Identification and Characterization of Myosin from Rat Testicular Peritubular Myoid Cells

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ABSTRACT

In the mammalian testis, peritubular myoid cells (PMCs) surround seminiferous tubules. These cells are contractile, express the cytoskeletal markers of true smooth muscle—alpha-isoactin and F-actin—and participate in the contraction of seminiferous tubules during the transport of spermatozoa and testicular fluid to the rete testis. Myosin from PMCs (PMC-myosin) was isolated from adult rat testis and purified by cycles of assembly-disassembly and sucrose gradient centrifugation. PMC-myosin was recognized by a monoclonal anti-smooth muscle myosin antibody, and the peptide sequence shared partial homology with rat smooth muscle myosin-II, MYH11 (also known as SMM-II). Most PMC-myosin (95%) was soluble in the PMC cytosol, and purified PMC-myosin did not assemble into filaments in the in vitro salt dialysis assay at 4°C, but did at 20°C. PMC-myosin filaments are stable to ionic strength to the same degree as gizzard MYH11 filaments, but PMC-myosin filaments were more unstable in the presence of ATP. When PMCs were induced to contract by endothelin 1, a fraction of the PMC-myosin was found to be involved in the contraction. From these results we infer that PMCs express an isoform of smooth muscle myosin-II that is characterized by solubility at physiological ionic strength, a requirement for high temperature to assemble into filaments in vitro, and instability at low ATP concentrations. PMC-myosin is part of the PMC contraction apparatus when PMCs are stimulated with endothelin 1.

male reproductive tract, myosin, peritubular myoid cells, seminiferous tubule, testis

INTRODUCTION

Testicular peritubular myoid cells (PMCs), the main cellular component of the seminiferous tube (ST) wall, are contractile cells that express cytoskeletal markers of true smooth muscle, such as alpha-isoactin, F-actin, and myosin. The contractile activity of PMCs is responsible for the contraction of STs underlying the transport of spermatozoa and testicular fluid, and at least partially for sperm release during spermiation [1, 2]. Previous work has shown that antibodies to smooth muscle myosin decorate myosin from PMCs [3]. Vertebrate smooth muscle myosin MYH11 (SMM-II) is a hexameric enzyme composed of two heavy chains (approximately 200 kDa) and two pairs of light chains (termed regulatory and essential light chains) of 20 and 17 kDa, respectively. Myosin heavy chains form a dimer consisting of two globular amino-terminal heads and carboxy-terminal, alpha-helical, coiled-coil tails of about 150 nm in length. Each head has an actin-binding site and an ATP hydrolysis site and is associated with one of each class of light chains. The carboxy-terminal portion of the MYH11 molecule associates with other myosin molecules and assembles into filaments. For this reason, in smooth muscle cells myosin is only found assembled. Myosin filaments are stabilized in vivo against disassembly by several proteins and by phosphorylation of the light chains [4, 5]. It has been demonstrated that MYH11 with phosphorylated light chains adopts a 6S conformation that is able to assemble into filaments, whereas MYH11 with unphosphorylated light chains adopts a 10S conformation where the tail is bent and does not assemble into filaments [5]. Proteins such as kinase-related protein MYLK (KRP), telokin, and a 38-kDa protein confer myosin stability in vitro under conditions where the level of myosin light chain phosphorylation is low and ATP is present [4, 6]. MYLK prevents myosin from adopting the 10S conformation by competing for the region on myosin where the tail folds over and which is thought to include the regulatory light chain-binding site [7, 8].

The current study was designed to isolate and characterize myosin from rat testis PMCs. For this purpose, we developed a method to isolate and purify myosin from PMCs (PMC-myosin). Peptides derived from purified PMC-myosin were analyzed using peptide mass fingerprinting and peptide fragmentation fingerprinting. The sizes of PMC-myosin tryptic peptides were compared with the tryptic peptides of MYH11 from spermatic artery and tunica albuginea. The solubility and the ability of PMC-myosin to assemble into filaments also were assayed.

MATERIALS AND METHODS

The animals used were 3-mo-old Wistar rats (Charles River Laboratories) born and housed in our animal colony, with a 12L:12D cycle and food and water ad libitum, until they were killed by cervical dislocation. Animals were...
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maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Unless stated otherwise, reagents were purchased from Sigma (St. Louis, MO).

Seminiferous Tubule and PMC Isolation

Testes without tunica albuginea were digested with 0.25% trypsin in PBS for 30 min at 32°C to detach the interstitium. The STs then were treated with 1 mg/ml collagenase A in PBS for 30 min at 32°C and allowed to settle under gravity. The supernatant was removed, and the STs were washed with PBS and allowed to settle under gravity. Peritubular myoid cell isolation was performed according to Palombi and Di Carlo [9]. In brief, the STs were digested partially in 0.1% trypsin in PBS, supplemented with 2% EDTA, at 37°C. The degree of PMC extraction from the STs by trypsin treatment was monitored by silver stain. Peritubular myoid cells marked by silver stain showed the typical polygonal shape. At the start of trypsin digestion, STs looked paled with PMCs. When trypsin detached PMCs, STs showed areas with epithelial seminiferous cells in place of PMCs. The areas without PMC increased with trypsin treatment, until few PMCs remained attached. The digestion was stopped with trypsin inhibitor when only 2%–5% of the ST surface remained paled with PMCs. After trypsin digestion, STs were allowed to settle under gravity, and the supernatant containing PMCs was removed and centrifuged at 40 × g to obtain the PMC fraction. To identify PMCs, the PMC fraction was incubated in MEM at 37°C and allowed to settle to the well bottom for 4 h, fixed with 4% paraformaldehyde in PBS, then assayed for alkaline phosphatase (see below) and the presence of alpha-isoactin (identified with alpha-smooth muscle actin kit IMM-H2; Sigma) [9]. Cells were visualized in a Nikon TE 2000-U microscope with a Plan Fluor 20× lens using phase contrast to visualize all of the cells, bright field to visualize alkaline phosphatase-positive cells, and fluorescence to visualize cells stained with alpha-isoactin. The total numbers of cells and cells stained with both alkaline phosphatase and alpha-isoactin in a ruled square-millimeter area were counted in 10 different areas of the well (average 300 cells/well). From these data the percentage of cells stained with both alkaline phosphatase and alpha-isoactin was calculated. To assay for the presence of myosin in ST, 1 g ST was digested in 0.1% trypsin and 2% EDTA in PBS at 37°C to obtain PMCs. After trypsin digestion, STs were allowed to settle under gravity, and the supernatant containing PMCs was removed and centrifuged at 40 × g to obtain the PMC fraction. Seminiferous tubules (1 g), T-ST fraction, and the PMC fraction were homogenized in 1 ml of 0.6 M NaCl in PMEE buffer (35 mM Pipes, 5 mM MgSO4, 1 mM EGTA, and 0.5 mM EDTA, pH 7.4), and centrifuged at 100 000 × g for 60 min. Myosin was assayed in the supematants.

Alkaline Phosphatase Assay

Alkaline phosphatase assay in the cell fractions was assayed according to Anthony and Skinner [10], with some modifications. The fractions were centrifuged, the supernatant removed, and the pellet sonicated in the presence of 145 mM NaCl and 10 mM sodium phosphate buffer, pH 7.5. An aliquot of the sonicate was assayed with the Wiener alkaline phosphate kit (Wiener Laboratories). In brief, 100 μl of the sonicate was immediately added to 25 μl of the alkaline phosphatase substrate: 1.4 M sodium phenyl phosphate in AMP buffer and 29 mM 4-aminoantipyrine in 3 M amino-methyl-propanol, pH 10 (NPP solution). The assay volume was adjusted to 0.5 ml with AMP buffer and incubated at 0°C for 1 h. The reaction product was quantified in a spectrophotometer (Shimadzu) at 520 nm.

Trypsin Digestion of Myosin

A total of 0.1 mg/ml PMC-myosin, tunica albuginea, and seminiferous artery from adult rat testis were incubated with 1 μg/ml trypsin in PMEE buffer for up to 40 min at 37°C. The digestion was stopped by boiling in Laemmli sample buffer.

ATPase Assay

The K2EDTA-ATPase activity was measured in 300 μl of 15 mM imidazole-HCl, pH 7.5, containing 0.6 M KCl, 5 mM EDTA, and 1 mM ATP at 20°C. The reaction was started by adding myosin to a final concentration of 0.2 mg/ml. Aliquots were removed at a minimum of four time points and quenched with a final concentration of 0.4 N perchloroacetic acid prior to spectroscopic analysis of phosphate with malachite green at 600 nm [12, 13].

Silver Stain of PMCs in Seminiferous Tubules

Seminiferous tubules were incubated with 1% AgCl in H2O for 15 min at 22°C, rinsed with H2O, and incubated with reduction solution (30% sucrose, 0.4 M HNO3, and 50% methanol) for 15 min at 22°C, and then rinsed with H2O. Seminiferous tubules were observed with a magnifying glass. Peritubular myoid cells were identified by the black-stained intercellular junctions.

Isolation and Purification of Myosin

Nonphosphorylated chicken gizzard MYH11 was prepared as described previously by Ikebe and Hartshorne [14] and maintained in storage buffer (50% glycerol in 15 mM NaHCO3, pH 7.5, and 0.5 M KCl) at −20°C until use. Myosin from PMC was purified using the following procedure: PMCs isolated by trypsin digestion of ST from 10–15 g of rat testes were homogenized in PMEE buffer with complete proteasine inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany) using a glass-Teflon pestle (protease inhibitor cocktail was used in all purification steps). The homogenate was centrifuged at 37 000 rpm at 4°C for 1 h in an SW 40 Beckman rotor (Beckman Instruments Inc., Palo Alto, CA) to obtain a supernatant (S1) and a pellet (P1). The S1 was incubated for 30 min at 37°C and centrifuged through a 4 mM ATP in PMEE buffer, incubated for 10 min at 20°C, and centrifuged at 32 000 rpm for 30 min at 4°C to obtain a supernatant (S2) and a pellet (P2). For myosin purification, S2 was applied to the top of a 5%–20% sucrose gradient in PMEE buffer and centrifuged at 37 000 rpm for 16 h at 4°C. Fractions of 0.5 ml were collected from the bottom of the tube and maintained in storage buffer (50% glycerol in PMEE buffer, 1 mM dithiothreitol [DTT]) at −20°C until use.

Myosin Solubility

One gram each of ST, tunica albuginea, and seminiferous artery from adult rat testis were pulverized in liquid N2, resuspended in 1 ml PMEE buffer, homogenized in a glass-Teflon pestle homogenizer, and centrifuged at 100 000 × g for 1 h. The supernatant (S1) was incubated with trypsin (0.25% trypsin) and the pellet (P1). The S1 was incubated for 20°C in PMEE buffer and centrifuged at 37 000 rpm for 30 min to obtain the supernatant (S2). S1 and S2 were mixed with sample buffer (1:1) and maintained at −20°C until use.

Gel Electrophoresis and Western Blot Analysis

All gel electrophoreoses were performed using the method of Laemmli [15] and using 7.5% SDS-PAGE subsequently stained with Coomassie blue. Monoclonal mouse anti-SMM-II heavy chain (MYH11) and myosin G-4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were tested by Western blot using myosin samples. Proteins run on SDS-PAGE were blotted onto nitrocellulose membranes. Anti-MYH11 antibody was used at a dilution of 1:1000. To compare the amount of loaded protein, parallel membranes were exposed to anti-mouse alpha-actin antibody (1:10,000 dilution). The membranes were incubated with anti-mouse antibody conjugated to alkaline phosphatase diluted 1:5000, and were developed with the Immuno-Blot assay kit (Bio-Rad, Hercules, CA) according to the manufacturer’s directions.

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FIG. 1. Immunohistochemistry of isolated PMCs. Fixed cells from a PMC fraction were assayed for both alkaline phosphatase activity and the presence of alpha-isoactin. A–C) Cells stained only with alpha-isoactin visualized with phase contrast (A), bright field (B), and fluorescence (C). D–F) Cells assayed for both endogenous alkaline phosphatase activity and alpha-isoactin visualized with phase contrast (D), bright field (E), and fluorescence (F). The figure shows cells from a representative experiment. Bar = 100 μm.

Protein Quantification

For quantitative analysis of proteins, samples were run on SDS-PAGE and gel bands compared with a standard curve of bovine serum albumin run on the same gel. The Kodak EDAD 120 system, consisting of a digital camera and software, was used for recording the gels bands. The bands were analyzed in the Scion Image program, and the optical densities of myosin bands were compared with those of bovine serum albumin known concentrations.

Peptide Mass Fingerprinting and Peptide Fragmentation

Fingerprinting Analysis

The excised spots were digested in gel using trypsin and desalted in line with liquid chromatography as described previously [16]. Spectra obtained in the positive ion mode with Q-ToF micro mass spectrometry (Micromass) were deconvoluted and analyzed using the MassLynx software (version 4.0; Micromass).

Mascot Server v2.2 (local host; Matrix Science) in MS/MS Ionic Search mode, and Protein Lynx Global Server v 2.2 (Micromass) were applied to conduct peptide matches (peptide masses and sequence tags) and fragmentation analysis. The searches were carried out in the NCBInr v20070504 and SwissProt v52.4 databases. The following parameters were set for the search: carboxyamidomethylation on cysteine was set as fixed; variable modifications included methionine oxidation, and asparagine and glutamine deamidation. Only one missed cleavage was allowed, monoisotopic masses were counted, and the precursor peptide mass and fragment mass tolerances were set at 100 ppm and 0.25 Da, respectively.

ST Contraction

Seminiferous tubule contraction was assayed by incubating isolated STs with or without 50 nM endothelin 1 or oxytocin in minimum essential medium (MEM) for 20 sec at 22°C, followed by immediate fixation with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. For the assay of alpha-isoactin, STs were fixed with 4% paraformaldehyde in PBS.

Seminiferous tubules for scanning electron microscopy were laid onto polylysine-coated coverslips and incubated for 1 h to ensure adherence. The samples were postfixed in 1% OsO4, dehydrated in acetone, critical point dried in acetone, coated with gold, and observed in a Siemens ETEC Autoscan Electron Microscope (Hayward, CA). Seminiferous tubules for immunofluorescence were incubated in 50 mM ammonium chloride in PBS for 30 min, in wash buffer (0.05% saponin and 0.2% bovine serum albumin in PBS) for 30 min, in anti-alpha-actin-fluorescein isothiocyanate (FITC)-conjugated antibody diluted 1:200 in wash buffer for 60 min, and rinsed with wash buffer. The samples were mounted with Mowiol and analyzed in a confocal system. For actin filament analysis, 10 sequential fluorescent images of the same field were acquired in successive 1-μm z-axis displacements as we scanned through the PMC layer in the wall of STs with a Plan Apo N 60× objective. The z-series of fluorescence sections was analyzed with the ImageJ 1.37a program (National Institutes of Health) to obtain a Z-stack image of maximal intensity projection. Additionally, isolated STs incubated with or without 50 nM endothelin 1 in MEM for 20 sec at 37°C were homogenized in PMEE, and the P1 pellet was prepared. The pellet was resuspended in Laemmli buffer, and the amount of myosin was assayed by Western blot.

Statistical Procedures

The data are expressed as the average of three experiments ± SEM. Statistical significance was assessed with one-way analysis of variance (ANOVA). P < 0.05 was considered significant.

RESULTS

Myosin from PMCs

To analyze PMC-myosin, STs were digested with trypsin, and PMCs were separated from germinal cells by centrifugation (PMC fraction). The percentage of PMC detachment from STs was 95.1% ± 9.1%. The alkaline phosphatase activity in the PMC fraction represented 89.1% ± 2.7% of the total ST fraction activity, and the percentage of cells positive for both alkaline phosphatase and alpha isoactin was 87.5% ± 3.3% (Fig. 1). To analyze myosin, the 0.6 M NaCl fraction of intact STs, trypsined STs, and PMC fraction were immunoblotted with anti-MYH11. The reaction revealed a reactive band of 205 kDa in intact STs and in the PMC fraction but not in trypsined STs (Fig. 2A). The optical density of the myosin band from the PMC fraction represents 80% of that of the myosin band from intact STs (Fig. 2B). For this reason, we deduced that the identified myosin comes from PMCs, and we named it peritubular myoid cell-myosin (PMC-myosin). Subsequently, intact STs or PMCs were used as a source of PMC-myosin.

PMC-Myosin Solubility

When STs were homogenized in PMEE buffer (a low-ionic strength solution) at 4°C and centrifuged to obtain cytosol and pellet, the distribution of PMC-myosin was 95.6% ± 5.1% in the cytosol and 5.2% ± 1.0% in the pellet (Fig. 3). Similar results were obtained when the distribution of PMC-myosin was analyzed from isolated PMCs (data not shown).

It is unusual to find myosin from smooth muscle cells in the cytosol, since it assembles into insoluble filaments at low ionic strength. It could be argued that the homogenization procedure disassembled PMC-myosin filaments. To evaluate this possibility, we compared the results with two tissues containing smooth muscle cells from the testis, the tunica albuginea and the spermatic artery (Fig. 3A). We observed that the distributions of MYH11 in both preparations were preponderantly insoluble—95.4% ± 7.3% and 93.5% ± 8.2% in the
tunica albuginea and the spermatic artery pellet, respectively (Fig. 3B)—a result that coincides with the behavior of MYH11 [17, 18].

Another possibility that we tested was that unknown factors coming from PMCs could solubilize PMC-myosin filaments. We used spermatic artery as a source of MYH11 assembled in filaments to homogenize with or without PMCs. The rationale for this experiment was that if the PMC homogenate contains a factor that induces myosin filament disassembly, the myosin filaments from artery tissue would be solubilized when homogenized in the presence of the PMC-homogenate. The assay indicated that 91.5% ± 4.2% and 90.1% ± 2.2% of MYH11 from the spermatic artery cells remained in the pellet when they were homogenized without and with PMCs, respectively (data not shown). From these results, we infer that neither the homogenization procedure used nor the presence of PMC factors solubilized myosin filaments from smooth muscle tissues. Hence, the presence of PMC-myosin in a soluble state in the cytosol could be due to PMC-myosin filament lability under the homogenization conditions used, or that PMC-myosin was not initially assembled into filaments. These results encouraged us to purify PMC-myosin and analyze its in vitro solubility.

**Purification of PMC-Myosin**

PMC-myosin was purified from low-ionic strength PMC cytosol (S1). The following strategy was used: 1) assemble PMC-myosin by warming S1 at 37°C for 30 min; 2) centrifuge the assembled PMC-myosin on a 20% sucrose cushion; and 3) solubilize PMC-myosin with ATP and obtain by centrifugation a supernatant (S2) rich in PMC-myosin. With this procedure, a suspension of proteins was obtained where 85%–90% corresponded to PMC-myosin (Fig. 4A). This partially purified PMC-myosin suspension was centrifuged on a 5%–20% sucrose gradient (Fig. 4B), yielding fractions with 95%–98% PMC-myosin purity (fractions 6–8; Fig. 4B), which were used...
to analyze ATPase activity, peptide composition, and in vitro solubility.

**ATPase Activity of PMC-Myosin**

To confirm that PMC-myosin has this inherent characteristic of myosin proteins, we assayed for K-EDTA ATPase activity. PMC-myosin hydrolyzed ATP at a rate of $0.8 \pm 0.01 \mu M$ ATP/mg per minute, similar to the rate reported for MYH11 [19].

**Myosin Tryptic Peptides**

Due to the fact that PMC-myosin is soluble under conditions where other testicular MYH11—from spermatogenic (SA-myosin) and tunica albuginea (TA-myosin)—is assembled into filaments (Fig. 3), we analyzed the peptides of the three myosins. SA-myosin and TA-myosin were digested with trypsin, and the sizes of the peptides obtained were compared with those of the PMC-myosin tryptic peptides. As shown in Figure 5, 20-min digestion of PMC-myosin resulted in a pattern of six bands with relative molecular weights (M_r) near 150, whereas TA-myosin showed a few bands of 150 and one with lower M_r. SA-myosin proteolysis additionally generated four bands of 120. The 40-min digestion of PMC-myosin produced several bands in the range of 150–45. Meanwhile, the TA-myosin digestion products remained near 150, and SA-myosin was reduced to bands of less than 45. These experiments show that the tryptic peptides generated from TA-myosin and SA-myosin are of different sizes than those from PMC-myosin, presumably reflecting differences in primary structure.

**PMC-Myosin Peptide Sequences**

As mentioned above, PMC-myosin is a protein of M_r 205,000 and cross-reacts with a monoclonal antibody to the smooth muscle myosin from *R. norvegicus* (GenBank accession no. GI 109489761). The peptides matching the reference sequence are in bold.

1. MAQQQRLOSDL RKLFLVDFKMR INSNAQADGW KAVKLWVTFPS RIKQGFSASSI
2. KEKOKKKEQSVK YLVDNQDPOQK AGDCLDAS
3. VNLNEKSVVY TLQIVYYGQLV FCYYNIVKTH LPIYSEKIVD MGYKSKRHEH
4. PFFYIAADT AYRMSLQDER QOQLCFTGSB AGKTYENTK VQYLYAVAS
5. SHEKEKSBSI TQERLFRQPK ANPPLQAEF ARTKVNDSBS RFPDKKPPHPH
6. DVTQTVSYAN LVANTLLAET AYQRTQETPH RPYYLCLDNAS
7. LENSWFRTP SNQFVPIAAP QDEQQFPOTL EALM135FSEB EQAILLKVUV
8. ZSLQVTCSN YPKKERTDQGA SDNMDPAQQK VCHLQIVNTF DFTTALILTPR
9. 1KQDVVQVQK AQTVQAFDAF IBALAKQTVY RLFRPMVSLW RMKALQHEHNO
10. GASPQLOQHMTL FFQLOQTPMT EPLQDOHFS NQOPQOHFQ
11. REIGEBNPV QDPLQICPTI LESRNQPFPV VLALQDQDLMF PKFAQKPSV
12. EKLSCEGGQH KPIKQKQLK DPKTPIHHLY AGQVKDNYQY MALTNPODPLN
13. DNYTSLNASN DQFKPADWLV DVQIVGQLQK MAKTSRESPL ASKEQFGTVQ
14. VTPQGQSYEQ TQFQKMTQPK MDFQKIKQKL PDAVLQPLQL
15. CMQVQXVIIRI CQQFQNPVRI PQFQPRYLLI LAHAIPIQK MGQPKCICLM
16. INALEQTLHL YRISQSKIFP VTQVLHAEEL EDBKIDTYYA MQAPMCNYQ
17. LARSERFFRTQ QTRLQNKLVU RAKYQVLKLM HNYQPLRFTK VQFLPQYTVQ
18. KEMQAEKQK HMKQPKQKKQK KQKRLEXTM KFPLAQKPSQT
19. ELYARARKRQ KRRQAKQKL EELKHEMPE LREBREEDEGQ LQAEREMEAQ
20. QMLOERGELE EEEAARQKQK LEQKTYBRAS KKLQDDIILQV DQONKQSLK
21. 1KQ1RLKRESD LITTHLARKF KAKNITLKLK KXHMSLQELK VLRKESKSHK
22. 1QELLEKRE KL EDDASDPHQ IADLQQAEBQ LMQMQAKLK QLEKALREK
23. 1KTEQGNML N KIRKIBQGK QSDQMQGDLK SAAANQAEKQ KQDLGQKMLA
24. 1LXCTELSTLD TATQGELRA KBKQVNLMLK KALDEBFRS QAQBERMQK
25. 1HTQAVERLHE QLQQERAKA NLQDMQERLK KIENADCLR PVLGQAKQKV
26. 1HEQELSGQF QLQGIQKOSD QGRATB TagR MHVLQYNEV STVGLTIAE
27. 1GRSLLAKVE ASLQEGQLOT QSLQERHKS KLYNSTELYK LEQINSDQD
28. 1QLRKRKQKQ KLEHHSVTLN IQLSQGKSL KQDLQTAQEV MLKQKQKQK
29. 1MECILQYQVR TAAAYDKLE RTRQRLQDDL DILVQIDQRQL QVLSNLHKEK
30. 1FKQDQARLE TKN9TQYRKQ KKBEBBRARK KRTXADLAR ALEEBRAKE
31. 1LQBQRTIPLK AMEDRLLRRK ERBQKQLQKK KEBQQPLTLD ACEPEHMLQ
32. 1LEDEQLELDAL ALEKLMRQMLNQK FQLEDRLRRK KQDIQKCLHH
33. 1YETELDERK QRALAARAK LQEDGKDQLE KQDASQVEKK EAAKNQKQK
34. 1QSKPLQERQ KIDGKQKQKQK QDIQKCLHHK EAKQIEMSEE CLQGDELAAR
35. 1RFQQADQRE NLBQRELALGQ LQMLQDPOQK KQBRLAQIAQ LBBELBQGQ
36. 1NMEAMSEVR KATLQAEQLS NLVTERJHSA QNQFSEQQLQ KQEBQKLKK
37. 1LQYKQGAVKE LKSTKVALAE AKRKLQEQI SQMRAROA QALQELQKD
38. 1LQEVYQVDQB RKLWQVTKR QAKQSMQKVT QLQRIERHE EBSRRQNENR
39. 1KQSGQLECM TSHSWAMHRD LQVNLQELSK PQFQPQSTQ

**FIG. 4.** Purification of PMC-myosin. PMC-myosin was isolated by assembly-disassembly (A), followed by purification on a sucrose gradient (B). Proteins (5 µg) were loaded in A, and 7.5 µl of each fraction were loaded in B, subjected to 7.5% SDS PAGE, and stained with Coomassie blue. Lanes 1–15: fractions 1–15 from the bottom of the gradient. We repeated the same experiments three times with reproducible results, and representative results from one experiment are shown. The 45–205 M_r values are in kDa.

**FIG. 5.** Myosin tryptic fragments. Myosin (0.1 mg/ml) from: PMCs (PMC-myosin), tunica albuginea (TA-myosin), and spermatic artery (SA-myosin)—is assembled into filaments (Fig. 3), we analyzed the peptides of the three myosins. SA-myosin and TA-myosin were digested with trypsin, and the sizes of the peptides obtained were compared with those of the PMC-myosin tryptic peptides. As shown in Figure 5, 20-min digestion of PMC-myosin resulted in a pattern of six bands with relative molecular weights (M_r) near 150, whereas TA-myosin showed a few bands of 150 and one with lower M_r. SA-myosin proteolysis additionally generated four bands of 120. The 40-min digestion of PMC-myosin produced several bands in the range of 150–45. Meanwhile, the TA-myosin digestion products remained near 150, and SA-myosin was reduced to bands of less than 45. These experiments show that the tryptic peptides generated from TA-myosin and SA-myosin are of different sizes than those from PMC-myosin, presumably reflecting differences in primary structure.

**FIG. 6.** PMC-myosin sequence covered by the matching peptide of smooth muscle myosin from *R. norvegicus* (GenBank accession no. GI 109489761). The peptides matching the reference sequence are in bold.
of MYH11 molecular mass and the isoelectric points are 224.242 kDa and 5.43, respectively. We also carried out an analysis of probable phosphorylation sites in the PMC-myosin tryptic peptides from the PMF and PFF data (searching for variable modification of the STY amino acids and also of the spectra), but no positive hits were generated.

**PMC-Myosin Assembly In Vitro**

As we show in Figure 2, PMC-myosin is isolated largely in a soluble form from PMCs; thus, we determined whether it is soluble in vitro in low-ionic strength solutions using dialysis against solutions of differing ionic strengths at 4°C and 20°C. A parallel assay was performed with purified gizzard MYH11. As can be seen in Figure 7A, the dialysis of PMC-myosin in buffer of low ionic strength (0–50 mM NaCl) at 4°C induced 10% assembly. For comparison, the dialysis of gizzard MYH11 in the same buffer induced 90% assembly (Fig. 7A), in accordance with previous results [20].

Because our previous results had shown that PMC-myosin assembled in PMEE buffer at 37°C (see above), we evaluated the ability of PMC-myosin to form filaments in saline buffers at 20°C. PMC-myosin in low-ionic strength buffer (0–50 mM NaCl) at 20°C showed 90% assembly (Fig. 7A).

The results shown above indicate that there is a significant impediment to PMC-myosin aggregation at 4°C, a situation in which gizzard MYH11 assembly occurs efficiently, but that dialysis at 20°C allows extensive PMC-myosin aggregation. The MYH11 filament shape showed characteristic side polar formation [21], as did the PMC-myosin filaments (data not shown). In order to compare the ability of PMC-myosin and gizzard MYH11 to form filaments at different temperatures, 0.1 mg/ml of each in PMEE buffer was incubated for 30 min at temperatures between 0°C and 37°C. Incubation at 0°C permitted 85.2% ± 5.1% of MYH11 to assemble into filaments, whereas only 2.3% ± 0.9% of PMC-myosin did. Incubation at 20°C allowed only 10.7% ± 3.3% of PMC-myosin to assemble in filaments, whereas at 37°C, 93.2% ± 7.1% of PMC-myosin assembled. It is clear that 37°C is the temperature that allows maximal PMC-myosin assembly. At this point, we asked whether PMC-myosin filaments are more labile than MYH11 filaments.

**PMC-Myosin Filament Stability**

Assembled gizzard MYH11 and PMC-myosin at 0.1 mg/ml were stimulated to disassembly by low ionic strength. As shown in Figure 7B, PMC-myosin solubility increased from 5.2% ± 0.3% at 100–200 mM NaCl, to 60.3% ± 8.1% at 300 mM NaCl, and finally 98.2% ± 3.1% at 400 mM NaCl. By comparison, the solubility of gizzard MYH11 filaments was 25% at 200 mM NaCl, 60% at 400 mM NaCl, and 100% at 400 mM NaCl. The results show that substantially, the same concentration of NaCl is needed to solubilize PMC-myosin as gizzard MYH11, indicating that the two proteins form filaments with the same stability to ionic strength.

It is known that ATP disassembles filamentous myosin [22]; thus, we assayed the sensitivity of the two forms of myosin to varying concentrations of ATP. As shown in Figure 7C, 2 mM ATP solubilized 80% of the PMC-myosin, whereas only an insignificant amount of gizzard MYH11 was solubilized at this concentration. It required 20 mM ATP to solubilize 20% of the gizzard MYH11, and higher concentrations gave no further disassembly. Thus, it is clear that PMC-myosin filaments are more labile to ATP than gizzard MYH11.

**Does PMC-Myosin Participate in Cell Contraction?**

Isolated STs were incubated with endothelin 1 for 20 sec or 1 min with oxytocin [23]. Scanning electron microscopy analysis of the STs showed that only endothelin 1 induced changes in PMC morphology, as previously demonstrated [24] (Fig. 8). To visualize whether the contraction observed with endothelin 1 corresponded to a specific mechanism involving the actin cytoskeleton, we compared the shape of PMCs and the actin filament organization in STs treated for 20 sec with endothelin 1 or 1 min with oxytocin [24]. To visualize actin filament organization, STs were stained with anti-actin-FITC-conjugated antibody and analyzed in a confocal microscope. The Z-stack image of PMCs from unstimulated STs (control) showed the characteristic orthogonal meshwork of actin filaments (Fig. 8A’) [24]. In STs stimulated with endothelin 1, the actin filament network evinced undulating actin filaments, losing the orthogonal organization characteristic of control PMCs (Fig. 8B’). This pattern of actin filament organization was observed in the whole ST wall, but differed from previous descriptions where endothelin 1 induced an actin filament network reduced in size and organized in bundles in centrally located regions of the cell [24]. To the best of our knowledge, this is the first time that a Z-stack image of the actin network from PMCs is described, and it likely shows different details of actin organization compared with conventional light microscopy. Meanwhile, PMCs from oxytocin-stimulated STs showed the typical orthogonal network of actin filaments found in PMCs of control STs (Fig. 8C’). From these observations, it seems that endothelin 1 might cause PMC-myosin to form actin–myosin complexes, which could be responsible for the changes in the actin cytoskeleton observed.
results, we could confirm that only endothelin 1 activated the actin filament network reorganization [24]. Consequently, it was interesting to determine whether PMC-myosin participates in PMC contraction. The amount of PMC-myosin was analyzed in the P1 pellet. As can be seen in Figure 9, a small amount of PMC-myosin was found in the pellet of control STs, whereas the amount of PMC-myosin in the P1 of endothelin 1 stimulated STs was three times higher.

DISCUSSION

In this paper, we isolated a protein with an $M_r$ of 205,000 from PMCs for the first time, which we named PMC-myosin. PMC-myosin cross-reacts with a monoclonal antibody to MYH11 heavy chain, and a polyclonal antibody raised against PMC-myosin cross-reacts with SA-myosin and TA-myosin (data not shown). Even though the anti-MYH11 antibody recognizes several peptides in PMC-myosin, many of them differ in $M_r$ from the peptides derived from SA-myosin and TA-myosin (Fig. 5), also indicating that PMC-myosin is not substantially contaminated with spermatic artery myosin.

The PMC-myosin peptide sequence showed homology with 38% of the MYH11 sequence from *R. norvegicus* (Fig. 6).

PMC-myosin has a high $K^+$-EDTA-ATPase activity inherent to the ATPase catalytic site in myosin molecules [25, 26]. PMC-myosin is 95% soluble in the cytosol and did not assemble in a standard assembly protocol for gizzard MYH11 [20]. However, an increase in temperature allows PMC-myosin to assemble into filaments with the same resistance to ionic strength as gizzard MYH11. Nonetheless, these PMC-myosin filaments are very unstable at low ATP concentrations.

Based on the above data, we assume that PMC-myosin is an isotype of MYH11. MYH11 heavy chains form a dimer consisting of two globular amino-terminal heads and carboxy-terminal, alpha-helical, coiled-coil tails. The $\alpha$-helical coiled-
coiled coil structure carries a characteristic seven-residue repeat pattern with hydrophobic residues that create a major stabilization energy for the coiled-coil structure [27]. The sequence alignment of the C-terminal end of the coiled-coil domain show that most of the charged residues are clustered on the outside of the coiled-coil dimer with alternate positive and negative charges [28–30].

Since PMC-myosin is found mostly soluble in the cell, it could be that the protein is really soluble at physiological ionic strength, or that it might be present in filaments that are labile to the tissue-processing protocol. The finding that the Triton X-100 cytosol from PMCs contains more than 90% of the total PMC-myosin reinforces the notion that PMC-myosin is highly soluble (data not shown). The solubility of PMC-myosin could be explained by a lack of necessary residues in the region critical for filament formation.

The phosphorylation of MYH11 regulatory light chains at physiological ionic strength [31] induces the assembly of filaments [32] that are not dissociated by ATP, whereas the filaments formed with unphosphorylated MYH11 regulatory light chains are dissociated by ATP [22, 33].

We observed that filaments formed in vitro with PMC-myosin are disassembled by lower ATP concentrations than filaments formed with unphosphorylated gizzard MYH11 (Fig. 7). Since we compared PMC-myosin filaments with gizzard MYH11 filaments with unphosphorylated light chains, the behavior of PMC-myosin cannot be explained unless PMC-myosin is more sensitive to disassembly by ATP than MYH11 for a reason unrelated to the state of light chain phosphorylation.

Additionally, phosphorylation of the cytoplasmic myosin-II heavy chain has been shown to be a critical regulatory signal for myosin-II assembly in Dictostelium [34, 35], but its role in vertebrate muscle cells is unclear. There is some indication that phosphorylation of heavy chains by protein kinase C can destabilize filaments of platelet myosin-II [36] and the B isoform of brain myosin II [37]. We did not find evidence that the PMC-myosin heavy chain could be phosphorylated, thus affecting its ability to assemble.

Although PMC-myosin is mostly soluble in low-ionic strength solutions and does not assemble easily in vitro, incubation at 37°C allowed it to form filaments. Endothelin-1 stimulated contraction of STs and induced the sedimentation of a significant amount of PMC-myosin. This change in PMC-myosin solubility could be attributed to an interaction with actin filaments, indicating that PMC-myosin participates in the contraction of PMCs. In addition, it could be inferred that PMC-myosin assembled into filaments before contracting but that the assay used did not discriminate between assembled and monomeric myosin attached to actin filaments.

There are several examples where nonmuscle myosin-II is found to alternate, in a cell state-dependent manner, between soluble and assembled forms. In chick embryo fibroblasts, the polarization of crawling movements is accompanied by the development of spatial asymmetry in the incorporation of cytoplasmic myosin-II into the cytoskeleton. Myosin-II in the anterior of migrating fibroblasts is immobile, whereas in the perinuclear cytoplasm it is largely freely diffusing [38, 39]. In motile endothelial cells, the front of the cell contains a high proportion of myosin-II that is associated with the insoluble cytoskeleton, whereas most of the myosin-II in the central part of the cell is preferentially released [40]. Furthermore, the formation of migrating cells and their subsequent disassembly in the central or posterior cytoplasm have been extensively documented by following the movements of fluorescently labeled myosin-II in living cells [19, 38, 41]. Thus, when cells undergo directional movement, they exhibit a clear asymmetry in the assembly of myosin-II into the locomotive cytoskeleton. In addition, in hepatic stellate cells, myosin-II participates in the cell contraction induced by endothelin 1 [42]. To initiate the contraction, myosin regulatory light chain must be phosphorylated, inducing myosin relocalization close to the actin cytoskeleton and filament assembly [42].

Our results indicate that a significant amount of PMC-myosin is found in the pellet when PMCs are stimulated to contract with endothelin 1. Thus, it appears reasonable to propose that PMC-myosin participates in the contraction mechanism, and that the active rearrangement of the actin-myosin cytoskeleton is necessary for normal PMC activity. It has been shown that a profound change in actin filament organization occurs when PMCs are in a contracted state [24]. Thus, we hypothesize that PMC-myosin is soluble in the cytoplasm and assembles into filaments in response to extracellular signals to induce proper cell contraction, and then is disassembled until the arrival of a new stimulus. Hence, the specific mechanism by which PMC-myosin cycles through assembly-disassembly might be critical for PMC contraction. Experiments are being carried out in our laboratory to test this hypothesis.
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