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Contraction Kinetics and Myosin Isoform Composition in Smooth Muscle From Hypertrophied Rat Urinary Bladder

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Abstract Mechanical properties and isoform composition of myosin heavy and light chains were studied in hypertrophying rat urinary bladders. Growth of the bladder was induced by partial ligation of the urethra. Preparations were obtained after 10 days. In maximally activated skinned preparations from the hypertrophying tissue, the maximal shortening velocity and the rate of force development following photolytic release of ATP were reduced by about 20 and 25%, respectively. Stiffness was unchanged. The relative content of the basic isoform of the essential 17 kDa myosin light chain was doubled in the hypertrophied tissue. The expression of myosin heavy chain with a 7 amino acid insert at the 25K/50K region was determined using a peptide-derived antibody against the insert sequence. The relative amount of heavy chain with insert was decreased to 50% in the hypertrophic tissue. The kinetics of the cross-bridge turn-over in the newly formed myosin in the hypertrophic smooth muscle is reduced, which might be related to altered expression of myosin heavy or light chain isoforms.

Key words: smooth muscle, urinary bladder, hypertrophy, myosin light chain, myosin heavy chain, force-velocity relationship

Distension and hypertrophy of the urinary bladder is a common response to a lower urinary tract obstruction in man. This condition has been studied in several different animal models and is associated with alterations in bladder contractility, detrusor instability, and with prolonged micturition time and incontinence [Malmgren et al., 1987]. One obvious alteration during bladder outflow obstruction and hypertrophy is an increase in the bladder circumference. This structural change can be associated with distension of the cells and their contractile components, with formation of new cells and new contractile units in series or with altered interrelationship between cells. Urethral obstruction in rat induces both hypertrophy and hyperplasia of the smooth muscle of the urinary bladder [Gabella and Uvelius, 1990; Uvelius et al., 1984] with changes in structural components such as collagen [Uvelius and Mattiasson, 1984] and increased cellular concentration of desmin [Malmqvist et al., 1991] in accordance with structural data from hypertrophic intestinal smooth muscle [Gabella, 1979]. The total amounts of the contractile proteins actin and myosin increase in hypertrophying bladder which suggest formation of new contractile components [Malmqvist et al., 1991b]. These changes were associated with isoform shifts towards more γ-actin and towards more of the 205 kDa myosin heavy chain form. Isoform composition of the 17 kDa myosin light chain has been correlated with the ATPase activity of smooth muscle myosin [Helper et al., 1988]. A relative increase of the basic isoform of 17 kDa light chain (LC_{17b}) is associated with a lowered ATPase activity. Comparative studies have shown that muscles with high LC_{17b} content have a lower shortening velocity [Malmqvist and Arner, 1991; Morano et al., 1993] and higher affinity for ADP-analogues [Fuglsang et al., 1993; Hasegawa and Morita, 1992]. Recently an insert of seven amino acids in a region near the ATP-binding site in the myosin heavy chain has been demonstrated and suggested to influence the acto-myosin ATPase

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activity and the velocity of movement of actin filaments in vitro [Babij et al., 1991; Babij, 1993; Kelley et al., 1993; White et al., 1993].

Although, the structural and biochemical changes during hypertrophy of the urinary bladder alter the mechanical working conditions for the smooth muscle cells in a pronounced way, little is known about the mechanical properties of the contractile system in hypertrophying detrusor muscle. Also, to our knowledge, no information regarding the LC_{17} isoform composition and distribution of the myosin heavy chain insert in hypertrophying smooth muscle is available. The objective of the present study was to investigate the force-velocity relation and the rate of force development of hypertrophied smooth muscle from rat urinary bladder. The mechanical data were combined with a biochemical analysis of the tissue content of the LC_{17} and of the heavy chain inserted myosin isoforms.

MATERIALS AND METHODS

Operative Procedure and Preparation

Adult female Sprague-Dawley rats weighing about 250 g were used. Bladder outflow obstruction was created by a partial ligation of the urethra [Uvelius et al., 1984]. The animals were killed after 10 days by cervical fracture and the bladders were quickly taken out and placed in ice cold Ca^{2+}-free physiological saline solution. The bladders were gently blotted between two sheets of filter paper and weighed. Smooth muscle strips from the mid-section of the bladder were carefully dissected and the mucosa was removed with scissors. The fibers were chemically skinned using Triton X-100 and stored at −15°C in a glycerol containing solution as described by Arner and Hellstrand [1985] before being mounted for isotonic quick release or caged-ATP experiments at 22°C as described below.

Isotonic Quick Release Experiments

Thin muscle strips (approximate length 3 mm and diameter 0.2 mm) were cut out and mounted with aluminum foil in an apparatus for isotonic quick release experiments as described by Arner and Hellstrand [1985]. One end of the muscle was attached to the arm of an AE 801 force transducer (SensoNor, Horten, Norway) and the other to a lever system. The muscles were stretched to a length where passive tension was just noticeable. The lever could be clamped or released with electromagnetic relays and the after-load on the preparation was adjusted by varying the load on the lever. Force and length were digitized at 1 kHz using a computer equipped with an Analog Devices (Norwood, MA) RTI-800F board. The shortening velocity decreases with time after the release [Arner and Hellstrand, 1985], and therefore the velocity was determined at fixed point in time (100 ms) after the release. As indicated in the text, measurements were also made at other points in time. Afterload (P) and velocity (v) data were fitted by the Hill [1938] equation in the form: $v = b(1\text{-}P/P_0)/(P/P_0 + a/P_0)$, where P_0 is isometric force and a and b are constants. The maximal shortening velocity (V_{max}) was calculated as bP_0/a. The shortening velocity is given as muscle lengths per second. The force-velocity relation was determined using maximally thiophosphorylated preparations [Arheden et al., 1988]. The muscles were incubated for 15 min in an ATP-free rigor solution containing Ca^{2+} (pCa 4.5), 0.5 μM calmodulin and 2 mM ATP-γ-S and thereafter contracted by introducing MgATP at pCa 9.0. Releases (20–25) to different afterloads were done at the plateau of the contraction. After each series of releases, the preparation was again thiophosphorylated for about 10 min. Two series of releases were done on each muscle preparation. For each release the series elastic recoil was determined as described by Arner [1982]. The relation between the relative length change (L in muscle lengths) and relative force (P/P_0) was fitted to the logarithmic function $L = 1/K \ln((P/P_0\text{-}B)/A)$ were A, B, and K are constants. The constant K (describing the dependence of stiffness = $\Delta P/\Delta L$ on force) was used to evaluate stiffness.

Caged-ATP Experiments

The skinned muscle strips were attached between a fixed steel rod and an AE 801 force transducer using aluminum foil wrapped around the ends of the preparation as described above. The muscles were initially held in ATP-free solution for 30 min to remove ATP in the fiber and then thiophosphorylated. After thiophosphorylation ATP-γ-S was washed out in ATP-free solution. The muscles were then transferred to a rigor solution containing 5 mM caged-ATP in a 50 μl cuvette equipped with a quartz window. Photolysis of caged-ATP was achieved by using a xenon flash tube system (Rapp Opto-

electronics, Hamburg, Germany), as described in Malmqvist and Arner [1991]. The force transients were digitized at 1 kHz and fitted to a mono-exponential function.

Determination of Myosin Light Chain Isoforms

Bladder tissue was frozen in liquid nitrogen and stored at −80°C. The isoform composition was determined as described by Malmqvist and Arner [1991]. The frozen tissue was crushed and extracted at a ratio of 10 μl buffer/mg wt weight in a solution containing: 9 M urea, 2% Nonidet-P40, 2% ampholytes (Pharmalyte pH 4.0–6.5, Pharmacia-LKB, Bromma, Sweden), 5% β-mercaptoethanol. Two-dimensional electrophoresis was performed using a Bio-Rad (Richmond, CA) minigel system. The isoelectric focusing gel-rods contained 4% acrylamide, 1.6% pH 4.5–5.5 ampholytes, 10% Nonidet-P40, 9 M urea. Focusing was performed at 750 V for 3.5 h at 18°C. The second dimension gels were prepared with 12% total acrylamide, 375 mM tris(hydroxymethyl)aminomethane (pH 8.8) and 0.1% SDS. Electrophoresis was carried out for 40 min at 60 mA. The gels were stained with Coomassie blue, destained, and scanned with a GS-30 densitometer (Hoefer, San Francisco, CA). Identification of the different LC_{17} isoforms was based on their different isoelectric points [Helper et al., 1988; Malmqvist and Arner, 1991].

Quantification of 5′-Inserted Myosin Heavy Chain Isoform

A polyclonal antibody against the 25K/50K insert (sequence QGPSFAY) [compare White et al., 1993], raised in rabbits and purified with a peptide affinity column was used to detect the amount of insert in smooth muscle tissue as described previously [Calovini et al., 1995]. Frozen samples of bladder tissue were obtained as described above, homogenized and separated on 5% polyacrylamide gels. Three different amounts of protein were run from each sample. Two lanes were run in parallel for each amount. One lane for each concentration was stained with Coomassie Blue to determine protein concentration in the myosin heavy chain band. The other lanes were transferred to nitrocellulose and stained with the antibody against the insert region. The antibody reaction was detected with a secondary peroxidase-conjugated antibody and by the enhanced chemiluminescence reaction (ECL, Amersham, U.K.).

Statistics

Values are given as means ± SEM with the number of observations within parenthesis. Comparisons were made using the Student's t-test for unpaired data with $P < 0.05$ as significance level.

RESULTS

Force-Velocity Relations and Stiffness

After the thiophosphorylation procedure the muscles contracted maximally after transfer to ATP-containing solution at pCa 9.0. No change in active force was observed when $[Ca^{2+}]$ was increased to pCa 4.5 at the plateau of the contraction, indicating that the muscle fibers were maximally activated. Figure 1A shows force-velocity relationships from a control and a hypertrophic bladder preparation. The maximal shortening-velocity at 100 ms after the release of the hypertrophic muscle was slightly lower than that of the control. Two determinations of the force-velocity relationship were performed on each preparation. The average value was used as a representative value for the muscle. Figure 1B shows the summarized data, for 6 preparations in each group, with a slightly, and significantly, reduced maximal shortening velocity of the hypertrophic preparations. Velocity (in percent of that at 100 ms) decreased with time after the release in a similar manner in the hypertrophic muscle (200 ms: 60 ± 3; 500 ms: 25 ± 3; 750 ms: 22 ± 2%, n = 5–8) and in the controls (200 ms: 57 ± 6; 500 ms: 25 ± 3; 750 ms: 19 ± 3%, n = 5–8), showing that the decreased velocity in the hypertrophic muscles was not due to a change in the shape of the shortening response. The decrease in V_{max} in hypertrophic muscles was associated with a decrease in both the parameters b (at 100 ms: controls: 0.143 ± 0.038, n = 7; hypertrophic: 0.058 ± 0.008 muscle lengths/s, n = 12) and a/Po (at 100 ms: controls: 0.705 ± 0.165, n = 7; hypertrophic: 0.321 ± 0.038, n = 12) in the Hill equation.

The stiffness constant K, describing the dependence of stiffness on force was 23.0 ± 5.6 (n = 6) and 24.3 ± 2.5 (n = 8) in the control and hypertrophic groups, respectively. Although these measurements, with relatively slow force-steps (complete within 5 ms), are not directly comparable with rapid stiffness measurements using fast length steps [compare Arheden et al., 1988], our data exclude the possibility that changes in the series elastic component are involved in the

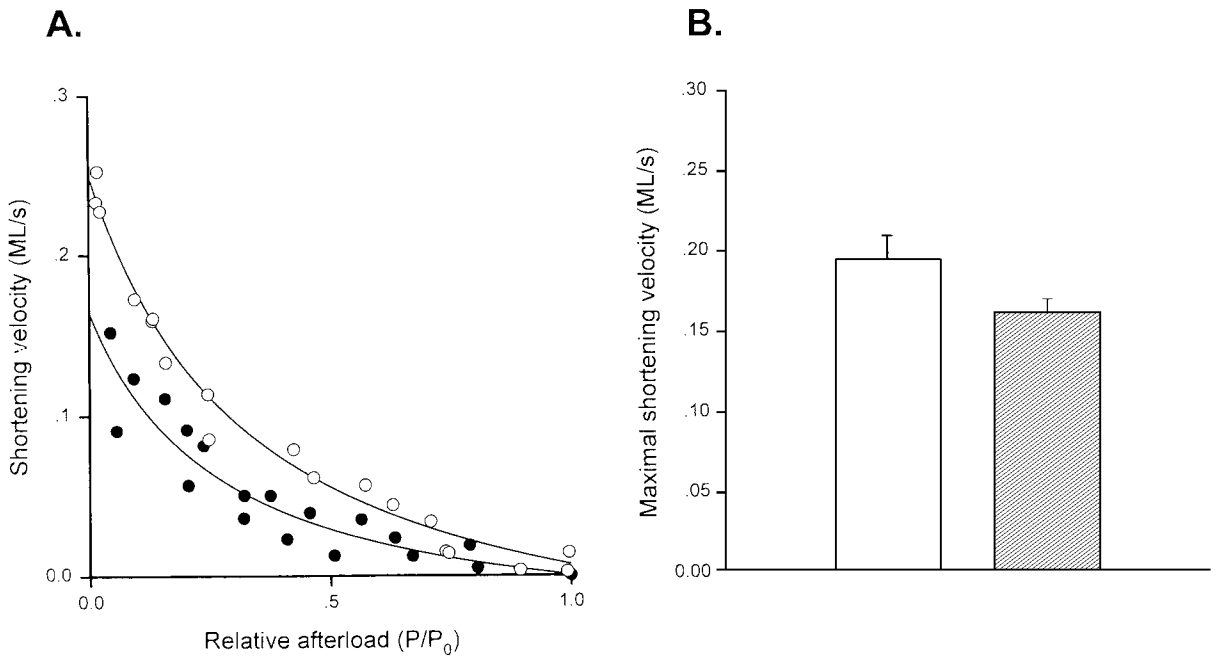

Fig. 1. Force-velocity relationships of control and hypertrophic bladder preparations. **A:** The shortening velocity, in muscle lengths (ML) per second, is plotted against the relative afterload (P/P_0). Open and filled circles show data from a control and a hypertrophic preparation, respectively. Fits to the Hill [1938] equation are shown. **B:** The summarized data of controls (n = 6, open bar) and hypertrophic muscles (filled bar, n = 6). Data are shown as means ± SEM. The difference between the groups was significant ($P < 0.05$).

altered contraction kinetics of the hypertrophied tissue.

Rate of Force Development After Photolytic Release of ATP

Figure 2A and B shows original records of force transients after photolytic release of ATP in thiophosphorylated muscles. After ATP release the muscles contracted rapidly, reaching half-maximal force after about 2–3 s. The hypertrophic muscle contracted somewhat slower than the control. The control and hypertrophic muscles contracted to 71 ± 5 and 70 ± 3% (n = 8, both groups) of the maximal ATP induced force, respectively. The mechanical transients were analyzed by fitting a monoexponential function to the force data. The average values for the rate constants are shown in Figure 2C. The rate of tension development of the hypertrophic muscles was significantly lower than that of the controls.

Isoform Composition of the 17 kDa Myosin Light Chain

Two protein spots representing the LC_{17a} and LC_{17b} isoforms of the essential myosin light chain were identified on the two-dimensional gels as described by Malmqvist and Arner [1991]. As shown in Figure 3 the relative content of the LC_{17b} isoform was doubled in the hypertrophic bladder tissue compared with the controls.

Relative Amount of 5′-Inserted Myosin Heavy Chain

Figure 4A shows the myosin heavy chain bands stained for protein content with Coomassie Blue and the immunoreactivity to the polyclonal antibody against the insert. In Figure 4B the intensity of the enhanced chemiluminescence reaction, used to detect the antibody binding, is plotted against the densitometrically determined intensity in the myosin heavy chain band on the Coomassie blue stained gels. For each amount of myosin heavy chain, the reactivity to the antibody against the insert was about 50% in the hypertrophic preparations compared to the controls. These data suggest that the amount of inserted myosin heavy chain relative to the total amount of heavy chain is reduced in the hypertrophic tissue.

DISCUSSION

Growth of the urinary bladder in response to urinary outflow obstruction is associated with

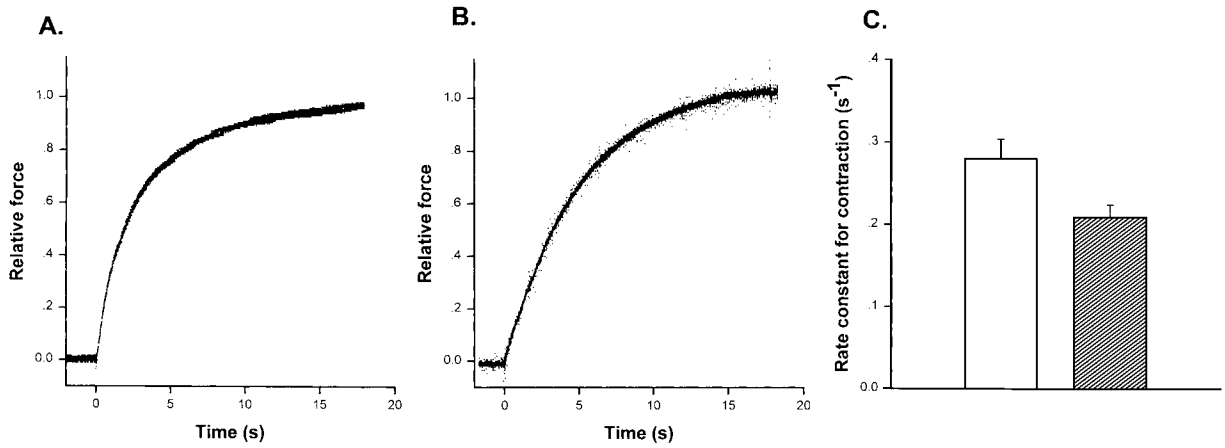

Fig. 2. Rate of contraction determined after photolytic release of ATP. **A** and **B:** Force traces from a control and a hypertrophic muscle, respectively. Force values are given relative to the maximal force recorded in ATP containing solution. **C:** Summarized data for the rate constant (controls: open bar, n = 6; hypertrophic: filled bar, n = 6). Data are shown as means ± SEM. The difference between the groups was significant ($P < 0.05$).

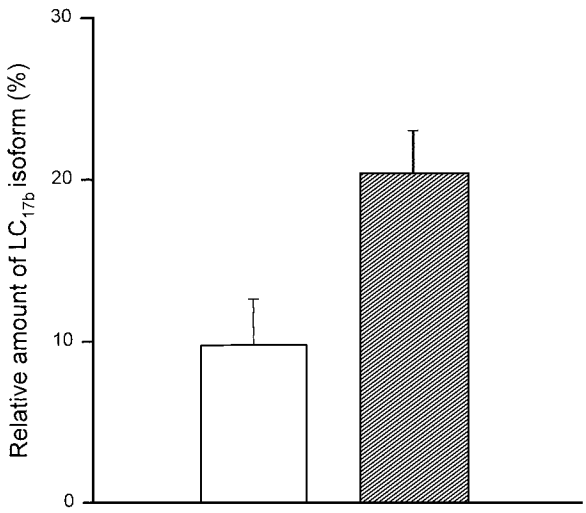

Fig. 3. Mean values for the relative content of the basic isoform of the 17 kDa myosin light chain. Open and filled bars show data from control (n = 6) and hypertrophic preparations (n = 11), respectively. Data are shown as means ± SEM. The difference between the groups was significant ($P < 0.01$).

pronounced structural changes in the bladder wall. The weight increases at least 5–6-fold [Sjuve et al., 1995; Uvelius et al., 1984]. The resting volume at zero pressure in the rat bladder has been found to increase from about 0.07 ml in the controls to 1 or 3.33 ml in the hypertrophic bladders [Damaser et al., 1996; Malmgren et al., 1987]. Since the increase in bladder volume is at least 14-fold, the circumference would increase more than 2.5-fold. If this change is associated with a pure stretch of pre-existing contractile elements, i.e., an increase in the length of "sarcomere equivalents," with unchanged shortening properties the unloaded maximal shortening velocity of the preparations (in muscle lengths per unit time) would decrease to about 40%. However, the increase in muscle mass in the growing rat urinary bladders is due to hypertrophy and hyperplasia of the smooth muscle cells [Uvelius et al., 1984] and with synthesis of myosin and actin [Malmqvist et al., 1991b] suggesting that new contractile elements are formed in the tissue. Since force per muscle area is unchanged or slightly reduced [Arner et al., 1990] the new contractile elements would be formed in series rather that in parallel with pre-existing ones; i.e., the number of series coupled sarcomere equivalents per unit length in the preparations would be similar in hypertrophic and control muscles.

Metabolic tension cost, considered to reflect the cross-bridge turnover under isometric conditions, does not change in hypertrophied rat urinary bladder [Arner et al., 1990], which might suggest that the cross-bridge turnover under isometric conditions of the newly formed contractile proteins is similar to that of the normal contractile system. However, these measurements were performed using intact tissue where metabolic events involved in cell metabolism and activation pathways may influence the determined metabolic tension cost. In a previous study [Sjuve et al., 1995] we found that the rate of contraction following rapid activation of purinoceptors in intact hypertrophied rat urinary bladders was slower than in controls. This change might reflect alterations both in activation mechanisms and in the kinetics of the contrac-

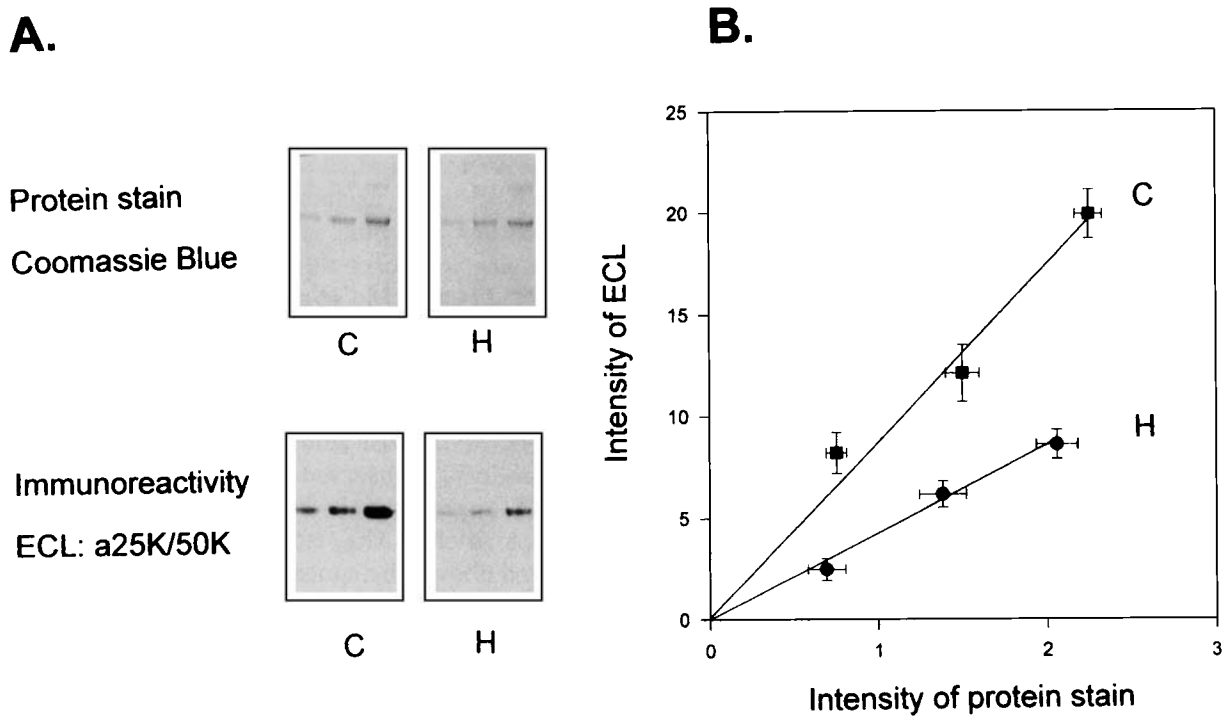

Fig. 4. Expression of myosin heavy chain with the seven amino acid insert in the a25K/50K region in control (C) and hypertrophic (H) tissue. The upper photographs in **A** show gels stained with Coomassie blue for determination of protein content in the myosin heavy chain band. The lower photographs show enhanced chemiluminescence (ECL) data from Western blots using the peptide-derived antibody against the insert for a typical experiment. **B:** ECL intensity is plotted against the protein content (n = 6).

tile system. In the skinned muscles where activation of the contractile machinery can be held constant we find that V_{max} decreases by about 15%. This was not due to a change in the curvature of the shortening response, since velocities at all investigated times after the release were equally decreased. It seems unlikely that the change in V_{max} was due to differences in muscle length since the preparations were stretched in a similar manner and the velocity of skinned urinary bladder shows little length dependence [Malmqvist et al., 1991a]. A frictional load as a cause for the decreased V_{max} is difficult to exclude. However, an increased internal load would be expected to decrease the curvature of the force-velocity relation (increase a/Po), which is contrary to our results. We thus consider the change in V_{max} in the hypertrophic muscles to reflect a change in the kinetics of the cross-bridge turnover under isotonic conditions. Also the rate of tension development after photolytic release of ATP was decreased (Fig. 2C). The rate of tension development is considered to be correlated with rates in the force generating transitions during cross-bridge turnover [Malmqvist and Arner, 1991]. The series elastic stiffness was unchanged in the hypertrophic muscles, which suggests that the changes in structural components such as collagen [Uvelius and Mattiasson, 1984] or desmin [Malmqvist et al., 1991b] are not associated with alterations in tissue series elasticity and excludes the possibility that alterations in series elasticity influence the measured rate of tension development. Thus the mechanical data speak in favor of the possibility that new contractile filaments are introduced and organized in series with pre-existing contractile units and that the newly formed contractile system exhibits a slight decrease, compared to control tissue, in the rate of cross-bridge turnover at conditions of maximal activation.

During growth in the rat urinary bladder the contractile proteins actin and myosin are synthesized in amounts leading to an unchanged actin concentration and a slightly reduced myosin concentration in the growing smooth muscle [Malmqvist et al., 1991b]. Actin, which in smooth muscle exists in four different isoforms [Vanderkerckhove and Weber, 1981], shows an

increase in the relative content of the γ-isoform and a decrease in the relative content of the α-isoform in the hypertrophied rat bladder [Malmqvist et al., 1991b]. The change in actin isoform towards more γ-actin has also been demonstrated in hypertrophied uterus from man and monkey [Cavaillé et al., 1986]. The physiological significance of these shifts in actin isoforms is unknown, and so far no correlation between actin isoform and actin-activated myosin ATPase or velocity of actin over myosin in in vitro motility has been demonstrated [Harris and Warshaw, 1993; Mossakowska and Strzlecka-Golaszewska, 1985]. We have previously reported that the relative amount of the 200 kDa SM2 myosin heavy chain isoform is decreased by about 25% in hypertrophic rat urinary bladder [Malmqvist et al., 1991b]. When different muscles are compared no clear correlation between the relative contents of the 205 kDa SM1 and the SM2 isoforms and maximal shortening velocity can be observed [Malmqvist et al., 1991b], consistent with results from in vitro motility experiments [Kelley et al., 1992]. This suggests that the expression of these two heavy chain variants is not a major factor controlling shortening velocity. The presence of a 7 amino acid insert region in the smooth muscle myosin heavy chain [Babij et al., 1991; Babij, 1993; Kelley et al., 1993; White et al., 1993] has been proposed to increase the velocity in in vitro motility assay and increase the ATPase activity. Bladder tissue from rabbit and rat [Babij, 1993; White et al., 1993] expresses relatively high contents of the inserted isoform compared to vascular and uterine smooth muscle. We report here, using a peptide derived antibody, that the relative amount of myosin heavy chain containing the insert is significantly decreased in the hypertrophic tissue. As discussed above we have found that the SM2 myosin isoform is decreased by about 25% during hypertrophy [Malmqvist et al., 1991b]. It is at present not clear whether the inserted region occurs in equal proportion in both the SM1 and SM2 isoforms in bladder tissue, and therefore the decrease in the relative amount observed in the hypertrophying bladders might reflect an altered distribution of the SM1 and SM2 isoforms.

We find a significant increase in the relative amount of the basic 17 kDa light chain isoform. Previous work comparing different smooth muscles has revealed a correlation between the ratio of the LC_{17a} and LC_{17b} isoforms and the ATPase activity [Helper et al., 1988] and the maximal shortening velocity [Malmqvist and Arner, 1991; Morano et al., 1993]. More of the basic form is associated with a slower ATPase turnover rate and shortening velocity. We cannot at present exclude that changes in other regulatory proteins, e.g., calponin, which can influence the force-velocity relation in skinned fibers [Jaworowski et al., 1995], are responsible for the change in V_{max}. The relation between V_{max} and the LC_{17} distribution for both controls and hypertrophic bladders falls close to the relation obtained when different muscles are compared [Malmqvist and Arner, 1991]. The decrease in V_{max} observed in hypertrophic muscles could quantitatively be explained by the increase in the LC_{17b} isoform. However, as discussed above, the content of the inserted heavy chain isoform also change. When the data regarding the insert from the study of White et al., [1993] is related to the V_{max} of muscle fibers [Malmqvist and Arner, 1991] a relation between the insert and V_{max} also seems to exist. The possibility thus exists that the expression of the two myosin isoforms, i.e., inserted myosin heavy chain and LC_{17}, is coordinated in smooth muscle. Until the function or content of these components can be differentially altered in the muscle, it is however difficult to determine which is the major determinant of cross-bridge turnover in smooth muscle fibers.

The distension and hypertrophy of the bladder alters the mechanical conditions for the bladder muscle during emptying in a drastic way. Less shortening is required for emptying a specific volume in the distended bladders and thus the volume flow can be increased at a given rate of shortening in the wall. This is counteracted by at least three factors (1) the load on the muscle is increased due to the increased pressure caused by the obstruction and the fact that tension at a given pressure is increased due to the law of LaPlace; (2) active force per area (active stress) is decreased [Arner et al., 1990]; (3), as shown here, the V_{max} of the force-velocity relation appears to be lower in the detrusor muscle in the wall, possibly related to expression of myosin with slower turn-over rate. The first two factors would, in addition, lead to an increased relative afterload during emptying, which would further decrease the shortening velocity of the muscles in the wall and reduce the rate of bladder emptying during micturition.

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