Nonmuscle Myosin motor of smooth muscle.

Löfgren, Mia; Ekblad, Eva; Morano, Ingo; Arner, Anders

Published in:
Journal of General Physiology

DOI:
10.1085/jgp.200208720

2003

Link to publication

Citation for published version (APA):

Total number of authors:
4

General rights
Unless other specific re-use rights are stated the following general rights apply:
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.
• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Nonmuscle Myosin Motor of Smooth Muscle

MIA LÖFGREN,1 EVA EKBLAD,1 INGO MORANO,2 and ANDERS ARNER1

1Department of Physiological Sciences, Medical Faculty, Lund University, SE-221 84 Lund, Sweden
2Max Delbrück Center for Molecular Medicine, Medical Faculty of the Charité, Humboldt University of Berlin, 13122 Berlin, Germany

ABSTRACT Nonmuscle myosin can generate force and shortening in smooth muscle, as revealed by studies of the urinary bladder from mice lacking smooth muscle myosin heavy chain (SM-MHC) but expressing the nonmuscle myosin heavy chains A and B (NM-MHC A and B; Morano, I., G.X. Chai, L.G. Baltas, V. Lamounier-Zepter, G. Lutsch, M. Kott, H. Haase, and M. Bader. 2000. Nat. Cell Biol. 2:371–375). Intracellular calcium was measured in urinary bladders from SM-MHC–deficient and SM-MHC–expressing mice in relaxed and contracted states. Similar intracellular [Ca2+] transients were observed in the two types of preparations, although the contraction of SM-MHC–deficient bladders was slow and lacked an initial peak in force. The difference in contraction kinetics thus do not reflect differences in calcium handling. Thick filaments were identified with electron microscopy in smooth muscle cells of SM-MHC–deficient bladders, showing that NM-MHC can form filaments in smooth muscle cells. Maximal shortening velocity of maximally activated, skinned smooth muscle preparations from SM-MHC–deficient mice was significantly lower and more sensitive to increased MgADP compared with velocity of SM-MHC–expressing preparations. Active force was significantly lower and less inhibited by increased inorganic phosphate. In conclusion, large differences in nucleotide and phosphate binding exist between smooth and nonmuscle myosins. High ADP binding and low phosphate dependence of nonmuscle myosin would influence both velocity of actin translocation and force generation to promote slow motility and economical force maintenance of the cell.

KEY WORDS: nonmuscle myosin • urinary bladder • ATP • ADP • phosphate

INTRODUCTION Nonmuscle myosins of the myosin II super family are ubiquitously expressed motor proteins in eukaryotic cells and are involved in a large variety of important physiological tasks, including cell migration and cytokinesis (Spudich, 1989). Mutations in a nonmuscle myosin heavy chain gene have been associated with severe disease affecting many organ systems (Kelley et al., 2000; Heath et al., 2001). Nonmuscle myosin expression is altered in several pathophysiological conditions, e.g., atherosclerotic plaque formation (Kuro-o et al., 1991).

The velocity of actin over nonmuscle myosin is slow compared with muscle myosin in vitro (Umemo and Sellers, 1990), suggesting differences in the kinetics of the cross-bridge interactions. In general, a relationship between the rate of the ADP release from the actin–myosin–ADP complex and the shortening velocity of muscle fibers has been proposed (Siemankowski et al., 1985; Weiss et al., 2001). Consistent with this, smooth muscle myosin has a higher ADP affinity in vitro (Cremo and Geeves, 1998) and in muscles fibers (Arheden and Arner, 1992; Nishiye et al., 1993) compared with the faster skeletal muscle myosin. This correlation is also valid within the smooth muscle group; higher ADP affinity has been demonstrated in slow compared with fast smooth muscle types (Fuglsang et al., 1993; Khromov et al., 1998; Löfgren et al., 2001). At present, information regarding the kinetics of the actin–nonmuscle myosin interaction of the organized contractile system is very sparse.

Recently, a transgenic mouse strain lacking the smooth muscle myosin heavy chain (SM-MHC)* expression has been introduced (Morano et al., 2000). These animals die a few days after birth but their urinary bladder smooth muscle obtained immediately postnatally can contract by the action of the nonmuscle myosins. This contraction has a slow onset in response to stimulation and is characterized by a low shortening velocity. The smooth muscle of these animals provide a unique model for studies of regulation and contractile kinetics of nonmuscle myosin.

In the present study we have used the urinary bladder from SM-MHC–deficient mice to answer the following questions: (a) Is the slow onset of the contraction of intact smooth muscle preparations from the SM-MHC–deficient mice due to altered Ca2+ handling? (b) Can nonmuscle myosins form thick filaments in the smooth muscle cells? (c) What is the shortening velocity of fully activated organized nonmuscle myosin? (d)

*Abbreviations used in this paper: NM-MHC, nonmuscle myosin heavy chain; SM-MHC, smooth muscle myosin heavy chain.
What are the binding characteristics of substrates and products (MgATP, MgADP, and inorganic phosphate) in the cross-bridge cycle of nonmuscle myosin?

Materials and Methods

Animals and Preparations

SM-MHC-deficient mice were generated using gene targeting as described previously (Morano et al., 2000). New-born mice were killed within 24 h of birth and their urinary bladders were dissected. Preparations were used for measurements of intracellular calcium in intact or chemically skinned preparations and used for mechanical measurements. Samples were frozen for gelelectrophoresis and fixed for immunohistochemistry and electron-microscopy. In some analyses, urinary bladder from 3-wk-old SM-MHC-expressing mice were used.

Homozygous SM-MHC-deficient mice were identified by the thin-walled and distended urinary bladder and the lack of an initial force transient in intact preparations (Morano et al., 2000). This definition of the phenotype was confirmed by gelelectrophoresis showing a low total myosin heavy chain content and a lack of the 205-kD SM-1-MHC band in these animals. It has been shown that the myosin content is essentially similar in the mice heterozygous and homozygous for the SM-MHC gene (Morano et al., 2000) and these animals were analyzed as one group denoted SM-MHC expressing.

Measurements of Intracellular Calcium in Intact Preparations

Small strips (2–3 mm long, ~500 μm wide) were prepared from the urinary bladder wall in the sagittal direction. The preparations were wrapped at each end with aluminum foil, and transferred to an oxygenated HEPES (N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid)) buffered solution containing, in mM: NaCl 118, KCl 5, NaH2PO4 1.2, MgCl2 1.2, HEPES 24 (pH 7.4), glucose 10, and CaCl2 1.5. Loading of Fura-2 was performed for 3–4 h at 22°C in the HEPES-buffered solution containing 10 μM Fura-2-AM (Molecular Probes), 1% DMSO, and 0.02% Pluronic F127. After an ~30-min wash in the HEPES buffer the preparations were transferred to a 1-mL bath on the stage of an inverted microscope. The muscles were kept in a Krebs-Henseleit solution of the following composition (in mM): NaCl 119, glucose 12.2, KCl 4.6, NaHCO3 25, KH2PO4 1.2, MgSO4 1.2, CaCl2 2 at 37°C, gassed with 95% O2/5% CO2 giving a pH of 7.4. Recordings of force and intracellular free Ca2+ concentration were made in the Krebs-Henseleit solution as described previously (Lucius et al., 1998) in the relaxed state (with and without 2 mM extracellular CaCl2) and after activation with 80 mM KCl. Force and fluorescence signals (at an emission wavelength of 510 nm) at excitation wavelengths 340 and 380 nm were recorded with a time resolution of 4–6 Hz. An internal calibration of the Ca2+ signal was made as described previously (Lucius et al., 1998), using 50 μM ionomycin for permeabilization, 20 mM MnCl2 for quenching, and an apparent dissociation constant of the calcium–fura-2 complex of 224 nM (Grynkiewicz et al., 1985).

Preparation of Permeabilized Tissue

Strips (2–3 mm long, ~500 μm wide) of urinary bladder were pinned to silica gel and permeabilized with 1% Triton X-100 as described previously (Arner and Hellstrand, 1985), and thereafter kept in a glycerol containing solution at −20°C and used for quick-release experiments and isometric force registrations, as described below, within a month. All experiments on skinned muscle preparations were performed at room temperature (22°C).

Quick-release Experiments

Maximal unloaded shortening velocity (Vmax) at maximal activation was examined with the isotonic quick-release method (compare Arner and Hellstrand, 1985). The preparations were attached with aluminum clips between a force transducer (AE 801; SensoNor) and an isotonic lever arm. The experimental procedure was executed as described previously (Löfgren et al., 2001). The preparations were kept maximally activated using repeated incubation with ATP-Py/S (Arheden et al., 1988). Contractions were elicited by transfer to an ATP-containing solution (see below) and at the plateau of each contraction, a series of 15–25 releases to different afterloads was performed. After each release, the length and afterload were recorded for 1 s. Velocity was determined 100 ms after release. Vmax was calculated by fitting the Hill equation (Hill, 1938; Eq. 1) to the force-velocity data and extrapolating to zero afterload:

\[ V = \frac{b(1 - P/P_0)}{(P/P_0 + a/P_0)}. \] (1)

In this equation, V is the velocity, P is isometric force, P is afterload, and a and b are constants. Two series of experiments were performed: (a) varied [MgATP] in the presence of an ATP-regenerating system and (b) varied [MgADP] at constant [MgATP] in the absence of an ATP-regenerating system. The ATP and ADP concentrations were applied at random order. At the end of the experiments in the first series, a contraction in 3.2 mM MgATP was followed by a 40 min incubation in rigor solution (0 MgATP and 0 phosphocreatine and creatine kinase with 50 U/ml hexokinase and 10 mM glucose) before a series of releases was performed, in order to determine the apparent Vmax in rigor (Löfgren et al., 2001).

Measurements of Isometric Force

Skinned preparations were mounted between a force transducer (AE 801; SensoNor) and a stainless steel pin and maximally activated using ATP-Py/S (Arheden et al., 1988). In the series of experiments where the effects of inorganic phosphate on isometric force was determined, the inorganic phosphate was introduced in the ATP-containing activating solution and the different concentrations of inorganic phosphate were applied at random order. In a separate series of experiments, the maximal force generation at 3.2 mM MgATP was determined. These preparations were fixed in 1% glutaraldehyde, embedded in Epon, and sectioned for light microscopy to determine preparation cross-sectional area.

Solutions Used in Experiments on Skinned Preparations

For the experiments on skinned preparations, a solution of the following composition was used: 30 mM TES, 4 mM EGTA, and 2 mM free Mg2+. The ionic strength and the pH were adjusted to 150 mM and 6.9, respectively, using KCl and KOH. The standard ATP-containing solution contained 3.2 mM MgATP and an ATP-regenerating system with 12 mM of phosphocreatine and 0.5 mg/ml of creatine kinase. In the ADP-containing solutions, the ATP-regenerating system was not used and 0.2 mM of the myokinase inhibitor ApA was added (Feldhaus et al., 1975).
antibody against nonmuscle myosin heavy chain A (NM-MHC-A), a gift from Dr. R. Adelstein (Kelley et al., 1996), or a polyclonal rabbit antibody against NM-MHC-B (Sjuve et al., 2001). Immunoreactivity was detected using Enhanced Chemiluminescence (ECL; Amersham Biosciences) and visualized with a Fluor-S Max (Bio-Rad Laboratories). For immunohistochemistry, urinary bladders were cut open, pinned to pieces of cork, fixed in 2% formaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer (pH 7.2) over night, rinsed in a Tyrode solution with 10% sucrose, and embedded for cryosectioning (Sjuve et al., 1998). Sections were stained with NM-MHC-A or NM-MHC-B antibodies. Double staining with a monoclonal antibody raised in mouse against smooth muscle alpha-actin (Cy3 conjugated; C6198, Sigma-Aldrich) was used to determine the colocalization of the proteins in cells/regions.

Electron Microscopy

Strips of urinary bladder tissue were pinned to silica gel and fixed using glutaraldehyde and paraformaldehyde as described previously (Sjuve et al., 1998). The preparations were stored at 4°C in cacodylate buffer (0.125 M) with 0.1% glutaraldehyde. Post fixation was in 1% OsO4, followed by contrasting with uranyl acetate and Reynolds’ lead citrate, and embedding in Epon. Sections, 50-nm thick, were examined using a Jeol JEM 1230 microscope equipped with a Gatan Multiscan CCD camera Model 791.

Statistics

Values are given as means ± SEM. Curve fitting was performed using routines implemented in Sigma Plot (SPSS Science). Statistical comparisons were made using Student’s t test or repeated measures analysis of variance (using SPSS 10.1.3).

R E S U L T S

Force and Intracellular Calcium Transients in Intact Preparations

Fig. 1 shows original recordings of force and intracellular [Ca2+] in relaxed and contracted urinary bladder tissue from a SM-MHC–expressing and a SM-MHC–deficient mouse. Activation with high K+ in the SM-MHC–expressing muscle resulted in a contraction with an initial force peak followed by a decay to a stable force level. In the preparation from the SM-MHC–deficient mouse, force increased gradually to a steady force level without an initial peak. In both preparations, a similar Ca2+ transient was observed with a peak reaching between 400–500 nM and a fall to a stable lower level during the sustained phase of contraction. Table I summarizes the results on intracellular [Ca2+] and force. Although force transients were different, no significant differences in calcium levels between the tissues were observed in the relaxed state (with and without external calcium) or during activation with high K+

Force-velocity Relation and Maximal Isometric Force

Original force-velocity relations of urinary bladder preparations from a new-born and a 3-wk-old SM-MHC–expressing and a new-born SM-MHC–deficient mouse are shown in Fig. 2 A. The SM-MHC–deficient preparation had markedly lower shortening velocity. In Fig. 2 B, mean values of maximal shortening velocity (Vmax) at 3.2 mM MgATP for the respective groups are shown. Vmax of the urinary bladder preparations of the SM-MHC–deficient mice was ~40% of that in the SM-MHC–expressing tissues. The Vmax values in Fig. 2 B were not corrected for the influence of velocity at zero MgATP (see below). If these corrections were made, the velocity of SM-MHC–deficient tissue would be ~30% of that of the new-born SM-MHC–expressing tissue at 3.2 mM MgATP. No significant difference in Vmax was observed between the new-born and the 3-wk-old SM-MHC–expressing tissues. The parameters a/Po of

Figure 1. Isometric force (A and B) and intracellular calcium (C and D) in intact urinary bladder preparations from new-born SM-MHC–expressing (A and C) and SM-MHC–deficient (B and D) mice. Preparations were activated with 80 mM K+ (arrows).
Relaxed, 2.5 mM CaCl₂ (nM) 284

MHC–deficient mice, no clear initial peak in force was detected. Calcium sustained (plateau after 300 s) force was evaluated. Note that for the SM-

Initial (maximal value measured within 60 s after activation) relative to

ences of P

Peak (nM) 414

F

Force and Intracellular Calcium Concentration in Urinary Bladder Prep-

arations from SM-MHC–expressing and SM-MHC–deficient Mice

<table>
<thead>
<tr>
<th></th>
<th>SM-MHC expressing</th>
<th>SM-MHC-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial/sustained force</td>
<td>2.57 ± 0.26</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Relaxed, calcium-free (nM)</td>
<td>181 ± 26</td>
<td>206 ± 44</td>
</tr>
<tr>
<td>Relaxed, 2.5 mM CaCl₂ (nM)</td>
<td>284 ± 35</td>
<td>302 ± 47</td>
</tr>
<tr>
<td>Peak (nM)</td>
<td>414 ± 46</td>
<td>428 ± 64</td>
</tr>
<tr>
<td>Plateau (nM)</td>
<td>313 ± 31</td>
<td>326 ± 41</td>
</tr>
</tbody>
</table>

Initial (maximal value measured within 60 s after activation) relative to sustained (plateau after 300 s) force was evaluated. Note that for the SM-

MHC–deficient mice, no clear initial peak in force was detected. Calcium was measured in the relaxed state (at 0 and 2.5 mM external CaCl₂) and during peak and plateau phase (after 300 s) of high K⁺-induced contractions. No significant differences in calcium transients were observed between the two muscle groups, although force transients were markedly different. n = 6–12.

Eq. 1 was: 0.426 ± 0.165 for SM-MHC–deficient mice, 0.144 ± 0.017 for new-born SM-MHC–expressing and 0.127 ± 0.024 for 3-wk-old SM-MHC–expressing mice (3.2 mM MgATP). The parameter \( b \) was: 0.028 ± 0.009 for SM-MHC–deficient mice, 0.027 ± 0.002 for new-born SM-MHC–expressing mice, and 0.021 ± 0.003 ML/s for 3-wk-old SM-MHC–expressing mice (3.2 mM MgATP). The low \( V_{\text{max}} \) of the SM-MHC–deficient mice was thus not associated with a lower value of parameter \( b \) in Eq. 1, instead the \( a/P \) value was increased, indicating a less concave force-velocity relation.

Fig. 2 C shows summarized data regarding force generation of maximally activated (thiophosphorylated), permeabilized preparations contracted at saturating MgATP (3.2 mM). Data are given as stress, i.e., force per cross-sectional area, calculated from maximal isometric force and stress (i.e., isometric force per cross-sectional area) of SM-MHC–expressing and SM-MHC–deficient tissues. These results suggest that no difference in the rate of Ca²⁺-induced light chain phosphorylation is present between the two groups. Experiments in which free Ca²⁺ level was increased (pCa 9–4.5) in the presence of calmodulin at the plateau of contractions after the 15- and 30-min thiophosphorylation periods were also performed. No further increase in force was detected, showing that phosphorylation was maximal and sufficient to fully activate the tissue.

Figure 2. Shortening velocity (\( V \), muscle lengths/s) and force generation of skinned preparations. A shows force-velocity relations at 3.2 mM MgATP of skinned urinary bladder preparations from a new-born SM-MHC–expressing mouse (open circles), a 3-wk-old SM-MHC–expressing mouse (open squares) and a SM-MHC–deficient mouse (filled circles). B and C show mean values of the maximal shortening velocity (\( V_{\text{max}} \)) and stress (i.e., isometric force per cross-sectional area) of SM-MHC–deficient (filled bars), new-born SM-MHC–expressing mice (open bars), and 3-wk-old SM-MHC–expressing mice (hatched bars). ** and *** indicate statistical differences of \( P < 0.01 \) and \( P < 0.001 \), respectively, compared with both SM-MHC–expressing groups, Bonferroni method. n = 5–8.
Ki values. show fits of Eq. 2 to the mean values. Lines in B and C show fits of Eq. 3 to the mean values. Thick lines indicate relations used to estimate the apparent maximal shortening velocity in rigor from the respective groups have been subtracted from the individual values. Lines in A and those in C with 10 mM MgATP and the values are given relative to velocities at 0 mM MgADP at the respective MgATP. Mean values of between 98 and 112% of Vmax at 10 mM MgATP with or without ATP-regenerating system were for all groups. The Vmax at 6 and 10 mM MgATP concentrations (6 and 10 mM MgATP) have to be performed in the absence of an ATP-regenerating system. As seen in Fig. 3, B and C, MgADP inhibited shortening velocity. The effects of MgADP were dependent on the MgATP level was saturating even in the absence of ATP-regenerating system. High MgATP concentrations (6 and 10 mM MgATP) were used in these experiments, to ensure that the ATP level was saturating even in the absence of ATP-regenerating system. The Vmax at 6 and 10 mM without ATP-regenerating system were for all groups between 98 and 112% of Vmax at 10 mM MgATP with ATP regeneration. As seen in Fig. 3, B and C, MgADP inhibited shortening velocity. The effects of MgADP were dependent on the MgATP (compare Fig. 3, B and C), suggesting a competition between ATP and ADP. For the urinary bladders from SM-MHC–expressing mice, minimal inhibition of shortening velocity was observed at 10 mM MgATP (Fig. 3 C) whereas at 6 mM MgATP (Fig. 3 B), velocity was inhibited in a dose-dependent manner by MgADP. In the SM-MHC–deficient tissue the dependence of Vmax on MgADP was very steep at 6 mM MgATP. At 10 mM MgATP for the SM-MHC–deficient tissues and at 6 mM MgATP for the SM-MHC–expressing tissues, a graded inhibition by MgADP was observed. As seen in Fig. 3, the maximal effect of MgADP seemed to level off at an inhibition of Vmax to ~50% of maximal. This is similar to previous observations in fast and slow smooth muscle from the guinea-pig (Löfgren et al., 2001). Since velocity could not be completely inhibited by MgADP, Michaelis-Menten’s kinetics for complete inhibition in its simplest form could not be applied. Since the data suggest an inhibition to ~50%, we used a modified equation (Eq. 3) to describe the inhibition of Vmax by MgADP at constant [MgATP].

\[
\text{Relative } V_{\text{max}} = 0.5 + 0.5^* \frac{[\text{MgATP}]}{K_m + [\text{MgATP}]}.
\] (3)

Using the K_m values for MgATP (compare text above and Fig. 3 A) we fitted Eq. 3 to the to the mean data in Fig. 3. B and C, to obtain an estimate of the apparent K_i values for MgADP. Best fits were obtained at 6 mM MgATP for the SM-MHC–expressing groups and at 10 mM MgATP for the SM-MHC–deficient tissue. The mean values for the K_i were for the new-born SM-MHC–expressing: 5.8; for the 3-wk-old SM-MHC–expressing: 8.8 and for the SM-MHC–deficient mice: 0.64 μM (n = 5–6), showing an ~10-fold lower K_i in the SM-MHC–deficient compared with the SM-MHC–expressing mice and 41.7 ± 7.0% (SM-MHC–deficient, n = 6) of the Vmax at saturating MgATP. Fig. 3 shows the influence of varied concentrations of MgATP (Fig. 3 A) and MgADP (Fig. 3 B) on Vmax. The data at different MgATP were fitted to a hyperbolic equation (Eq. 2), giving an apparent binding constant (K_m) for MgATP and Vmax.

\[
V_{\text{max}} = \text{Max}^* \frac{[\text{MgATP}]}{(K_m + [\text{MgATP}])}.
\] (2)

The parameter “Max” denotes the estimated maximal shortening velocity at saturating [MgATP]. This parameter was in the curve fitting not different from the measured maximal velocities at 3.2 and 10 mM MgATP for each group. The mean K_m values for MgATP and Vmax were for the new-born SM-MHC–expressing: 0.013 ± 0.004, for the 3-wk-old SM-MHC–expressing: 0.024 ± 0.009, and for the SM-MHC–deficient mice: 0.023 ± 0.017 mM, n = 5–6. In view of the low velocities, especially in the SM-MHC–deficient tissue, the number of data points below maximal were limited and therefore we cannot exclude a small systematic error in the determination of K_m. However, based on the values from the individual experiments presented above and the mean data presented in Fig. 3 A, we conclude that the K_m values for the three groups were similar.

Experiments with varied concentrations of MgADP have to be performed in the absence of an ATP-regenerating system. High MgATP concentrations (6 and 10 mM) were used in these experiments, to ensure that the ATP level was saturating even in the absence of ATP-regenerating system. The Vmax at 6 and 10 mM without ATP-regenerating system were for all groups between 98 and 112% of Vmax at 10 mM MgATP with ATP regeneration. As seen in Fig. 3, B and C, MgADP inhibited shortening velocity. The effects of MgADP were dependent on the MgATP (compare Fig. 3, B and C), suggesting a competition between ATP and ADP.
ing tissues. If the data were analyzed using a standard Michaelis-Menten’s equation for competitive inhibition using the lowest concentrations of MgADP only, an ~10-fold lower $K_i$ for MgADP for the SM-MHC–deficient compared with the SM-MHC–expressing tissue was also observed.

**Phosphate Dependence of Force**

The effects of varied concentrations of inorganic phosphate ($P_i$) on active force of skinned preparations are shown in Fig. 4. Force was inhibited by $P_i$ in a dose-dependent manner in all preparations. The 3-wk-old SM-MHC–expressing group was most sensitive to $P_i$, with ~50% inhibition of force at 40 mM $P_i$. The SM-MHC–deficient group was markedly less sensitive compared with the SM-MHC–expressing groups.

**Tissue Expression of Actin and Nonmuscle Myosin**

Immunohistochemistry showed that the majority of the cells in the muscular layers of the bladder wall were smooth muscle cells as indicated by the smooth muscle α-actin staining. The NM-MHC-B antibody stained α-actin–negative cells located in the interstitium between the muscle bundles and in the submucosa and serosa. These cells comprised <5% of the wall cross-sectional area. NM-MHC-B immunoreactive interstitial cells were small and stellate. They were more numerous and smaller in the new-born mice, both SM-MHC–deficient and SM-MHC–expressing, and less frequent in 3-wk-old SM-MHC–expressing mice (Fig. 5 A). In the new-born mice, the NM-MHC-B antibody gave a very weak signal also from the smooth muscle cells. The NM-MHC-A antibody showed a weak staining of α-actin positive cells in all groups of tissues (unpublished data).

Coomassie-stained gels showed that the intensity of the myosin heavy chain was lower (~1/5) in the SM-MHC–deficient preparation compared with the SM-MHC–expressing tissue (Fig. 5 B). Extracts of bladders from new-born SM-MHC–expressing and SM-MHC–deficient mice reacted with the NM-MHC-A and NM-MHC-B antibodies on Western blots. For the 3-wk-old SM-MHC–expressing bladders NM-MHC-A was present. Also NM-MHC-B could be detected on the Western blots, but the staining was much weaker than in the new-born bladders (Fig. 5 B).

**Figure 4.** Measurements of active force at different concentrations of inorganic phosphate. Filled circles show results from SM-MHC–deficient mice, open circles show results from new-born SM-MHC–expressing mice, and open squares from 3-wk-old SM-MHC–expressing mice. Force is normalized to the value at zero $[P_i]$. $n = 6–8$. Using repeated measures analysis of variance and correction for multiple comparisons (Bonferroni), all three groups differed ($P < 0.001$) relative to each other.

**Figure 5.** Immunohistochemistry and gel electrophoresis. A shows a immunohistochemical section from a 3-wk-old mouse bladder stained with the NM-MHC-B antibody. Immunoreactivity is observed in the serosa and submucosa and in small stellate cells in the muscle layer. Bar, 0.1 mm. B shows Coomassie-stained SDS-PAGE and corresponding Western blots with NM-MHC-A and -B antibodies of extracts from the muscle layer of new-born SM-MHC–deficient and SM-MHC–expressing mice and from a 3-wk-old SM-MHC–expressing mouse.
Ultrastructure

Transmission electron microscopy was performed on transverse sections of the urinary bladder preparations. The main focus of these experiments was to identify myosin filaments in SM-MHC-deficient smooth muscle cells. Structures with sizes corresponding to cross sectioned actin and myosin filaments were observed. The measured diameter of the latter was $14.3 \pm 0.41$ nm, $n = 36$. In smooth muscle cells from SM-MHC-expressing mice regions with comparatively abundant thick filaments could easily be identified. Regions with thick filaments (Fig. 6 B) were observed also in smooth muscle cells of the SM-MHC-deficient mice; however, such regions were very sparse and contained fewer thick filaments compared with those in SM-MHC-expressing tissue.

DISCUSSION

We present data on cross-bridge kinetics of filamentous nonmuscle myosin in smooth muscle using a novel transgenic mouse model where the SM-MHC expression is ablated (Morano et al., 2000). To date, two main nonmuscle myosin heavy chain variants have been described in smooth muscle tissue, NM-MHC-A and NM-MHC-B (Kawamoto and Adelstein, 1987, 1991; Katsumura et al., 1989; Kuro-o et al., 1991; Simons et al., 1991). Recently, a third nonmuscle myosin heavy chain has been proposed on the basis of gencan predictions (Berg et al., 2001). The structure and role of this putative novel nonmuscle myosin is unknown at present; an ~75% homology with the motor domain of the nonmuscle myosin B isoform seems to exist. A previous study (Morano et al., 2000) has shown that both nonmuscle myosin A and B are expressed in smooth muscle cells from SM-MHC-deficient transgenic mice. In this study we examined the tissue expression of the NM-MHC-B and found that this type was mainly expressed in smooth muscle α-actin negative cells in the interstitium, similarly to observations from adult rat bladders (Sjuve et al., 2001). In the new-born SM-MHC-deficient tissue, both NM-MHC-A and -B are identified with Western blots. NM-MHC-B seemed to be mainly localized to the interstitial cells, but the smooth muscle cells, identified by the α-actin antibody, also expressed a very weak staining. NM-MHC-A did not appear to be expressed in the interstitial cells, but rather showed a weak distribution in the bladder wall. Our conclusion is thus that the smooth muscle tissue express nonmuscle myosins consistent with the previous report by Morano et al. (2000). The main isoform seems to be NM-MHC-A, but a contribution from NM-MHC-B and possibly also a third, yet uncharacterized, NM-MHC isoform cannot be excluded. The nonmuscle myosins belong to the myosin II family and has previously been shown to form filaments in vitro (Schloey et al., 1980; Smith et al., 1983). Using electron microscopy we demonstrated myosin filaments in the urinary bladder smooth muscle cells of the SM-MHC-deficient mice, showing that the NM-MHC form thick filaments in vivo, at least in tissues lacking SM-MHC. The size of these filaments in cross section appears to be similar to that of smooth muscle myosin filaments, although the detailed structure remains to be clarified.

We interpret the contractions of bladder muscle from SM-MHC-deficient mice to reflect active force generation from a contractile apparatus containing smooth muscle actin and NM-MHC. It should, however, be noted that a very small number of interstitial cells expressing NM-MHC and most likely nonmuscle actin isoforms are present in the tissue. We do not have data suggesting myosin filaments in these cells and cannot at present determine if they contribute to force generation and shortening of the tissue. Different actin iso-
forms (skeletal and smooth) have been shown to have similar function in the actin–myosin interaction (Mosakowska and Strzelecka-Golaszewska, 1985; Harris and Warshaw, 1993) and cytoplasmic and smooth muscle specific actin isoforms can coexist in the same thin filament (Drew and Murphy, 1997). This suggests that our data, independent of cell type, reflect properties of the nonmuscle myosin.

The nonmuscle myosin IIs are considered to be regulated by myosin light chain phosphorylation (Bresnick, 1999), mainly via myosin light chain kinase (MLCK) activated by Ca\textsuperscript{2+}–calmodulin. We show that the activation by depolarization was associated with a transient in intracellular [Ca\textsuperscript{2+}], exhibiting an initial peak and a plateau during the sustained phase of the contraction. The transients in [Ca\textsuperscript{2+}] after activation were similar in SM-MHC–expressing and SM-MHC–deficient muscles. In the SM-MHC–expressing muscles the force responses to high K\textsuperscript{+} showed an initial transient and a sustained tonic phase while in the SM-MHC–deficient tissue the initial force transient is absent (compare Mosakowska and Strzelecka-Golaszewska, 1985; Harris and Warshaw, 1993) and cytoplasmic and smooth muscle myosins. If we use the in vitro motility data, in combination with our determinations of V\textsubscript{max}, we can estimate the length of the contractile unit in the smooth muscle (compare half sarcomere length in striated muscle) to =1.7 μm in control and 1.2 μm in SM-MHC–deficient tissue. Although these values are very crude estimates, they seem reasonable in view of the estimated thick filament length (2.2 μm) in smooth muscle (Ashton et al., 1975).

In general, differences in shortening velocity between muscles are considered to reflect properties of the myosin molecules. Shortening velocity is considered to be rate-limited by the rate of cross-bridge dissociation (Weiss et al., 2001). In smooth muscle cells, the second-order rate constant of the ATP-induced dissociation of the rigor complex has been reported to be lower than that of skeletal muscle (Nishiye et al., 1993) and lower in slow compared with fast smooth muscles (Somlyo et al., 1988; Khromov et al., 1996). The K\textsubscript{m} values for ATP and V\textsubscript{max} of the SM-MHC–deficient and SM-MHC–expressing urinary bladders were approximately equal. The ratio between V\textsubscript{max} and K\textsubscript{m} for ATP are correlated with the binding constant for ATP to the actin–myosin complex (Stienen et al., 1988). Since the V\textsubscript{max} differed by a factor of ~3 at similar K\textsubscript{m}, this could suggest that the binding of MgATP to the NM-MHC is weaker compared with that of the SM-MHC. However, the ATP-induced dissociation is fast at saturating MgATP and is thus most likely not limiting the maximal velocity of sliding in smooth muscles.

The ADP-release reaction preceding the cross-bridge dissociation is considered to be important for the velocity of filament sliding (Siemankowski et al., 1985; Weiss et al., 2001). In the slow smooth muscle, the binding of ADP is generally stronger than for the faster skeletal muscle (Arheden and Arner, 1992; Nishiye et al., 1993; Cremo and Geeves, 1998). This relation between velocity and ADP binding is also observed within the smooth muscle group, where the fast and slow muscle types exhibit differences in binding of ADP to both the rigor state (Fuglsang et al., 1993) and to phosphorylated cy-
clinging cross-bridges (Löfgren et al., 2001). Here we present data on the effects of ADP on organized nonmuscle myosins. ADP inhibited the $V_{\text{max}}$ in both SM-MHC–expressing and SM-MHC–deficient tissue. Inter­estingly, the velocity could not be completely inhibited with ADP within the range studied, but the effect satu­rated at a relative velocity of $\sim 50\%$ of that in the absence of ADP, a phenomenon also observed in fast and slow smooth muscles of the guinea-pig (Löfgren et al., 2001). One possible interpretation of this is that the in­complete inhibition is due to differential effects of ADP on the two heads in the myosin molecule during cy­cling (Löfgren et al., 2001). Due to the complexity of the incomplete inhibition by MgADP, an apparent inhi­bition constant ($K_i$) could not be directly derived using Michaelis-Menten’s kinetics. Using a modified model assuming 50% maximal inhibition, a 10-fold lower $K_i$ in the SM-MHC–deficient tissues was found. This suggests that the maximally phosphorylated NM-MHC has a stronger ADP binding during cross-bridge cycling.

Fast and slow smooth muscles also differ significantly regarding the inhibitory effect of inorganic phosphate (Pi) on isometric force. Force of smooth muscle is less sensitive to Pi than force of skeletal muscle (Österman et al., 1995) and slow smooth muscle is less influenced by Pi than fast smooth muscle (Löfgren et al., 2001). Our data from the SM-MHC–deficient mice show that the force of nonmuscle myosin has a lower sensitivity to Pi compared with the smooth muscle myo­sin. This shows that the NM-MHC in comparison to the SM-MHC, not only differs in ADP and cross-bridge dis­sociation reactions, but also in reactions associated with force generation. The Pi effects also provide a measure of cross-bridge kinetics different from the shortening velocity parameter. The results also argue that the difference in $V_{\text{max}}$ is not only due to structural differences in, for example, filament length.

NM-MHCs have important functions in several nonmuscle contractile systems and experiments on the orga­nized NM-MHC in the smooth muscle tissue can pro­vide a tool for analysis of the general contractile function of this myosin type. In summary, our results show that the NM-MHC can form filaments in smooth muscle cells and that the NM-MHC has a lower shortening velocity, and has different kinetics of reactions involved in cross-bridge dissociation and in force generation compared with the smooth muscle myosin.

We thank Rita Wallén for kindly helping with preparations for electron microscopy and Doris Persson for helping with the immuno­histochemistry.

The study was supported by grants from Swedish Medical Research Council (A. Arner: 04x-13406), Medical Faculty Lund University, Crafoord foundation, Swedish Heart Lung Foundation, and Deutsche Forschungsgemeinschaft (I. Morano: Mo. 362/16-2).

Olaf S. Andersen served as editor.

Submitted: 24 September 2002
Revised: 27 February 2003
Accepted: 28 February 2003

REFERENCES


Kawamoto, S., and R.S. Adelstein. 1991. Chicken nonmuscle myo-