showing the MLCK, MYPT1, and β-Actin bands of 3 bladder and 3 urethra samples. **B.** Graph representation of the compiled densitometry data from A. Expression level is relative to β-Actin expression and based on 3 independent experiments.

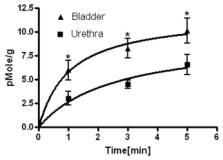

FIG. 3.

Rat bladder and urethra (n=6 each) were analyzed for MLCK activity at 1, 3, and 5-min time points. MLCK activity was calculated as pMole of ^{32}P bound to MLC20 per g of cell extract protein. * indicates $p<0.01$.

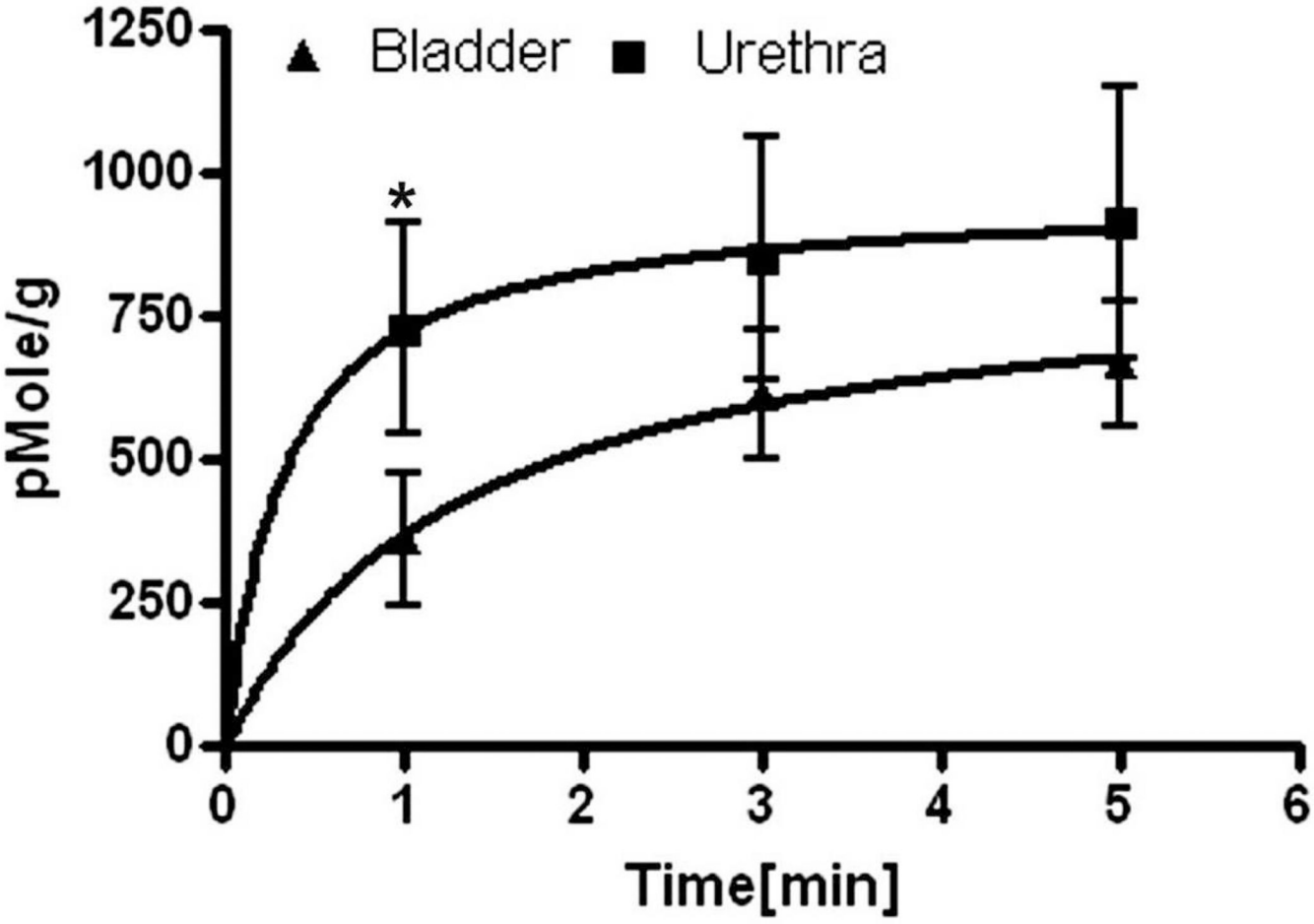

FIG. 4.

Rat bladder and urethra (n=6 each) were analyzed for MLCP activity at 1, 3, and 5-min time points. MLCP activity was calculated as pMole of ^{32}P released from ^{32}P-labled MLC20 per g of cell extract protein. * indicates $p<0.05$.

Table 1

Primer sequences

Gene	Primer sequence
MLCK	F: AATGGTGTTGCTGGAGATCGAGGT
	R: GCTGGATCAAATTGCGGTGGTTCA
MYPT1	F: CGCCGACATCAATTACGCCAATGT
	R: TTGTCATCAATGCAAGCCTGGTGC
GAPDH	F: ATGATTCTACCCACGGCAAG
	R: CTGGAAGATGGTGATGGGTT