Antibodies Probe for Folded Monomeric Myosin in Relaxed and Contracted Smooth Muscle

Arie Horowitz, Kathleen M. Trybus, Douglas S. Bowman,* and Fredric S. Fay*

Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254; and *The Biomedical Imaging Group and the Department of Physiology, University of Massachusetts Medical Center, Worcester, Massachusetts 01605

Abstract. Regulatory light chain phosphorylation is required for assembly of smooth and non-muscle myosins in vitro, but its effect on polymerization within the cell is not understood. Relaxed smooth muscle cells contain dephosphorylated thick filaments, but this does not exclude the presence of a pool of folded myosin monomers which could be recruited to assemble when phosphorylated, thus forming part of smooth muscle's activation pathway. To test this hypothesis, relaxed and contracted avian gizzard cryosections were labeled with a fluorescently conjugated monoclonal antibody specific for the folded monomeric conformation, or with an antibody against the tip of the tail whose epitope is accessible in the monomeric but not the filamentous state. Fluorescence intensity observed in the two physiological states was quantitated by digital imaging microscopy. Only trace amounts of folded monomeric myosin were detected in both the relaxed and contracted states. The amount of monomer also did not increase when α-toxin permeabilized gizzard was equilibrated in a solvent that disassembles filaments in vitro. Assembly/disassembly is therefore unlikely to play a major role in regulating the contraction/relaxation cycle in smooth muscle cells.

MYOSIN filaments were initially observed by electron microscopy only in contracted smooth muscles, lending support to the idea that smooth muscle thick filaments were much less stable than those isolated from skeletal muscle (Kelly and Rice, 1969; Shoenberg, 1969). Subsequent studies (e.g., Devine and Somlyo, 1971; Cooke and Fay, 1972; Small and Sobieszek, 1979) found thick filaments both in relaxed and contracted muscle, however, suggesting that the earlier negative observations resulted from the fixation techniques. Nevertheless, the discovery that smooth muscle myosin light chain phosphorylation promoted myosin assembly in vitro (Suzuki et al., 1978) renewed interest in the correlation between myosin assembly/disassembly and the physiological state of smooth muscle. It was soon shown (Somlyo et al, 1981), however, that relaxed smooth muscle cells in which the myosin is more than 95% dephosphorylated contain numerous thick filaments.

The findings of Somlyo et al. (1981) do not rule out the possibility of partial thick filament disassembly upon relaxation. A pool of disassembled myosin could exist in equilibrium with the dephosphorylated thick filaments. These monomers could be recruited to incorporate into existing or new filaments upon muscle contraction. Support for such a mechanism came from studies which showed that thick filaments in glycerinated smooth muscle cells are unstable under conditions resembling the relaxed state (presence of MgATP and absence of Ca^{2+}; Cande et al., 1983), and from electron microscopy where a higher density of thick filaments was found in contracted than in relaxed smooth muscle cells (Gillis et al., 1988).

The previous structural studies examined and quantitated only the filamentous myosin. To fully test whether partial filament disassembly occurs, it is advantageous to use probes for detecting the presence of monomeric myosin in the cytoplasm of smooth muscle cells. Here we employed a monoclonal antibody specific for the folded monomeric myosin conformation as such a probe (Ab 10S.1; Trybus and Henry, 1989). Alternatively, we used an antibody specific for the tip of the myosin rod (Ab LMM.4), whose epitope is buried in the filamentous state, but accessible in monomers. The degree of labeling of relaxed and contracted gizzard cryosections by these antibodies was then quantitated by digital immunofluorescence microscopy. The results indicate that only low amounts of folded monomeric myosin are present in both the relaxed and contracted gizzard, suggesting that assembly/disassembly does not play a major role in regulating contraction in a smooth muscle cell.

Address all correspondence to Arie Horowitz, Program in Smooth Muscle Research, Division of Cardiology, Beth Israel Hospital, Harvard Medical School, 330 Brookline Ave., Boston, MA 02215. Tel.: (617) 735-3022. Fax: (617) 735-4833.

Materials and Methods

Tissue Preparation and Force Measurement

Gizzards excised from freshly slaughtered chickens (Eastern Live Poultry Co., Boston, MA) were transported in previously air-bubbled ice-cold relaxing avian Hank's buffer (137 mM NaCl, 5.5 mM dextrose, 5 mM KCl, 5 mM EGTA, 5 mM Pipes, pH 6.8, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 1.1 mM Na_2HPO_4, and 0.4 mM KH_2PO_4). 10–15 mm long and ~1 mm^2 thick strips were sliced by razor from the superficial layers of the gizzard and mounted between two metal wires by cyanoacrylate glue (Borden, Columbus, OH). One of the wires served as the lever of a force transducer (KG3; Scientific Instruments, GMBH, Heidelberg, Germany). Strips were briefly incubated in the relaxing Hank's buffer at 20°C and stretched to ~1.25 × their initial length. When passive tension stabilized, typically to 20–50 mg, the gizzard strips were transferred to a previously air-bubbled activating Hank's buffer (137 mM K_2SO_4, 6 mM $CaCl_2$, 5.5 mM dextrose, 5 mM NaCl, 5 mM Pipes, pH 6.8, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 1.1 mM Na_2HPO_4, and 0.4 mM KH_2PO_4). When active tension reached a steady level (typically 3 min after start of activation), strips were quickly frozen by plunging into liquid nitrogen. Tissue for determining phosphorylation levels was stored in 10% TCA-acetone (at liquid nitrogen temperature), while tissue for cryosectioning was stored in liquid nitrogen. Relaxed tissue was obtained by plunging gizzard strips incubated in relaxing Hank's buffer into liquid nitrogen.

Embryonic gizzards were excised from 14-d-old incubated eggs (Spafas, Norwich, CT) and were dissected similarly to adult gizzards.

Tissue Permeabilization

Adult or embryonic gizzard strips prepared as described above were incubated in relaxing Hank's buffer containing 2500 U/ml of Staphylococcus aureus α-toxin (Calbiochem-Novabiochem, La Jolla, CA) for 1 h at 20°C. After washing in relaxing Hank's buffer, strips were incubated for 30 min at 20°C either in 10S-forming (150 mM KCl, 10 mM NaPi, pH 7.0, 5 mM EGTA, 4 mM MgATP, and 1 mM DTT) or in filament-forming buffer (135 mM KOH, 2.5 mM $MgCl_2$, 2.2 mM $CaCl_2$, 2 mM MgATPγS, 2 mM EGTA, and 1 mM DTT, titrated to pH 6.5 with propionic acid). The terms 10S-forming and filament-forming derive from the in vitro use of such buffers to effectively promote either disassembly of myosin filaments into 10S folded monomers or assembly of monomers into filaments (Trybus and Lowey, 1984). Strips were then plunged in liquid nitrogen and stored as described above.

Quantitation of Myosin Light Chain Phosphorylation

Relaxed or contracted gizzard strips were pulverized by pestle and mortar in 10% TCA-acetone at dry ice temperature and brought to 20°C. The pulverized tissue was washed in 0.24 M Tris, 1.67 M glycine, then in H_2O and finally in acetone by spinning in a microfuge for 1 min after each wash. The pellet was dispersed and extracted for 3 h at 20°C in 8 M urea, 34 mM Tris, 236 mM glycine, 0.17 mM EDTA. After spinning for 1 min as above, the pellet was discarded and 0.1% β-mercaptoethanol and bromphenol-blue/glycerol were added to the supernatant. Dephosphorylated and phosphorylated light chains were separated on glycerol/acrylamide gels (Perrie and Perry, 1970), transferred onto nitrocellulose for 1 h at 60 volts (Towbin et al., 1979), reacted with 5 μg/ml anti-regulatory light chain monoclonal antibody (clone 5B1.2) for 1 h at 20°C, and detected by goat anti-mouse antibody conjugated to horseradish peroxidase (Bio-Rad Labs, Richmond, CA). The extent of light chain phosphorylation was quantitated by densitometry (E-C Apparatus Corp., St. Petersburg, FL) of photographic film images of the immunoblotted bands.

Antibody Labeling by Fluorophores

Antibodies were reacted with an equal weight of TRITC on celite or twice the weight of FITC on celite (Research Organics Inc., Cleveland, OH) for 5 min at 20°C. The reaction buffer was PBS titrated to pH 9.3 with carbonate-bicarbonate. The celite was pelleted in a microfuge for 15 min at 4°C, and the labeled antibody in the supernatant was immediately separated from free fluorophore on a Sephadex G-50 (Pharmacia, Uppsala, Sweden) column. The antibody was concentrated by ammonium sulfate precipitation and resuspended in PBS. Antibody concentrations were determined by the Bradford (1976) method with myosin as a standard. The degree of conjugation was determined by spectrophotometry using the formulas (Hudson and Hay, 1980): mol rhodamine/mol antibody = $OD_{515} \times 3.73/(OD_{280} - 0.56 \times OD_{515})$; mol fluorescein/mol antibody = $OD_{495} \times 4.48/(OD_{280} - 0.35 \times OD_{495})$.

Preparation of Purified Myosin in Agarose for Cryosectioning

Purified turkey gizzard myosin (Sellers et al., 1981) was mixed with an equal volume of 4% low melting point agarose at 40°C. The buffer concentrations after mixing were: dephosphorylated monomeric extended myosin–0.5 M KCl, 10 mM NaPi, pH 7.0, 1 mM EGTA, 1 mM MgATP; dephosphorylated folded monomeric myosin–0.15 M KCl, 10 mM NaPi, pH 7.0, 2 mM $MgCl_2$, 1 mM EGTA, and 1 mM MgATP; filamentous phosphorylated myosin–0.15 M KCl, 10 mM imidazole, pH 6.5, 10 mM $MgCl_2$, and 1 mM EGTA. Folded monomeric myosin was cross-linked with ethyl-3-(3-dimethyl-aminopropyl) carbodiimide as described in Trybus and Lowey (1988) before embedding.

Cryosectioning and Immunolabeling

Tissue or agarose blocks were embedded in 2 M sucrose/PBS and cut to 8-μm-thick sections at −20 to −35°C on a cryomicrotome (FC 4E; Reichert-Jung, Vienna, Austria). Sections were thawed to 20°C on glass slides (Superfrost/Plus; Fisher, Pittsburgh, PA) and stored in PBS (pH 7.0) or, alternatively, fixed in 0.01% or 0.1% glutaraldehyde, 20 mM ethyl acetimidate for 15 min. The fixed sections were washed 2 × 5 min in PBS, quenched in 0.1% $NaBH_4$ for 15 min, and washed again as before. Tissue sections were blocked with 100 μg/ml affinity-purified goat IgG (Bio-Rad Labs) in PBS for 30 min. Agarose sections were blocked with 1% gelatin/PBS for 1 h. After washing 4 × 5 min in PBS, sections were labeled by 10 μg/ml rhodamine or fluorescein-conjugated monoclonal antibodies for 75 min. The anti-gizzard myosin monoclonal antibodies used here were characterized in Trybus and Henry (1989). An anti-rod skeletal muscle myosin monoclonal antibody (5C3) that does not cross-react with smooth muscle myosin was used to correct for nonspecific labeling (Winkelmann et al., 1983). Finally, sections were washed 4 × 5 min in PBS, fixed with 1% paraformaldehyde/PBS for 5 min, mounted with 2.5% wt/vol 1,4-diazabicyclo-[2.2.2]octane (Sigma Chem. Co., St. Louis, MO) in PBS/90% glycerol, and covered by glass coverslips.

Preparation of Myosin Minifilaments and Folded Monomers for Rotary Shadowing

Myosin minifilaments were prepared by dialyzing dephosphorylated turkey gizzard myosin against 5 mM pyrophosphate, pH 7.5 (Trybus and Lowey, 1987). Minifilaments at 0.5 mg/ml were cross linked by 0.01% or 0.1% glutaraldehyde for 15 min and quenched by 0.1% $NaBH_4$, similar to the fixation of gizzard tissue. Folded monomers were prepared by dilution of dephosphorylated myosin to 0.25 mg/ml in filament-disassembling buffer, followed by dialysis and fixation similar to minifilaments. Both minifilaments and folded monomers were reacted with Ab LMM.4 at a molar ratio of 1:1 for 15 min at 20°C. Minifilaments and monomers were diluted to 25 or 50 μg/ml, respectively, in a 5 mM pyrophosphate, pH 7.5/66% glycerol, sprayed onto mica and rotary shadowed with platinum as described (Trybus and Lowey, 1984).

Quantitative Fluorescence Microscopy

Sections were observed by the digital microscopy system described in Fay et al. (1989). Four images, each measuring 107 × 76 μm, were acquired per labeling condition. Each image represented a single focal plane viewed through a Nikon PlanApo ×60, numerical aperture 1.4 objective in combination with a ×10 eye piece (Nikon Inc., Melville, NY). Typical image acquisition time was 1 s. After digitization and subtraction of the CCD dark current, the mean fluorescence intensity was sampled in two 12 × 12 μm subfields in each image, using software written by The Biomedical Imaging Group, University of Massachusetts Medical Center, on a Silicon Graphics (Mountainview, CA) 4D/GTX work station. In total, 8 12 × 12-μm subfields were sampled for each labeling condition. Calculation of the fluorescence intensity of myosin embedded in agarose was based on sampling five images of 47.6 × 47.6 μm for each labeling condition. The values shown in the corresponding figures are means of these measurements and their standard errors (calculated as: $SEM = [\Sigma(I_i - M)^2/n(n-1)]^{1/2}$; I_i, ith intensity measurement; $i = 1, 2, 3, \ldots, n$; M, mean; n, number of measurements).

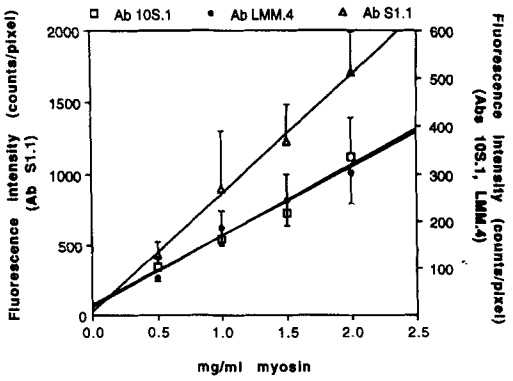

Figure 1. Antibody detection of folded monomeric myosin cross-linked by ethyl-3-(3-dimethyl-aminopropyl) carbodiimide and embedded in agarose. Sections were either labeled with anti-head Ab S1.1-rhodamine, Ab 10S.1-fluorescein, or Ab LMM.4-fluorescein. Bars denote standard deviations.

The mean intensity of specific labeling was calculated by subtracting the intensity obtained with a control nonspecific antibody (anti-skeletal myosin Ab 5C3-fluorescein) from that obtained with antibodies that bind preferentially to monomers (Abs 10S.1-fluorescein and LMM.4-fluorescein) or with a conformation-independent antibody (Ab S1.1-rhodamine). Before subtraction, the fluorescence obtained with each antibody was multiplied by a scaling factor to correct for slightly different degrees of conjugation with fluorophore. When nonspecific labeling by Ab 5C.3-fluorescein was subtracted from the mean labeling intensity of Ab S1.1-rhodamine, the former was also multiplied by the ratio between the rhodamine to fluorescein optical efficiencies of the microscope. This ratio was determined by placing a cell containing 50 μM aqueous solution of either rhodamine or fluorescein on the microscope objective, and measuring the corresponding fluorescence intensity.

Results

Antibodies Used to Detect Monomeric Myosin In Situ

Monoclonal Ab 10S.1, which binds to the folded monomer where the rod forms a hairpin bend, has a 100-fold higher affinity for myosin in the folded than the extended conformation (Trybus and Henry, 1989). Here the affinity of Ab 10S.1 for different myosin conformations was examined under conditions that more closely resemble the tissue. Agarose embedded with purified myosin (1.4 mg/ml) in the folded monomeric, extended monomeric, or filamentous state was cryosectioned and immunolabeled with Ab 10S.1-fluorescein. The fluorescence intensity counts of sections containing extended monomers (224 ± 150; mean ± SD, n = 5) or filaments (229 ± 55) were low and indistinguishable from that obtained by labeling with a nonspecific antibody (323 ± 74). A significantly higher fluorescence signal was obtained when the folded monomer was embedded in agarose (1202 ± 296). In a separate experiment the intensity of the fluorescence signal was proportional to the concentration of folded monomeric myosin embedded in the agarose (Fig. 1, *squares*). Fixation with 0.01% or 0.1% glutaraldehyde decreased the fluorescence signal to 74 or 33%, respectively, of that obtained with unfixed material.

Ab LMM.4 preferentially detects monomers since its epitope at the tip of the myosin tail is relatively inaccessible in filamentous myosin. This Ab was only used with fixed cryosections, to prevent Ab induced disassembly of thick filaments (Trybus and Henry, 1989). To determine the concentration of fixative necessary to prevent disassembly by Ab LMM.4, myosin minifilaments were fixed with either 0.01 or 0.1% glutaraldehyde, and then reacted with Ab LMM.4. Minifilaments were chosen as a test system since the tip of the rod should, if anything, be more accessible than in the larger native filaments found in situ. At 0.01% glutaraldehyde, most minifilaments frayed and were partially disassembled (Fig. 2 *a*). At 0.1% glutaraldehyde, disassembly was prevented, and Ab LMM.4 did not bind to the minifilament bare zone (Fig. 2 *b*). When folded monomeric myosin was similarly fixed with 0.1% glutaraldehyde, 80% of the monomers (n = 206) retained the ability to bind Ab LMM.4 (Fig. 2 *c*). These results establish that LMM.4 should react only with monomeric myosin in tissue fixed with 0.1% glutaraldehyde.

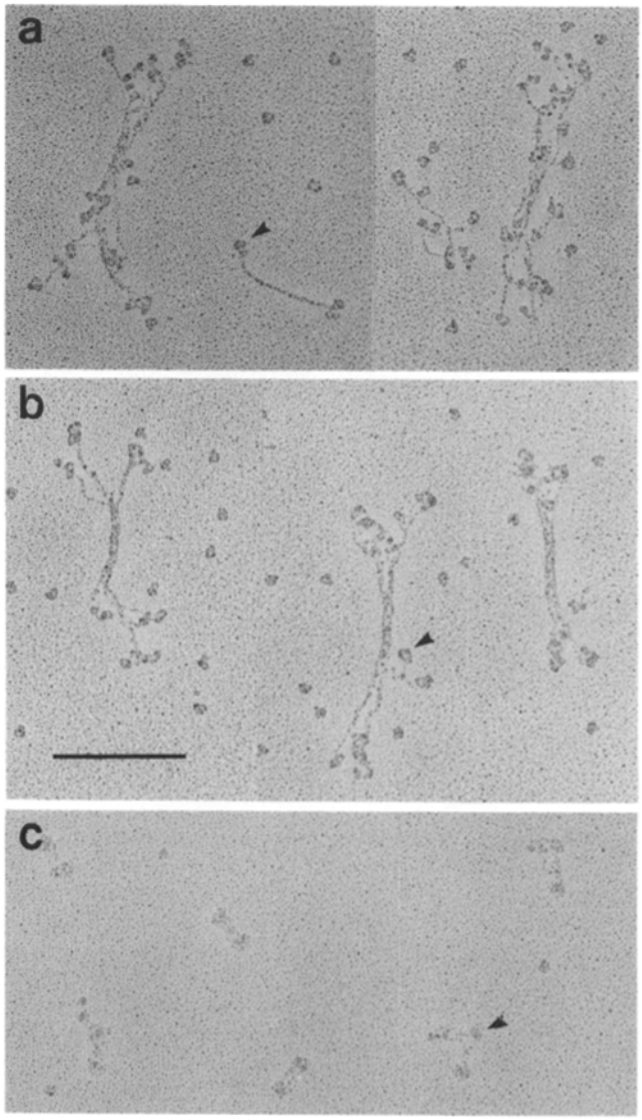

Figure 2. Reaction of Ab LMM.4 with myosin minifilaments fixed with (*a*) 0.01% or (*b*) 0.1% glutaraldehyde. (*c*) Binding of Ab LMM.4 to folded myosin monomers fixed with 0.1% glutaraldehyde. Arrowheads point to antibody. Bar, 200 nm.

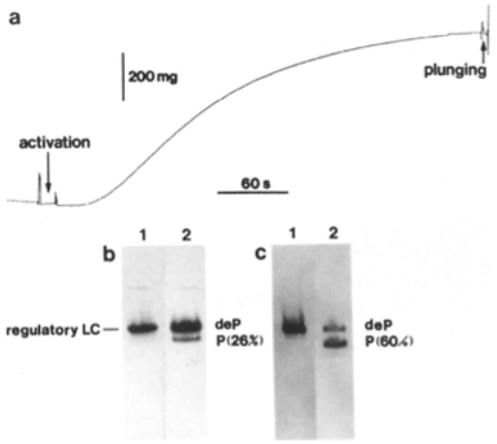

Figure 3. Force and phosphorylation of intact gizzard activated by potassium depolarization. (*a*) Force record indicating when contracted tissue was plunged into liquid nitrogen. Relaxed tissue was obtained by plunging prior to activation. (*b* and *c*) The extent of regulatory light chain phosphorylation of relaxed gizzard (*b*, lane *1*) and of the activated strip (*b*, lane *2*) was measured by densitometry of the immunoblotted light chain bands. Similar phosphorylation measurements were done on a permeabilized gizzard strip incubated in 10S-forming buffer (*c*, lane *1*) or filament-forming buffer (*c*, lane *2*).

Immunolabeling of Intact Gizzard Tissue

Cryosections were cut from unfixed relaxed or contracted gizzard tissue strips. The physiological state of the tissue was assayed by measuring force and the extent of light chain phosphorylation in the same tissue strip. In 1-mm²-thick gizzard strips activated by potassium depolarization, force climbed to a plateau typically in 3 min, reaching ~800 mg (Fig. 3 *a*). The force development rate was relatively slow probably because the experiments were carried out at 20°C and not at avian body temperature. The light chain phosphorylation level in the contracted tissue during the force plateau was 26% in one experiment (Fig. 3 *b*) and 29% in another (data not shown). The relaxed tissue was completely dephosphorylated.

Tissue cryosections were simultaneously labeled with Ab 10S.1-fluorescein and anti-head Ab S1.1-rhodamine which has high and similar affinities for all myosin conformations (Trybus and Henry, 1989) (Fig. 4 *a*). As expected, there was no significant difference in the mean fluorescence intensity of relaxed and contracted sections labeled with Ab S1.1 (Fig. 5). ($P > 0.05$, as determined by a two-tailed Student's *t* test.) Nonspecific labeling was determined with an anti-skeletal myosin antibody. With conformation-specific Ab 10S.1 as the probe, the highest fraction of folded monomeric myosin was detected in unfixed sections of relaxed and contracted gizzard (Fig. 5 *a*). Once sections were fixed with 0.01 or 0.1% glutaraldehyde, the signal obtained with Ab 10S.1 was not distinguishable from background (Fig. 5, *b* and *c*).

Similar experiments were performed with the high affinity anti-rod Ab LMM.4. In this case, only tissue fixed with 0.1% glutaraldehyde was used to prevent Ab-induced filament disassembly, and to allow reaction only with monomeric myosin. Consistent with the results obtained with Ab 10S.1, the fluorescence intensity of cryosections labeled with Ab LMM.4 was very low and comparable to background (Fig. 5 *d*).

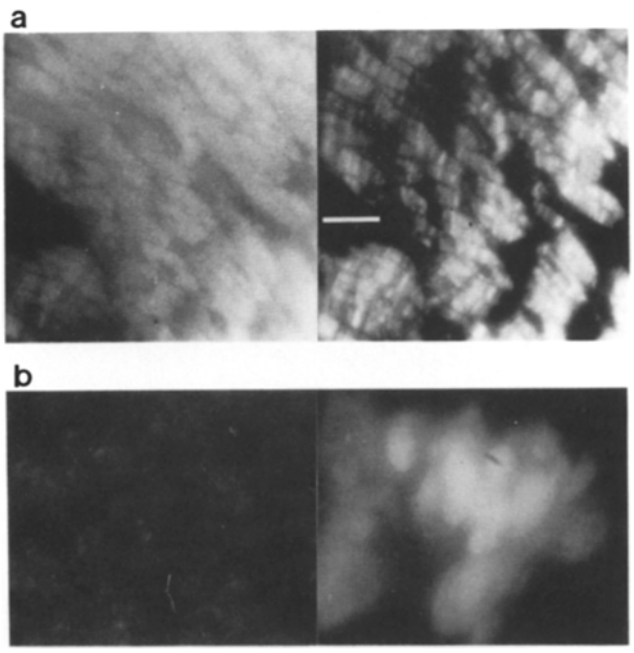

Figure 4. (*a*) Fluorescence images of a contracted unfixed gizzard cryosection labeled with Ab 10S.1-fluorescein (*left*) and Ab S1.1-rhodamine (*right*). Note the contraction bands (Bennet et al., 1988) in the S1.1-rhodamine image. A comparable section labeled with a nonspecific antibody would be black (data not shown). (*b*) Fluorescence images of permeabilized embryonic gizzard cryosections equilibrated in 10S-forming buffer (*right*) or filament-forming buffer (*left*) and labeled with Ab LMM.4-fluorscein. The left hand image was printed at half the exposure time of the right hand one, otherwise it would appear completely dark. Bar, 10 μm.

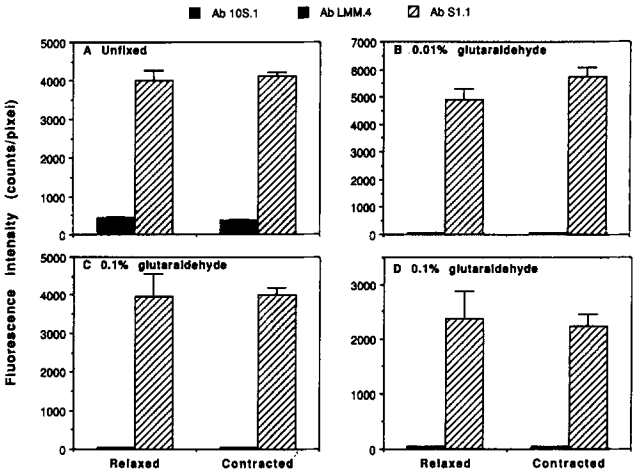

Figure 5. The immunofluorescence intensity of relaxed and contracted gizzard tissue doubly labeled with Abs S1.1 and 10S.1 (*a–c*), or by Abs S1.1 and LMM.4 (*d*). The tissue was (*a*) unfixed, (*b*) fixed with 0.01% glutaraldehyde, or (*c* and *d*) fixed with 0.1% glutaraldehyde. Sections were labeled by Abs 10S.1 or LMM.4 for detection of monomeric myosin, by Ab S1.1 for labeling the total myosin pool, and by a nonspecific antibody. The labeling intensity of the nonspecific antibody is subtracted from the intensities of the specific labeling. Each column in this figure and in Fig. 6 represents the mean and the SEM of eight measurements of 12 × 12 μm squares in four different gizzard sections.

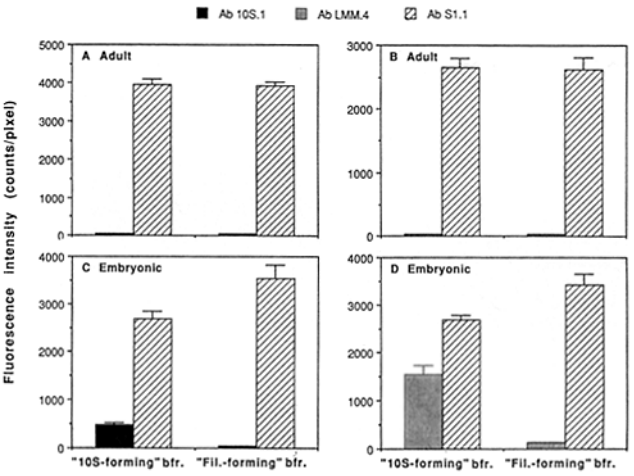

Figure 6. Permeabilized adult (*a* and *b*) or 14-d-old embryonic gizzard tissue (*c* and *d*) was equilibrated in 10S-forming buffer or filament-forming buffer (see Materials and Methods) and doubly labeled by Abs 10S.1 and S1.1 (*a* and *c*), or by Abs LMM.4 and S1.1 (*b* and *d*). Tissue was fixed with 0.1% glutaraldehyde.

Immunolabeling of Permeabilized Gizzard Tissue

Permeabilized gizzard tissue was used to test whether the amount of folded monomer could be increased by equilibrating the cells in a nonphysiological buffer known to induce filament disassembly in vitro. The gizzard strips were permeabilized with α-toxin, since the 1–2-nm membrane pores that are produced (Bhakdi and Tranum-Jensen, 1991) are too small to permit diffusion of myosin out of the cell. To induce filament disassembly, the permeabilized strips were incubated in a calcium-free buffer containing MgATP, while in order to induce formation of phosphorylated filaments, the strips were incubated in a buffer containing calcium and MgATPγS (see Materials and Methods). In vitro, these two solvent conditions would produce either all dephosphorylated folded monomers (provided that the myosin concentration is lower than the critical concentration) or all phosphorylated filaments. The degree of light chain phosphorylation in gizzard strips incubated in 10S-forming buffer was too low to be detectable, while the strips incubated in filament-forming buffer were 60% phosphorylated (Fig. 3 *c*). Similar to the fixed intact tissue, the degree of labeling by Ab 10S.1 and by Ab LMM.4 did not exceed background levels (Fig. 6, *a* and *b*).

To investigate further why the critical concentration of monomer could not be detectably increased in adult gizzard tissue, and also to test the sensitivity of our Abs as probes for monomeric myosin, we repeated the experiment described above on embryonic tissue. The rationale for choosing this tissue was that the concentration of myosin and other proteins that might stabilize filaments should be lower in 14-d-old embryonic tissue than in the adult one. In the embryonic tissue, myosin filaments are localized to discrete patches throughout the cell, with large regions of cytoplasm having no observable polymer (unpublished results). Under these cellular conditions, it may be possible to induce disassembly.

α-toxin permeabilized 14-d-old embryonic tissue was treated in the same way as adult tissue. The degree of regulatory light chain phosphorylation was undetectable in tissue incubated in 10S-forming buffer, while tissue incubated in filament-forming buffer was 67% phosphorylated (data not shown). After cryosectioning and fixation with 0.1% glutaraldehyde, the tissue was doubly labeled with Abs LMM.4 and S1.1, or with Abs 10S.1 and S1.1. The fluorescence signal obtained with Ab LMM.4 was 12-fold higher in tissue incubated in 10S-forming buffer than in filament-forming buffer (Figs. 4 *b* and 6 *d*). Since we have shown that 0.1% glutaraldehyde is sufficient to prevent filament disassembly by Ab LMM.4 (Fig. 2 *b*), it is likely that we are detecting an increase in monomer concentration. Qualitatively similar results were obtained with Ab 10S.1 as the probe, where labeling intensity did not exceed background in the solvent favoring filament assembly, but was significantly higher in the solvent favoring filament disassembly (Fig. 6 *c*).

Discussion

The Monomer Pool Is Very Small in Both Relaxed and Contracted Gizzard Tissue

The aim of this study was to determine if myosin assembly/disassembly contributes to regulation of a smooth muscle contractile cycle. Digital imaging microscopy was used to quantitate the fluorescence intensity obtained when gizzard muscle cryosections were reacted with monoclonal antibodies capable of detecting folded monomeric myosin. Ab 10S.1 preferentially binds to the bend in the folded monomer, whereas Ab LMM.4 preferentially binds monomers because its epitope is buried in fixed filaments. Neither antibody detected a significant pool of monomeric myosin in relaxed or contracted fixed tissue. Only in unfixed tissue did the fluorescence intensity observed with Ab 10S.1 exceed background levels. Since the sensitivity of Ab 10S.1 for folded myosin embedded in agarose was shown to decrease with fixation, the signal obtained in unfixed cryosections probably represents a real, but small pool of folded monomer. Even in this case, however, there was no increase in signal in the relaxed versus contracted tissue. The observed signal could also be due in part to some amount of antibody-induced disassembly in the unfixed tissue. We conclude that the pool of monomeric myosin in the cell is too small relative to the concentration of filamentous myosin for assembly/disassembly to significantly contribute to a contraction/relaxation cycle in gizzard smooth muscle.

The amount of myosin that might be expected to be in the monomer pool can be indirectly estimated from other data. Based on the fact that nonarterial smooth muscle contains ~20 mg myosin/g wet weight tissue (Cohen and Murphy, 1978), and that the critical concentration for assembly of dephosphorylated myosin under solvent conditions approximating physiological is ~2.5 mg/ml (Kendrick-Jones et al., 1987), <15% of the myosin might be unassembled. The fluorescence intensity we obtained for 2.5 mg/ml purified myosin (Fig. 1) is in fact similar to that observed in unfixed relaxed gizzard tissue (Fig. 5 *a*).

Quantitation of thick filament density by electron microscopy of relaxed and contracted rat anococcygeus muscle led to a different conclusion (Gillis et al., 1988). Their results showed a 1.6-fold increase in thick filament density upon contraction, implying a fairly large recruitment of folded monomers into the filamentous state upon phosphorylation.

Our immunofluorescence techniques should have detected a change of this magnitude, if it had occurred in the gizzard.

Filament Disassembly Could Not Be Induced in Permeabilized Adult Gizzard

We were unable to detect an increase in the monomeric pool of myosin by equilibrating permeabilized cells in a buffer where >2 mg/ml dephosphorylated folded monomer can be formed in vitro. When the same procedure was done with 14-d embryonic gizzard tissue, Ab LMM.4 showed a 12-fold higher intensity under relaxing conditions compared with activating conditions, and Ab 10S.1 showed a similar difference in intensities. This observation establishes, at a minimum, that our antibodies are capable of detecting an increase in the monomeric pool under some conditions. The extent of myosin light chain phosphorylation was higher in the permeabilized embryonic tissue than in the intact adult tissue (67 vs. 26%). However, since the partially formed thick filaments in the 14-d-old embryos fill only about half of the cell's cross section (unpublished data), the change in the size of the putative adult monomer pool would be comparable to the change in the size of the embryonic one, and, likewise, should be detectable by our methods.

Our ability to promote myosin disassembly in embryonic gizzard raises the question of why the same conditions did not produce similar results in the adult gizzard. One possibility is that the disassembly we detected was either partially or totally of non-muscle myosin, instead of smooth muscle myosin, since we cannot rule out that our antibodies cross-reacted with non-muscle myosin. In addition, specific proteins that may stabilize filaments in the adult might not yet be synthesized in the embryo. A recent report (Shirinsky et al., 1993) suggests that a low molecular weight protein, with sequence identity to the COOH-terminal portion of myosin light chain kinase, promotes assembly of dephosphorylated myosin in the presence of MgATP. Such a protein could stabilize filaments in the cell under conditions where they would disassemble in vitro.

Summary

These results rule out a large scale recruitment of folded monomers into filaments upon contraction of gizzard smooth muscle cells, and suggest that dephosphorylated thick filaments are relatively stable within the cell. The pool of unassembled myosin is very small, and the high effective myosin concentration in smooth muscle favors assembly even under relaxing conditions. If thick filaments primarily remain assembled because of mass action, myosin in non-muscle cells would be expected to undergo a much more sizable flux between the monomeric and polymeric states since the total myosin concentrations in these cells are closer to the critical concentration for assembly of dephosphorylated myosin.

The fact that smooth muscle myosin has retained the ability to form the enzymatically inert, folded monomeric conformation suggests that there is a yet undiscovered functional role for this conformation in the smooth muscle cell. One possibility is that newly synthesized myosin adopts the folded monomeric conformation until it diffuses to an existing thick filament (Ankrett et al., 1991). Such a role can be tested in developing gizzard cells, where myosin is being actively synthesized.

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