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Alternative Splicing of Smooth Muscle Myosin Heavy Chains and Its Functional Consequences

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Abstract The aim of our study was to determine the relation between alternatively spliced myosin heavy chain (MHC) isoforms and the contractility of smooth muscle. The relative amount of MHC with an alternatively spliced insert in the 5′ (amino terminal) domain was determined on the protein level using a peptide-directed antibody (a25K/50K) raised against the inserted sequence (QGPSFAY). Smooth muscle MHC isoforms of both bladder and myometrium but not nonmuscle MHC reacted with a25/50K. Using a quantitative Western-blot approach the amount of 5′-inserted MHC in rat bladder was detected to be about eightfold higher than in normal rat myometrium. The amount of heavy chain with insert was found to be decreased by about 50% in the myometrium of pregnant rats. Although bladder contained significantly more 5′-inserted MHC than myometrium, apparent maximal shortening velocities (Vmax) were comparable, being 0.138 ± 0.012 and 0.114 ± 0.023 muscle length per second of skinned bladder and normal myometrium fibers, respectively. Phosphorylation of myosin light chain 20 induced by maximal Ca^{2+}/calmodulin activation was the same in bladder and myometrial fibers. These results suggest that the amount of 5′-inserted MHC is not necessarily associated with contractile properties of smooth muscle.

Key words: myosin heavy chains, smooth muscle, alternative splicing, contractility, myosin light chains

In adult smooth muscle cells (SMC), two different genes coding for the myosin heavy chain (MHC) are expressed, namely one smooth-muscle-specific (SM) MHC and one nonmuscle (NM) MHC [for review see Somlyo, 1993]. The single SM-MHC gene transcript is alternatively spliced at both the 3′-carboxyl terminus (myosin tail) and at the 5′ amino terminus. Inclusion of the 39 nucleotide (nt) exon at the 3′-terminus generates two SM-MHC: SM2 (200 kDa) by inclusion and SM1 (204 kDa) by exclusion [Rovner et al., 1986; Nagai et al., 1989; Hamada et al., 1990]. In rat urinary bladder, both smooth-muscle-specific isoforms SM1 and SM2 are expressed [Malmqvist et al., 1991]. Myometrial cells express SM1 and NM-MHC of 196 kDa [Morano et al., 1993; Calovini et al., 1995]. 5′ splicing is accomplished by excision of 21 nt (seven amino acids) near the ATP-binding 25K/50K junction [White et al., 1993; Babij, 1993; Kelley et al., 1993]. SM-MHC with the 5′-insert were designated as the "B-forms" (SM1B and SM2B), while those without 5′ insertion as the "A-forms" (SM1A and SM2A). As demonstrated by mRNA analysis, about 85% of rat bladder MHC mRNA contained the 25K/50K insert, while rat aorta and myometrium contained very low levels of SM-MHC mRNA with the 25K/50K insert [White et al., 1993].

Little is known about regulation of alternative splicing as well as of the functional consequences of alternatively spliced MHC isoforms in the SMC. MHC containing the 25K/50K insertion revealed a higher ATPase activity and moved actin filaments faster in in vitro motility assays than MHC without the 5′ insert [Kelley et al., 1993]. We could recently demonstrate that alternative splicing of SM-MHC was regulated by steroid hormones (e.g., estrogen decreased while testosterone increased 5′-inserted SM-MHC [Calovini et al., 1995].

SMC express two essential myosin light chain (MLC) isoforms of 17 kDa by alternative splicing, namely MLC17a and MLC17b [Lenz et al., 1989; Hasegawa and Morita, 1992]. Two isoforms of the 20 kDa phosphorylatable MLC, designated as the MLC20A and MLC20B with increasing acidity (Erdödi et al., 1987; Inoue et al., 1989), have been identified in SMC. Phosphorylation of the MLC20 by Ca^{2+}-calmodulin-dependent myosin light chain kinase is a key

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event in the initiation of smooth muscle contraction, and the extent of MLC20 phosphorylation determines tension development and shortening velocity [for review see Kamm and Stull, 1985].

Here we compared the relative amounts of 5′-inserted MHC in rat bladder and rat myometrium of nonpregnant (normal) and late pregnant rats on the protein level using a quantitative Western-blot approach and a peptide-directed antibody. We found that MHC of the bladder contained significantly more 5′-inserted MHC than MHC from the myometrium. The low expression level of 5′–SM-MHC isoforms in the rat myometrium was further reduced during pregnancy. Vmax of both uterus and bladder of nonpregnant rats was identical. We conclude that the presence of high amounts of 5′ SM-MHC is not necessarily associated with high speed of smooth muscle shortening.

MATERIALS AND METHODS

Treatment of Tissue

Twelve-week-old female rats (Sprague-Dawley) were killed by cervical dislocation, and the bladder and myometrium were removed. Tissue specimens were either immediately frozen in liquid nitrogen and stored at −80°C or chemically skinned according to the following method: excised tissue was incubated for 30 min in (mM) EGTA (5), imidazole (20), KCl (50), sucrose (110), and DTE (2) pH 7.4, on ice. Tissue samples were subsequently incubated in the same solution containing in addition 1% Triton X-100 for 4 h on ice. Skinned fibers were then incubated in a solution containing (mM) Imidazole (20), EGTA (4), ATP (7.5), $MgCl_2$ (10), and NaN_3, pH 6.7, and 50% glycerol for 1 h on ice and subsequently stored at −20°C. All skinning solutions contained 10 μM leupeptine as protease inhibitor.

Analysis of In Vivo Myosin Heavy Chain (MHC) Isoform Expression

Samples (20–40 mg) of bladder and myometrial tissues were homogenized in SDS-sample buffer (5% SDS, 50 mM Tris/HCl, pH 7.5, 250 mM sucrose, 75 mM urea, 60 mM β-mercaptoethanol), boiled for 2 min, and cleared by centrifugation. Aliquots from the supernatant were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Morano et al. [1993]. Briefly, we used a 4% stacking gel and a 5% separation gel both containing 25% glycerol. The gels ran overnight (5 mA constant current) at 15°C. Following SDS-PAGE the gels were stained for protein with Coomassie blue R250, and the MHC were evaluated densitometrically (ScanPack; Biometra, Germany).

Antibody Production and Western-Blot Analysis

The peptide antibody specific for the 25K/50K insertion of rat smooth muscle MHC (5′ insert) was generated based on the deduced amino acid sequence QGPSFAY [White et al., 1993] and purified as described previously [Calovini et al., 1995]. The antibody, designated as a25K/50K, was used to quantify 5′-inserted MHC isoforms on Western blots. Proteins were electrophoretically transferred from SDS gels to nitrocellulose (Hybond-C, 45 μm; Amersham) in a buffer containing 25 mM Tris, 192 mM glycin, 0.1% SDS, 20% (v/v) methanol (90 min, 0.8 mA/cm^2) using the Bio-Rad Mini-Protean II system. The nitrocellulose was blocked with 3% ovalbumin and subsequently incubated with the a25K/50K antibody (2 h at room temperature) at a concentration of 3 μg IgG/ml and the secondary peroxidase-conjugated antibody (anti-rabbit IgG; Sigma) for 1 h at room temperature. Proteins were visualized by enhanced chemiluminescence reaction kit (ECL; Amersham) using an X-ray film (X-Omat; Kodak). The signals were scanned densitometrically with an Epson GT 8000 (ScanPack; Biometra).

Analysis of Myosin Light Chain (MLC) Phosphorylation

Phosphorylation of the 20 kDa MLC of skinned bladder and myometrium fibers was analyzed by two-dimensional gelelectrophoresis (2D-PAGE). Isoelectric focussing (first dimension) and SDS-PAGE (second dimension) were performed as described recently [Calovini et al., 1995]. To measure Ca^{2+}-activated phosphorylation, skinned fibers were incubated under isometric conditions for 30 min at room temperature in pCa 4.5 and 1 μM calmodulin (maximal activation solution) or 30 min in pCa 8.0 and 1 μM calmodulin at room temperature (relaxing solution; for solution composition, see below). The gels were stained with silver (BioRad), and the MLC were scanned densitometrically.

Mechanical Analysis

All mechanical experiments were performed with bladder and myometrium skinned fibers.

Fibers were mounted horizontally between a force transducer (AME-AE 801; SensoNor, Horten, Norway) and a length step generator with a celluloid acetone glue. Relaxation solution contained (mM) HEPES (50), ATP (10), creatine phosphate (10), $MgCl_2$ (12.5), NaN_3 (5), EGTA (5), DTE (1), KCl (12.5), and calmodulin (0.001), pH 7.0. Contraction solution was the same as relaxation solution except that EGTA was substituted by 5 mM CaEGTA. Free Ca^{2+} concentrations were calculated using the dissociation constant given by Fabiato and Fabiato [1979]. Ionic strength was adjusted to 130 mM with KCl. The fibers were mounted in relaxation solution, and length was adjusted such that resting tension was just threshold. This was about 0.75 L_0 of both bladder and myometrial fibers (L_0 is the length where maximal force is generated). Subsequently the fibers were transferred into maximal Ca^{2+} activation solution (pCa 4.5). Force-velocity relations were determined by isotonic quick releases under constant load at 22°C. Load clamping for isotonic shortening was achieved by rapidly changing the mode of operation of the puller from fiber-length control to force control during isometric steady-state tension. The force during isotonic contraction was held constant by the controlled motion of the puller which followed the contracting fiber with the appropriate velocity. The force-control mode was maintained for 250 ms. The velocity of the length step was determined with an optoelectronical position detector (Hamamatsu, Japan). Both force and velocity signals were displayed on a storage digital oscilloscope and analyzed with an IBM compatible PC. The relation between shortening velocity determined between 25 and 50 ms after release and force during isotonic contraction was analyzed using a linearized form of the Hill equation:

$$(P + a)(v + b) = (P_0 + a)b$$

where v is the shortening velocity, P is the force during isotonic contraction, P_0 is the maximal isometric steady-state force, and a and b are constants [Hill, 1938].

Statistics

Statistical analysis was performed using the commercially available statistic program on an IBM-compatible PC (means, standard deviations (SD), regression analysis, Student's *t*-test for unpaired values). All values are expressed as means ± SEM.

RESULTS

In Vivo Expression of Myosin Heavy Chain (MHC) Isoforms

MHC isoform expression of bladder and myometrium of nonpregnant and pregnant rats was analyzed by SDS-PAGE and immunoblotting using a peptide-directed antibody specific for the 5′ insert sequence (QGPSFAY) of rat smooth muscle MHC. Generally, three MHC isoforms could be differentiated on the protein level in the smooth muscle tissues due to their apparent molecular mass having Mr of 204 kDa (SM1), 200 kDa (SM2), and 196 kDa (NM) (Fig. 1). Rat bladder expressed SM1 and SM2 but no NM-MHC; the relative portion of SM1 was 71 ± 3%. Myometrium of nonpregnant rats expressed SM1 and NM-MHC but no SM2. The relative amount of SM1 was 63 ± 5% (Fig. 1). The antibody a25K/50K differentiated additional splice variants within MHC. It reacts with both SM1 and SM2 of bladder and SM1 of myometrium (Fig. 1). In the myometrium, however, immunorecognition by a25K/50K was much less pronounced than that in the bladder (Fig. 1). The immunoreactivity was completely blocked by addition of excess peptide during incubation of a25K/50K, indicating specific antigen recognition (data not shown). Moreover, nonmuscle MHC revealed no cross-reactivity with a25K/50K (Fig. 1).

To quantify the relative proportions of 5′-inserted MHC in bladder and myometrium, we used a quantitative Western-blot approach. Four different protein concentrations from bladder as well as from myometrial tissue of nonpregnant rats were run from each sample. Two identical gels were run in parallel. One gel was stained with Coomassie blue to determine protein concentration in the SM-MHC band. The corresponding gel was transferred to nitrocellulose and probed with a25K/50K. In Figure 2 the intensity of the immunorecognition by a25K/50K (ECL signal) is plotted against the densitometrically determined intensity in the SM-MHC band on the Coomassie blue–stained gels. A strong ECL signal was obtained with bladder SM-MHC at low protein concentrations, whereas virtually no immunoreactivity was detectable in the SM-MHC from myometrium. At higher protein concentration the ECL signal of myometrium MHC increased linearly, while that of bladder MHC exceeded the linear range of detec-

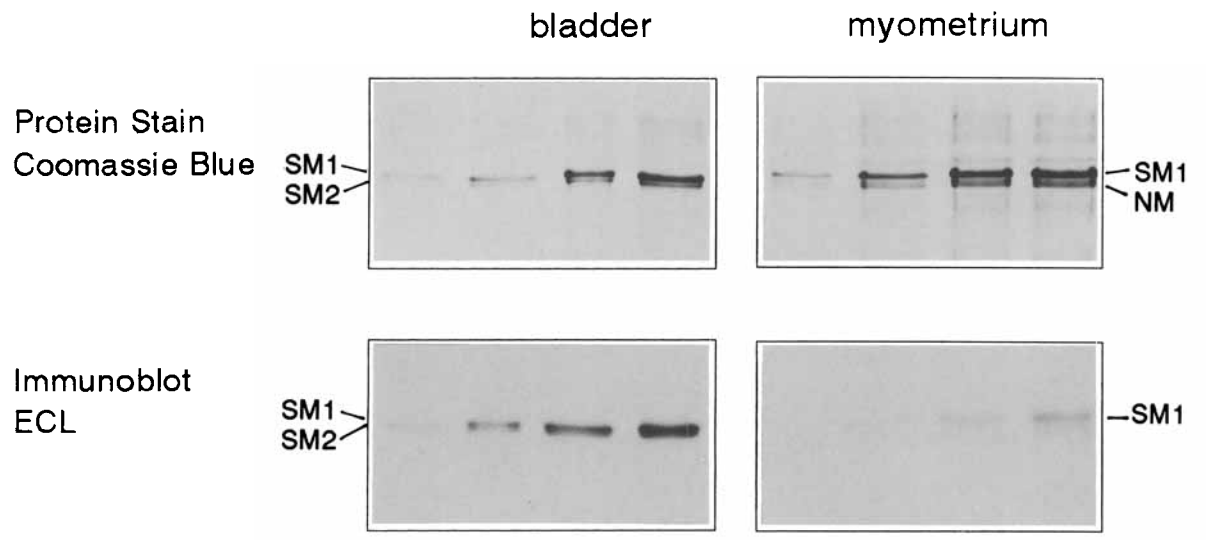

Fig. 1. Analysis of myosin heavy chain (MHC) isoform expression in smooth muscle tissue of bladder and myometrium. *SM1, SM2,* smooth muscle isoforms; *NM,* nonmuscle isoform. **Top:** MHC as detected by protein staining (Coomassie blue). **Bottom:** Western-blot analysis (ECL signal) of the respective MHC bands using the antipeptide antibody a25K/50K. Increasing concentrations of protein of bladder (5, 10, 15, 20 μg) and myometrium (10, 20, 30, 40 μg) were loaded on the gel.

tion. Comparing the ECL signals for an intermediate protein concentration around 1.5 (relative OD of Coomassie staining), the amount of 5′-inserted MHC was about eightfold higher in bladder than that in the myometrium (Fig. 2). Both SM1 and SM2 of rat bladder reacted with a25K/50K. Densitometric evaluation of the ECL signal revealed relative proportions of 70/30, which corresponds to the protein signal obtained with Coomassie blue.

In myometrium, the expression of 5′-inserted MHC was low. Besides this fact, we recently demonstrated that it is regulated by steroid hormones [Calovini et al., 1995]. We employed, therefore, the immunorecognition by a25/50K to study differences in the expression of 5′-inserted MHC in the myometrium of pregnant and nonpregnant rats. As demonstrated (Fig. 3), pregnancy was associated with a significant downregulation of MHC1B isoform expression. For each amount of MHC, the immunorecognition by the antibody a25K/50K was about 50% in the pregnant preparations compared to the controls of nonpregnant myometrium (Figs. 3, 4). The expression of SM1 was found to be increased during pregnancy, amounting to 81 ± 3% of the total MHC (Fig. 3, upper panel).

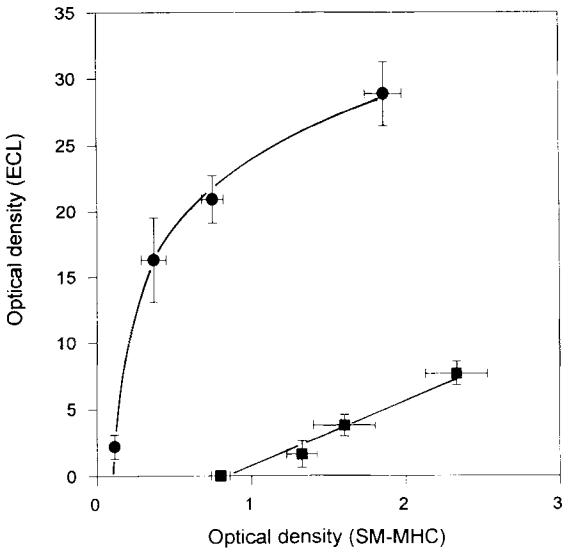

Fig. 2. Quantitative analysis of the expression of 5′-inserted myosin heavy chain (B-forms of SM-MHC) in bladder and myometrium of rat. ECL signals were plotted vs. protein signals (cf. Fig. 1) of bladder MHC (●) and myometrium MHC (■). Values are means ± SEM for three independent experiments.

Force-Velocity Relations

To investigate whether the different expression level of 5′-inserted MHC affects cross-bridge cycling kinetics, force-velocity relations of bladder and myometrial fibers were determined. This was obtained by applying isotonic quick releases under constant load conditions at slack length during steady-state maximal isometric tension (pCa 4.5). Shortening velocities were measured between 25 and 50 ms after the quick release. As extrapolated from the force-velocity relations (Fig. 5) to zero load, the apparent maximal shortening velocity ($Vmax_{app}$) of blad-

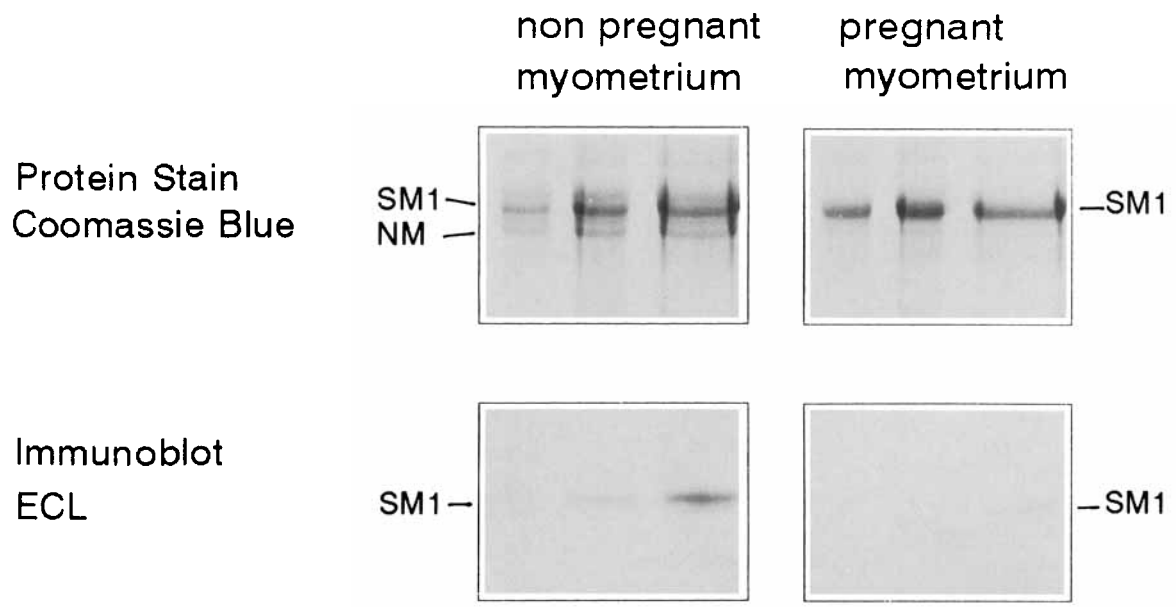

Fig. 3. Analysis of myosin heavy chain (MHC) isoform expression in the myometrium of nonpregnant and pregnant rats. SM1 and NM correspond to smooth-muscle- and nonmuscle-specific isoforms, respectively. **Top:** MHC as detected by protein staining (Coomassie blue). **Bottom:** Western-blot analysis (ECL signal) of the respective MHC bands using the antipeptide antibody a25K/50K. Increasing amounts of protein (10, 20, 30 μg each for nonpregnant and pregnant myometrium) were loaded on the gel.

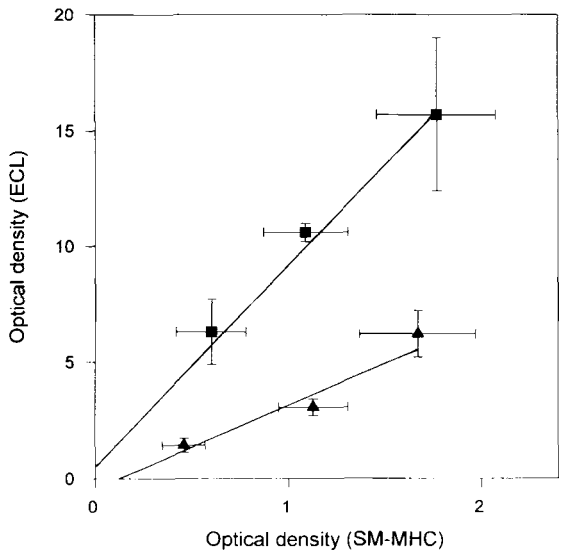

Fig. 4. Quantitative analysis of the expression of 5′-inserted myosin heavy chain (B-form of SM-MHC) in the myometrium of nonpregnant and pregnant rats. ECL signals were plotted vs. protein signals (cf. Fig. 3) of MHC from nonpregnant (■) and pregnant myometrium (▲). Values are means ± SEM for four independent experiments.

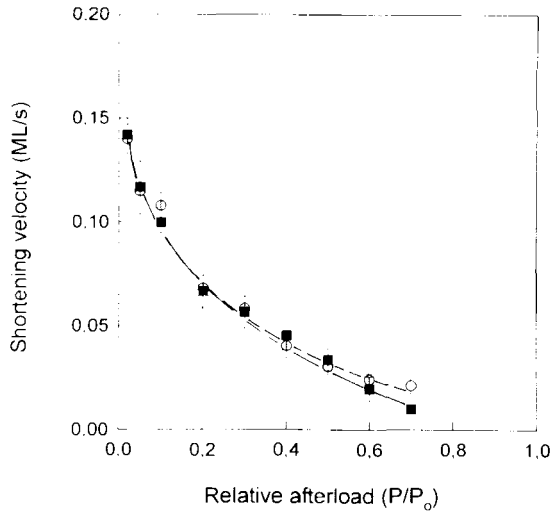

Fig. 5. Force-velocity relations of chemically skinned fibers of nonpregnant rats from bladder (*shaded circles*) and myometrium (■). P is force during isotonic contraction; P0 is the maximal isometric force. Values are Means ± SEM (six fibers per tissue).

der fibers was 0.138 ± 0.012 muscle length per second (ML s^{-1}, n = 14). Skinned fibers obtained from normal rat myometrium had 0.114 ± 0.023 ML s^{-1} (n = 9) (i.e., not significantly different from bladder fibers).

Myosin Light Chain Phosphorylation Pattern

To exclude the possibility that different myosin light chain (MLC) phosphorylation levels of bladder and myometrial fibers balanced the putative physiological effects of inserted MHC, we studied phosphorylation of MLC20. Skinned bladder and myometrial fibers were incubated

for 30 min in relaxation (pCa 8.0) or maximal Ca^{2+}/calmodulin activation solution (pCa 4.5). Subsequently, MLC20 forms were analyzed by 2D-PAGE. Since phosphorylation of the MLC introduces negative charges, analysis by 2D-PAGE leads to different MLC20 forms having almost the same molecular weights but different isoelectric points. Figure 6 demonstrates a quantitative analysis of these differentially phosphorylated forms as obtained by densitometric scanning. Three MLC20 forms (1–3 with increasing acidity) could be resolved in bladder fibers. The most basic MLC20 form, 1, is the unphosphorylated form comprising 74% of whole MLC20 forms. Forms 2 and 3 represent the phosphorylated forms of MLC20. Ca^{2+}/calmodulin activation changed this pattern, increasing the more acidic (phosphorylated) MLC20 forms. Thus, the unphosphorylated MLC20 form decreased significantly by a factor of 1.6, while the most acidic biphosphorylated MLC20 form, 3, increased significantly by a factor of 2.5.

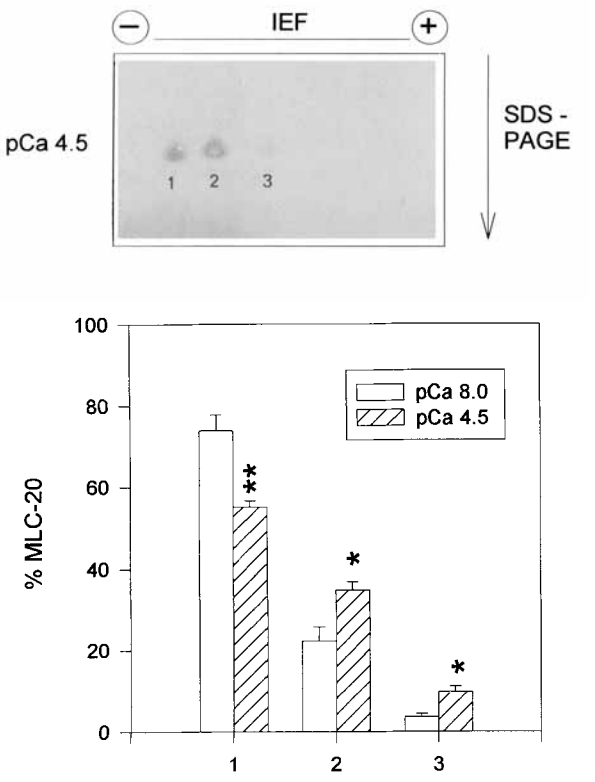

Fig. 6. Analysis of myosin light chain phosphorylation of skinned rat bladder fibers. **Top:** Original gel of Ca^{2+}-activated MLC20 (pCa 4.5). **Bottom:** Relative amounts of the unphosphorylated (*1*) and mono- (*2*) and bisphosphorylated (*3*) forms of MLC20 are shown under relaxation conditions (pCa 8.0) and after maximal Ca^{2+}/calmodulin activation (pCa 4.5). Values are means ± SEM of five fibers each.

The situation is more complex in the myometrial fibers (Fig. 7) since there are two different MLC20 isoforms (MLC20A and MLC20B). In the relaxed state (pCa 8.0), four MLC20 forms exist. MLC20 form 1 represents the unphosphorylated MLC20A, while form 2 represents the second unphosphorylated MLC20 isoforms (MLC20B). Forms 3 and 4 represent the phosphorylated MLC20 forms. Ca^{2+}/calmodulin activation (pCa 4.5) decreased the unphosphorylated MLC20A form 1 significantly by a factor of 1.4, while the most acidic, biphosphorylated form, 4, increased significantly by a factor of 2.0. Comparing changes of unphosphorylated and phosphorylated MLC20 forms in bladder and myometrium, we found no significant differences between both fiber types.

DISCUSSION

The smooth muscle MHC gene product is alternatively spliced at both the 3′ and the 5′ terminus [for review see Somlyo et al., 1993]. Splicing at the 5′ terminus leads to exclusion of a 21 nt exon (seven amino acids) at the 25K/50K boundary close to the ATP-binding domain. It is, therefore, reasonable to suggest that splicing in that region changes the enzymatic and mechanical properties of smooth muscle myosin [White et al., 1993; Babij, 1993; Kelly et al., 1993]. In fact, actin-activated ATPase activity and the velocity of actin filament sliding in the in vitro motility assay was higher in SM-MHC with 5′ insert than in that without 5′ insert [Kelley et al., 1993]. However, the behavior of alternatively spliced SM-MHC in a functionally intact muscle has not been investigated. The positive correlation between maximal shortening velocity (Vmax) and ATPase activity [Swynghedauw, 1986] predicts that fibers with high amounts of 5′-inserted MHC display a higher Vmax than fibers with low amounts of 5′-inserted MHC. To test this hypothesis, we compared the force-velocity relation of chemically skinned fibers of bladder and myometrium which are reported to differ in their amounts of 5′-spliced MHC mRNA [White et al., 1993]. Our results demonstrate, however, that there was no significant difference in maximal shortening velocity (Vmax) between both smooth muscle types. Relative increases in phosphorylation levels of MLC20 isoforms upon Ca^{2+}/calmodulin activation were also in the same range for bladder and myometrial fibers, excluding perturbations

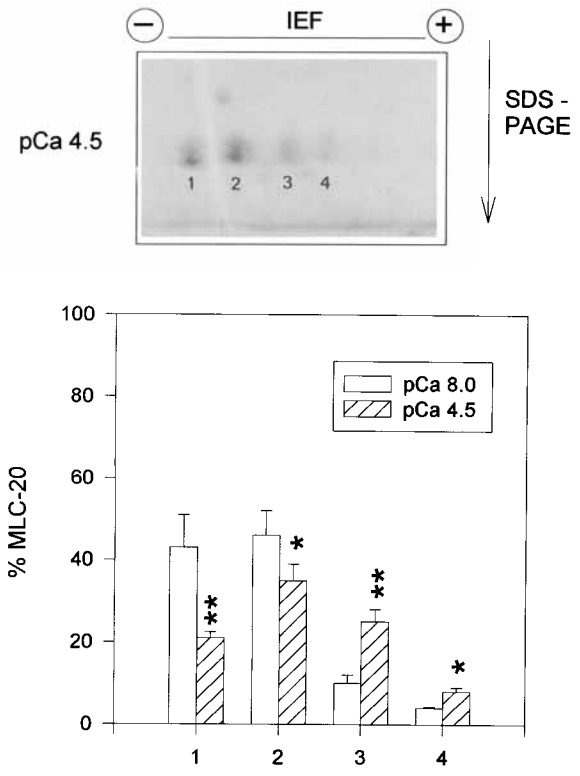

Fig. 7. Analysis of myosin light chain phosphorylation of skinned myometrial fibers of nonpregnant rats. **Top:** Original gel of Ca^{2+}-activated MLC20 (pCa 4.5). **Bottom:** Relative amounts of the unphosphorylated (*1*) and mono- (*2*) and bisphosphorylated (*3,4*) forms of MLC20 are shown under relaxation conditions (pCa 8.0) and after maximal Ca^{2+}/calmodulin activation (pCa 4.5). Values are means ± SEM of five fibers each.

of the putative effect of 5′-spliced MHC on Vmax by MLC20 phosphorylation. However, we cannot exclude the possibility that an effect of 5′ MHC on Vmax could be masked by different MLC20 isoforms present in both tissues.

To demonstrate that the MHC expression pattern predicted from mRNA studies is indeed realized on the protein level, we generated an antipeptide antibody (a25K/50K) directed against the 5′ insert sequence (QGPSFAY). In accordance with the mRNA study [White et al., 1993], we found an about eightfold higher immunoreactivity with a25K/50K in bladder compared to myometrium by Western-blot analysis. In addition, we could demonstrate that both SM1 and SM2 reacted with a25K/50K, the relative proportions being equal to that of the Coomassie-stained SM1 and SM2. These results suggest that alternative splicing of both smooth-muscle-specific MHC isoforms is regulated in parallel in the bladder. Thus, our data demonstrate that in rat bladder SM1B and SM2B are the dominant isoforms, while myometrial SMC expressed predominantly the SM1A splicing variant.

Interestingly, the low amounts of SM1B present in rat myometrium became downregulated in the pregnant state. This alteration was not associated with decreased shortening velocity as expected from the initial hypothesis. Rather, Vmax of skinned pregnant myometrium fibers increased [Morano et al., 1993]. This again suggests that there is no positive correlation between Vmax and 5′ MHC expression.

Although positive correlations between MLC17a and ATPase activity as well as Vmax have been reported [Helper et al., 1988; Malmqvist and Arner, 1991; Morano et al., 1993], differences in the MLC17a expression cannot explain the contractile behavior of bladder and myometrium of rats. Rat bladder is reported to contain higher amounts of MLC17a than rat myometrium [Malmqvist and Arner, 1991].

Thus, the physiological importance of 5′-spliced smooth muscle MHC isoenzymes still needs to be elucidated. At present we are investigating whether MHC polymorphism in smooth muscle is involved in the modulation of the mechanical responsiveness to different physiological and pharmacological stimuli in intact preparations.

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