Smooth and Skeletal Muscle Actin Are Mechanically Indistinguishable in the In Vitro Motility Assay

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Smooth muscle produces as much stress as skeletal muscle with less myosin. To determine if the actin isoforms specific to smooth muscle contribute to the enhanced force generation, the motility of actin filaments from smooth and skeletal muscle were compared in an in vitro assay in which single fluorescently labeled actin filaments slide over a myosin-coated coverslip. No difference was observed between the velocity of smooth versus skeletal muscle actin filaments over either smooth or skeletal muscle myosin over a large range of assay conditions (changes in pH, ionic strength, and [ATP]). Similarly, no difference was observed between the two actins when the filaments moved under load over mixtures of phosphorylated smooth and skeletal muscle myosin. Thus, it appears that the actin isoforms of smooth and skeletal muscle are mechanically indistinguishable in the motility assay and that smooth muscle's enhanced force generation may originate within the myosin molecule specific to smooth muscle.

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**KEY WORDS** • actin • smooth muscle • motility • protein isoform

The basic mechanism of smooth muscle contractionı and the biochemical steps of the smooth muscle actomyosin ATPase cycleı are thought to be qualitatively similar to those of skeletal muscle. Nevertheless, smooth muscle's mechanical properties distinguish it from skeletal muscle. One obvious difference is smooth muscle's slower maximum velocity of shortening. More interestingly, both vascular and visceral smooth muscle generate as much force per cross-sectional area as skeletal muscle with only 20% as much myosin. This apparent enhancement in smooth muscle myosin's force production may simply reflect greater numbers of crossbridges working effectively in parallel. Such a mechanical advantage could result from longer (i.e., 2.2-μm) myosin filaments or a more parallel arrangement of contractile units within the smooth muscle cell.

At the actomyosin level, however, differences in kinetics of the smooth muscle crossbridge cycle could result in crossbridges spending a higher fraction of their cycle in the active force-generating state (i.e., higher duty cycle) under isometric conditions. The distinct mechanical properties of smooth and skeletal muscle have been primarily explained on the basis of differences in the myosin molecule, with little attention given to the role of actin. Interest in actin's contribution to the force-generating event has gained momentum now that the primary structure of actin can be correlated to its tertiary structure, derived from actin:DNase I cocrystals. G-actin is a bilobed structure consisting of large and small domains, with the myosin binding site contained within the small domain. Given that a single genetically engineered amino acid change to actin, at its putative myosin binding site, can result in altered crossbridge kinetics in Drosophila flight muscle, actin's contribution to force generation must be considered. This is important, since smooth and skeletal muscle express different actin isoforms, which, in part, may account for smooth muscle's enhanced force-generating ability.

There are two smooth muscle actin isoforms (α-smooth muscle actin and γ-smooth muscle actin), one or both of which are found in every smooth muscle tissue studied. The α-smooth muscle actin isoform predominates in vascular tissue, whereas visceral smooth muscle tissue expresses γ-smooth muscle actin. Extensive amino acid homology exists between the smooth and skeletal muscle actins, with only six differences between chicken gizzard (γ-smooth muscle) and chicken pectoralis (α-skeletal muscle) actin. However, two of these changes occur within the cluster of acidic residues at the N-terminal end of actin (residues 1 and 3), believed to be part of the myosin binding site. In vitro motility can be altered in actins with reduced acidity in this cluster, including wild-type yeast actin and mutated Dictyostelium actin. Thus, it is possible that the naturally occurring amino acid differences between smooth and skeletal muscle actin, which include the absence of the initial aspartic acid found in skeletal muscle actin, may contribute to smooth muscle's unique mechanical properties.

The goal of this study was to determine if, in an in vitro motility assay in which single fluorescently labeled...
actin filaments were observed sliding over a myosin-coated coverslip, the characteristics of actin motility were dependent on actin isoform. Specifically, we wished to determine if the motility of smooth muscle actin differed from that of skeletal muscle actin over either phosphorylated smooth or skeletal muscle myosin over a wide range of assay conditions. The assay conditions studied were chosen so that direct comparisons could be made to the extensive body of literature on skeletal muscle actin motility in vitro.

**Materials and Methods**

### Contractile Protein Isolation and Preparation

Chicken skeletal pectoralis muscle myosin and turkey gizzard smooth muscle myosin were prepared as previously described. Smooth muscle myosin was 100% thio-phosphorylated by the addition of myosin light chain kinase, calmodulin, CaCl₂, and ATP-γ-S. Skeletal muscle actin was isolated from chicken pectoralis acetone powder. Smooth muscle actin was purified from chicken gizzard acetone powder as described, except that, before the final polymerization, size fractionation was carried out with a Sephacryl S300 column (Pharmacia). The purity of all protein-containing fractions was assessed on a 12% polyacrylamide gel, and the purest fraction was retained for polymerization and use. Both smooth and skeletal muscle actin were stored in filamentous form at 4°C and fluorescently labeled with tetramethylrhodamine phalloidin (Sigma Chemical Co., St. Louis, Mo.).

Actin purity and composition were determined by one- and two-dimensional gel electrophoresis (isoelectric focusing followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]), as previously described. One-dimensional SDS-PAGE of both smooth and skeletal muscle actin showed a single major protein band with minor contaminants visible in both preparations only when the actin was overloaded. Two-dimensional electrophoresis revealed that the smooth muscle actin focused overwhelmingly as a single species (data not shown). This is in agreement with previous determinations that chicken gizzard actin is 80% γ-smooth muscle actin with minor contributions from the nonmuscle actin species.

### In Vitro Motility Assay

The in vitro motility assay was carried out essentially as described previously. Briefly, flow-through chambers were constructed from a nitrocellulose-coated coverslip and a glass microscope slide. Then the following solutions were introduced into the chamber: 30 μl myosin (250 μg/ml) in a 300 mM KCl buffer, which allowed the myosin to adhere to the nitrocellulose without forming filaments (i.e., in monomeric form); 60 μl bovine serum albumin (0.5 mg/ml) in the same buffer to wash out unbound myosin and block any uncovered nitrocellulose; 60 μl fluoresecently labeled actin (0.5 μg/μl) in ATP-free assay buffer; 60 μl ATP-free assay buffer to remove unbound actin; and 90 μl assay buffer to initiate motility. Under standard conditions, the assay buffer contained 25 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl₂, 1 mM dithiothreitol, 0.5% methylcellulose, and 1 mM ATP, pH 7.4, with an enzymatic oxygen scavenger system (0.1 mg/ml glucose oxidase, 0.018 mg/ml catalase, and 2.3 mg/ml glucose) added to retard photobleaching. The content of this buffer was adjusted depending on the variable being examined (e.g., changes in [KCl], pH, and [ATP]). Where required, smooth and skeletal muscle myosin were mixed before introduction into the flow-through chamber. Temperature was controlled at 30°C by an objective heater (Vermont Technologies, Burlington, Vt.).

Fluorescently labeled actin filaments were viewed through an inverted microscope equipped for epifluorescence. Intensified video images were digitized and analyzed for actin filament velocity by computer.

### Statistics

All velocity measurements were obtained from at least 10 actin filaments and are presented as mean ± SD. Since the velocities that contribute to each mean value were all obtained from the actin population in a single flow-through chamber, mean velocities were considered an n of one for subsequent statistical analysis.

The effect of actin filament type as a function of assay condition (Figures 2–5) was determined by fitting velocity data for each filament type by polynomial regression (order of polynomial determined by analysis of variance [ANOVA]) and then comparing the quality of the regressions by F test. To allow comparison to previously published data in which no difference (p > 0.05) was found between actin filament types, data were combined and fit to the most appropriate polynomial as determined by ANOVA. Curve fitting was performed with the TABLECURVE and SIGMAPLOT software packages (Jandel Scientific, Corte Madera, Calif.).

### Results

#### Smooth and Skeletal Muscle Actin Filament Motility With Changes in Assay Condition

Under standard assay conditions (see “Materials and Methods”), skeletal and smooth muscle actin filament velocities over monomeric skeletal muscle myosin were not significantly different (p > 0.05), as was the case with monomeric smooth muscle myosin (Figure 1). As previously reported for filamentous myosin, the velocity of actin filament motion over monomeric skeletal muscle myosin was approximately an order of magnitude faster than over monomeric phosphorylated smooth muscle myosin.

To more rigorously probe for differences between myosin’s interaction with smooth and skeletal muscle actin, the motilities of the two actin filament types were compared over a wide range of assay conditions. Starting from standard assay conditions, each of the following parameters was varied independently: pH, [ATP], and [KCl]. These comparisons were performed over both smooth and skeletal muscle monomeric myosin.

When ionic strength was varied by changing [KCl], both smooth and skeletal muscle actin filament velocities increased with increasing ionic strength ([KCl], 12.5–75 mM) when observed over either phosphorylated smooth muscle (Figure 2A) or skeletal muscle (Figure 2B) myosin. No significant difference (p > 0.05) was observed between the ionic strength dependence of skeletal versus smooth muscle actin motility over either smooth or skeletal muscle myosin.
Smooth and skeletal muscle actin motility reacted similarly to changes in pH when measured over either smooth or skeletal muscle myosin. Actin filament velocity on both phosphorylated smooth (Figure 3A) and skeletal (Figure 3B) muscle myosin reached a broad peak between pH 7.0 and pH 8.0.

As [ATP] was reduced, actin filament velocity slowed for both skeletal and phosphorylated smooth muscle myosin (Figure 4). Since no difference was observed between the velocity of skeletal versus smooth muscle actin over either smooth or skeletal muscle myosin ($p>0.05$), actin filament types were combined for subsequent analysis.

Double reciprocal (Lineweaver-Burk) analysis showed that the ATP-dependent $K_m$ of actin filament velocity on smooth muscle myosin ($46 \mu$M) was not significantly different from that on skeletal muscle myosin ($53 \mu$M).

The ionic strength, pH, and [ATP] dependence of actin filament velocity over both phosphorylated Smooth Muscle Myosin

Skeletal Muscle Myosin

**FIGURE 1.** Bar graphs showing the velocity of smooth muscle versus skeletal muscle actin filaments with smooth muscle myosin (left panel) and skeletal muscle myosin (right panel). Data are mean ± SD of six independent experiments. The comparison between actins was made by t test.

**FIGURE 2.** Graphs showing smooth muscle actin filament ($\triangle$) and skeletal muscle actin filament (○) velocity as a function of [KCl] with monomeric phosphorylated smooth muscle myosin (panel A) and skeletal muscle myosin (panel B) at pH 7.4, 1 mM ATP, and 30°C. Data are mean ± SD. Solid lines are polynomial fits to data from both actin filament types: for panel A, $y=-0.00014x^2+0.02x+0.27$ ($r^2=0.92$); for panel B, $y=0.02x+5.08$ ($r^2=0.94$).

**FIGURE 3.** Graphs showing smooth muscle actin filament ($\triangle$) and skeletal muscle actin filament (○) velocity as a function of pH with monomeric phosphorylated smooth muscle myosin (panel A) and skeletal muscle myosin (panel B) at 25 mM KCl, 1 mM ATP, and 30°C. Data are mean ± SD. The solid lines are polynomial fits to data from both actin filament types: for panel A, $y=-0.33x^2+5.0x-18.03$ ($r^2=0.94$); for panel B, $y=-2.55x^2+38.89x-141.63$ ($r^2=0.86$).
smooth and skeletal muscle myosin monomers reported here (Figures 2–4) are in agreement with previous results from the motility assay.16,17,23,24

**Smooth and Skeletal Muscle Actin Filament Motility With Monomeric Myosin Mixtures**

Actin filament velocity over mixtures of myosins having different cycling rates may be determined by mechanical interactions between myosins.12,25,26 If so, comparison of smooth and skeletal muscle actin filament motility over mixtures of monomeric phosphorylated smooth and skeletal muscle myosins may reveal differences between the two actins under loaded conditions.

When increasing amounts of monomeric phosphorylated smooth muscle myosin were mixed with skeletal muscle myosin, the velocity of both smooth and skeletal muscle actin filament velocity dropped precipitously (Figure 5). By increasing the proportion of smooth muscle myosin to 32%, actin filament velocity was reduced by 50% of the velocity for skeletal muscle myosin alone. No significant difference (p > 0.05) was observed between smooth and skeletal muscle actin velocities in this experiment.

**Discussion**

Actin's role in muscle contraction has been viewed generally as both a cofactor to enhance myosin ATPase activity and a passive mechanical structure that transmits force generated by myosin to the ends of the contractile unit (i.e., sarcomere in striated muscle). However, a series of recent studies on nonmammalian actin isoforms14 and actins altered by enzymatic cleavage,27 chemical cross-linking,28 and genetic engineering8,15 suggests that actin may play a significant role in the chemomechanical transduction process (see Morel and Mereh29 for review). In fact, models in which actomyosin force generation originates within the actin filament itself have been proposed.30 Is it possible that smooth muscle's enhanced force-generating capacity is related in part to the smooth muscle-specific actin isofor?

Previous biochemical studies, designed to address this question, have shown that both smooth and skeletal muscle actin are equally capable of activating skeletal myosin ATPase activity.31 However, actomyosin ATPase measurements in solution merely reflect the enzymatic properties rather than the mechanical characteristics of the actomyosin interaction. Therefore, the in vitro motility assay provides a unique opportunity to assess the mechanical consequences of actin isoform on the actomyosin interaction through alterations in actin filament motility.

At least three studies using the in vitro motility assay have attempted to characterize whether the velocity of actin motility is related to either the actin or the myosin isoform.16,24,32 Kron and Spudich16 demonstrated that Dictyostelium and rabbit skeletal muscle actin filament velocity are similar over both Dictyostelium and rabbit skeletal muscle myosin. Similarly, Umemoto and Sellers24 and Okagaki et al32 found no difference between the velocity of gizzard and skeletal muscle actin over smooth muscle myosin. These investigators concluded that actin filament motility was independent of actin isoform and that the velocity of movement was governed by the myosin isoform. However, this conclusion was drawn in each case from a very limited experimental protocol performed at only one ionic strength, pH, and...
[ATP]. Given that both muscle fiber mechanics and in vitro actin filament motility are exquisitely sensitive to the assay conditions, we believe that a comparison of smooth versus skeletal muscle actin motility over a far wider range of assay conditions was necessary before a definitive conclusion could be drawn about the effect of actin isoform on the actomyosin interaction in vitro.

The results of the present study suggest that, over the wide ranges of pH and [KCl] investigated in which actin filaments are presumed to be under no load, the γ-smooth muscle and α-skeletal muscle actin isoforms are mechanically indistinguishable throughout both smooth and skeletal muscle myosin. Even though the two actin isoforms reacted similarly to changes in assay conditions, actin isoform may still affect actin filament motility when actin filaments experience a load. We approached this problem by studying the motility of actin filaments with mixtures of smooth and skeletal muscle myosin.

Mechanical interactions between myosin species occur in the motility assay whenever two myosin populations having different cycling rates attach to the same actin filament.\textsuperscript{17,25,26} The resultant actin filament velocity is dependent on the force-generating capacity of the faster cycling myosin and the magnitude of the load created by the slower cycling species as it is negatively strained by the faster cycling crossbridges.\textsuperscript{17,21} Therefore, actin filaments moving over a myosin mixture must be experiencing the opposing forces created by the mechanical interactions between the two myosin species.

Our laboratory has previously characterized the dependence of actin filament velocity on the proportion of phosphorylated smooth and skeletal muscle myosin.\textsuperscript{17} In these earlier studies in which skeletal muscle actin was used, actin filament velocity for an equal mixture of smooth and skeletal muscle myosin was more similar to the velocity over smooth muscle myosin alone. If actin isoform is crucial to force production, then one would predict that the shape of the relation between actin filament velocity and myosin mixture content might shift if the smooth muscle actin were used. However, no difference was observed between smooth versus skeletal muscle actin filament velocity over mixtures of smooth and skeletal muscle myosin (Figure 5). Once again, it appears that actin isoforms do not alter or modulate the force produced by the actomyosin interaction. This conclusion is supported by the finding that no difference was observed between the velocities of smooth and skeletal muscle actin filaments with either smooth or skeletal muscle myosin at low [ATP] (Figure 4), where slowing is presumably caused by the load imposed by rigor bridges. Therefore, differences in myosin isoforms between smooth and skeletal muscle may be the predominant cause for smooth muscle’s enhanced stress production. These results argue against models proposing that force generation originates in the actin filament.\textsuperscript{30}

The fact that actin isoforms are mechanically indistinguishable in the motility assay may relate to the conserved nature of actin. In fact, the naturally occurring amino acid substitutions that do exist between chicken α-skeletal and chicken γ-smooth muscle actin are quite conservative with respect to amino acid charge and polarity.\textsuperscript{12} Therefore, the present results may not be too surprising. It still remains to be determined whether, under truly isometric conditions, changes in actin isoform can modulate actomyosin force production. Future studies that include isometric force measurements on single actin filaments may reveal a mechanical difference between smooth and skeletal muscle actin.\textsuperscript{33} Alternatively, differences between actin isoforms may only be revealed when a more native smooth muscle thin filament, containing tropomyosin and possibly caldesmon and calponin, is reconstituted.

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