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Are Actin Filaments Moving under Unloaded Conditions in the in vitro Motility Assay?

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ABSTRACT With sliding actin-filament motility assays, filament velocity should be independent of changes in the level of actomyosin activation under unloaded conditions. Using a simple modification of the motility assay to measure relative changes in isometric force (activation), we determined that isometric force increased 200-fold with thiophosphorylation of the myosin regulatory light chain, and that with thiophosphorylated myosin, isometric force was further increased by the addition of saturating smooth-muscle tropomyosin (100%) or tropomyosin plus calponin (500%), and decreased with the addition of saturating caldesmon (−100%). Under "reducing conditions," filament velocity (2.0 μm/s) was constant for mixtures of dephosphorylated and thiophosphorylated myosin containing >5% thiophosphorylated myosin, and was unaffected by the addition of saturating concentrations of tropomyosin or caldesmon. In contrast, "standard assay conditions" were found to be nonreducing. With fully thiophosphorylated smooth-muscle myosin, saturating smooth-muscle tropomyosin increased velocity to 150% of control, and caldesmon halted all filament motion; with fully dephosphorylated myosin (<0.002 mol/mol) filaments only moved when tropomyosin or tropomyosin plus calponin was added. Taken together, these observations suggest that under "standard conditions" a mechanical load is present that is eliminated by "reducing conditions." Filament velocity was insensitive to changes in cross-bridge density, under all conditions, suggesting that noncycling cross-bridges, generated by photochemical oxidation of myosin, is a likely source of mechanical loading.

INTRODUCTION

Sliding filament motility assays provide a method for measuring unloaded actin filament velocity under a variety of well controlled experimental conditions (see Kron et al., 1991 for review). Unloaded velocity is sensitive to the concentration of MgATP and MgADP (Warshaw et al., 1991), but not to the level of filament activation (Haeberle et al., 1992). These observations are consistent with a two-state cross-bridge model such as that described by Huxley (1957), in which unloaded velocity is determined primarily by the rate of cross-bridge dissociation from actin (off-rate), and is relatively independent of the rate of cross-bridge attachment (on-rate). In the presence of any mechanical loading, however, the apparent unloaded velocity would change in parallel with any change in the fraction of force-producing cross bridges (which is determined by both the on- and the off-rate), as illustrated in Fig. 1.

METHODS FOR EVALUATING MECHANICAL LOADING IN ACTOMYOSIN-BASED MOTILITY ASSAYS

An established approach to demonstrating the absence of internal mechanical loads is to show that velocity is constant when the number of force-producing cross-bridges is altered. This can be accomplished either by varying the density of myosin on the nitrocellulose surface or by changing the level of activation by altering the on- or off-rate for cross-bridges.

In the motility assay, the density of myosin on the nitrocellulose surface can be varied by changing the concentration of the myosin solution applied to the nitrocellulose. A number of studies have generally shown that velocity is independent of myosin density over a relatively broad range (Warshaw et al., 1991; Haeberle, 1994).

The weakness of this test is that it would be, in theory, completely insensitive to mechanical loading by oxidized or denatured cross-bridges that bind to actin but do not cycle, because the load would vary in exact proportion to the number of cycling cross-bridges. We have previously shown that noncycling cross-bridges (i.e., NEM-modified myosin) slow filament velocity; and, in agreement with theoretical predictions, the extent of slowing is dependent only on the ratio of NEM-modified to -unmodified myosin, and not the myosin density.

In contrast, altering the level of activation (i.e., level of force production) should provide a very sensitive test for mechanical loading, even by noncycling (and presumably unregulated) myosin. Consequently, if filament velocity can be shown to be independent of filament activation, there is little chance that the filaments are subject to mechanical loading either by myosin or by other factors. If, however, velocity changes in parallel with the level of activation, then either a mechanical load is present, or the regulatory mechanism modulates the off-rate for cross-bridges. If regulation of the cross-bridge off-rate can be ruled out by other means, the source of mechanical loading (i.e., myosin versus nonmyosin) can be determined by looking at the effect of myosin density on velocity. If velocity is insensitive to changes in myosin density, then myosin is the most likely source.

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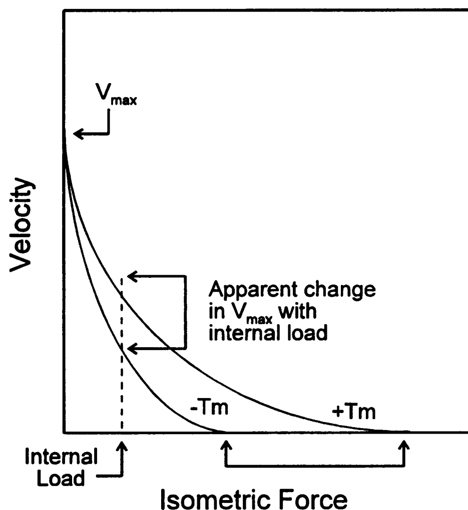

FIGURE 1 Activation by tropomyosin (Tm) will result in an apparent change in V_{max} only in the presence of mechanical loading.

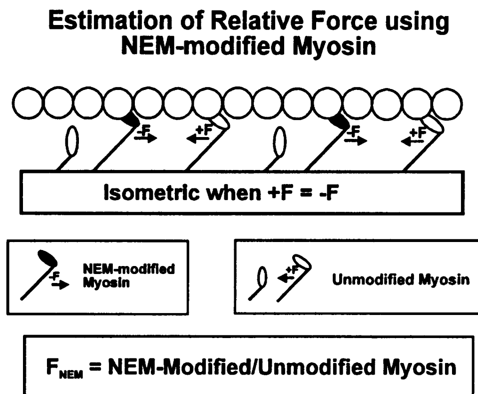

FIGURE 2 Estimation of relative force using NEM-modified myosin (NEM-modified skeletal muscle myosin forms high-affinity complexes with myosin but does not cycle or hydrolyze MgATP). NEM-modified skeletal muscle myosin is mixed at different molar ratios with unmodified myosin and applied to the nitrocellulose-coated coverslip at a concentration of 50–200 μg/ml. F_{NEM} is determined as the molar ratio of NEM-modified/unmodified myosin at which motility stops (Haeberle, 1994).

Ruling out the possibility that a regulatory factor is altering velocity by changing the off-rate of the cross-bridge is, nonetheless, a challenging problem. One approach is to eliminate potential sources of loading and show that this both increases velocity and eliminates the dependence of velocity on the level of activation. This is the approach we have taken with several smooth-muscle regulatory proteins.

Demonstrating that a regulatory protein alters the level of activation and the force applied to isolated thin filaments is essential for interpretation of its effects on filament velocity. A number of relatively direct mechanical methods have been described for measuring the force generated by a single actin filament in contact with a myosin-coated surface (Nishizaka et al., VanBuren et al., Mehta et al., Molloy et al., and Yanagida et al., this issue). These methods have the advantage that they can be readily calibrated in terms of absolute units of force (pN), but have the disadvantages of requiring specialized, and frequently expensive, equipment, and of being fairly time-consuming measurements. We have developed an alternative approach to measuring relative changes in isometric force production that is very inexpensive, technically undemanding, and relatively fast.

MEASUREMENT OF RELATIVE CHANGES IN ISOMETRIC FORCE PRODUCTION ON ISOLATED THIN FILAMENTS USING NEM-MODIFIED MYOSIN

As illustrated in Fig. 2, this method is based on the use of NEM-modified skeletal muscle myosin to mechanically load thin filaments (Haeberle, 1994). NEM-modified skeletal muscle myosin forms high-affinity complexes with myosin but does not cycle or hydrolyze MgATP.

Increasing amounts of NEM-modified myosin result in a progressive increase in the number of nonmoving filaments, an overall slowing of the filament motion, and a progressive increase in the rate of filament fragmentation. Filament fragmentation is very pronounced just before filament motion is completely halted. At slightly higher ratios of NEM-modified/unmodified myosin, there is no motion and no fragmentation. The dependence of fragmentation on filament motion and the relatively abrupt transition from complete filament fragmentation to no fragmentation makes it relatively easy to quickly identify the approximate ratio at which filament motion stops by casual observation of filament length. The nonmoving filaments showed no obvious Brownian-like motions and resembled filaments bound to myosin under rigor conditions. The ratio of NEM-modified/unmodified myosin at which motion ceased (F_{NEM}) was independent of the total myosin concentration applied to the coverslip from 50–200 μg/ml myosin. This confirmed that the amount of NEM-myosin required to stop filaments was linearly related to the density of unmodified myosin, and therefore, linearly related to the force produced on thin filaments. This was also confirmation that changing the myosin concentration was not a sensitive method for detecting the presence of loading by noncycling cross-bridges. An added advantage of this approach, from the standpoint of evaluating changes in the relative level of activation, is that F_{NEM} is also independent of filament length except at very low myosin densities and for very short filaments (<1 μm).

REGULATION OF ISOMETRIC FORCE BY SMOOTH-MUSCLE REGULATORY PROTEINS

When this method was used to evaluate effects of myosin light-chain phosphorylation, tropomyosin, and calponin on isometric force production, the results shown in Fig. 3 were obtained. Mixing increasing amounts of thiophosphorylated with dephosphorylated myosin resulted in a linear increase in force. The addition of saturating smooth muscle Tm (1 mg/ml) to the assay buffer increased the maximum force, and the recruitment of force became somewhat cooperative. If in addition, calponin was added, there was a dramatic increase

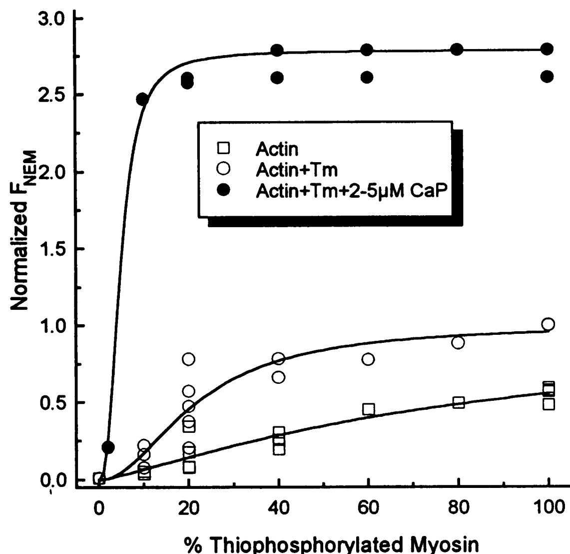

FIGURE 3 Effect of LC20 phosphorylation, tropomyosin (Tm), and calponin (CaP) on isometric force. Normalized FNEM is a measure of relative isometric force (see Fig. 2). %Thiophosphorylated myosin refers to the fraction of thiophosphorylated myosin in a mixture of thiophosphorylated and dephosphorylated chicken gizzard smooth-muscle myosins. The motility buffer contained: 1 mM ATP, 4 mM Mg^{2+}, 1 mM EDTA, 80 mM KCl; Tm (1 mg/ml) and CaP were added to the motility buffer.

in both the cooperativity of activation and of the maximum level of force. If caldesmon rather than calponin was added, there was a concentration-dependent inhibition of force (Fig. 4).

PHOTOCHEMICAL MODIFICATION OF PROTEINS IN THE MOTILITY ASSAY

Our results indicate that under "standard motility assay conditions" there is an internal load due primarily to noncycling

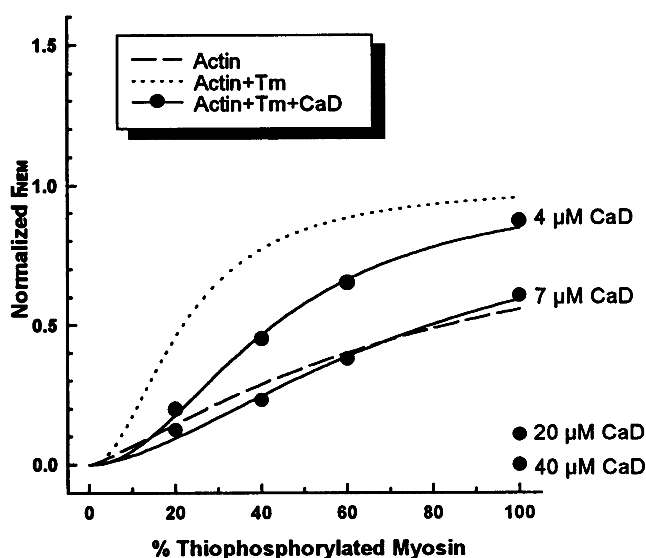

FIGURE 4 Inhibition of isometric force by caldesmon (CaD). Conditions are identical to those in Fig. 2 except that Tm and CaD were added to the motility buffer. Actin and Actin+Tm curves are the same as in Fig. 3.

cross-bridges. We use the phrase "standard motility assay conditions" here to refer to conditions similar to those described by Kron et al. (1991). The specific features of the assay that we have focused our attention on are 1) the concentration of dithiothreitol (DTT or Cleland's reagent), 2) the use of an oxygen-scavenger system, 3) the use of carrier proteins (e.g., BSA), and 4) the intensity of illumination. The standard motility buffer contained 1 mM DTT, a glucose/glucose oxidase/catalase oxygen scavenger system (2.3 mg/ml glucose, 0.1 glucose oxidase, 0.018 catalase), no carrier protein, and full-intensity illumination with a 100-W mercury arc lamp.

The basic picture that has emerged from these studies is that reactive free radicals are generated during photoillumination and the effects of these free radicals can be reduced by 1) reduced illumination to minimize the creation of free radicals, 2) addition of carrier molecules (e.g., BSA) at concentrations greater than or equal to the concentrations of regulatory proteins in the motility buffer, and 3) addition of high concentrations of DTT to reverse the oxidation of reactive sulfhydryls on contractile proteins.

An early observation was that the oxygen scavenger system could promote oxidation and sulfhydryl-dependent cross-linking of caldesmon, even in the absence of photoillumination. This is not surprising in retrospect, considering the fact that glucose oxidase generates peroxide, and the fact that catalase is typically added in nM concentrations compared with regulatory proteins, such as caldesmon, which are typically present at 1–10 μM. Although catalase has a very high V_{max}, there will be some steady-state peroxide concentration unless the motility buffer is completely isolated from atmospheric oxygen (Englander et al., 1987). Even with a low peroxide concentration, the 1000-fold concentration difference favoring the formation of collision complexes with added regulatory proteins may explain the observed oxidation of caldesmon in the presence of this scavenger system.

Along these same lines of thinking, we have found that "reducing conditions" (i.e., 5 mg/ml BSA plus 10 mM DTT or 1 mg/ml BSA plus 100 mM DTT) are much more effective at preventing photobleaching than the "standard conditions." We have monitored single flow cells for up to 90 minutes with continuous low level illumination under reducing conditions. Moreover, the addition of the oxygen scavenger system does little to further reduce photobleaching. Our general observation has been that a decreased rate of photobleaching is always associated with less actin filament fragmentation, fewer nonmoving filaments, and higher filament velocity. This is consistent with the argument that reactive intermediates generated by photoillumination result in oxidative modifications of myosin to produce noncycling, high-affinity cross-bridges that impose a mechanical load on actin filaments.

EVIDENCE FOR MECHANICAL LOADING WITH UNPHOSPHORYLATED SMOOTH-MUSCLE MYOSIN

A single cross-bridge generates sufficient force to move an actin filament, and consequently, filament velocity is in-

dependent of myosin concentration, except at very low myosin densities and actin filament lengths where cross-bridge interaction with the filaments becomes discontinuous (Uyeda et al., 1990). Because phosphorylation stimulates the on-rate for cross-bridges, but not the off-rate (Sellers, 1985), it was surprising to us that we had not observed motility with dephosphorylated myosin, even for very long filaments at higher myosin densities. Our first evidence that this might be due to the presence of mechanical loading was the observation that calponin, which decreases the velocity of filament motion and increases the level of force with phosphorylated myosin, causes nearly all filaments to move in the presence of fully dephosphorylated smooth muscle myosin (the level of phosphorylation was shown by direct measurement to be <0.002 mol/mol).

FILAMENT MOTILITY WITH DEPHOSPHORYLATED MYOSIN

We have since been able to show in preliminary studies that the regulatory proteins that increase isometric force (i.e., calponin and tropomyosin) also stimulate motility with dephosphorylated myosin (Haeberle, 1993). In contrast, caldesmon, which decreases isometric force, does not stimulate motility. This strongly argues that motility with dephosphorylated myosin is prevented by mechanical loading of the filaments. The observation that increased myosin density does not enhance motility, whereas "reducing conditions" do (Haeberle, 1993), strongly implicates oxidized myosin as the source of mechanical loading.

EFFECTS OF TROPOMYOSIN ON FILAMENT VELOCITY

Using standard motility buffers, we previously found that smooth-muscle tropomyosin increased filament velocity from 1.5 μm/s to 2.0 μm/s. Others have reported similar effects of tropomyosin (Shirinsky et al., 1992; Okagaki et al., 1991). Under "reducing conditions" filament velocity is 2.0 μm/s with or without tropomyosin. These observations, in light of the previously discussed activation of force in the presence of tropomyosin, are quite consistent with the argument illustrated in Fig. 1. Thus, smooth-muscle tropomyosin appears to regulate the on-rate exclusively, and increases activation (i.e., isometric force) but does not affect velocity. On this basis, we have suggested that smooth-muscle tropomyosin provides a nearly ideal probe for mechanical loading in smooth-muscle myosin-based motility assays.

EFFECTS OF CALDESMON ON FILAMENT VELOCITY

We have previously reported that caldesmon under standard conditions completely inhibits filament velocity, whereas there is no effect on velocity except at the highest concentration (10 μM) under reducing conditions (Haeberle et al., 1992). Because caldesmon reduces force production (Fig. 4), these results are also consistent with the notion that filaments are loaded under standard conditions. There may, however, be additional factors contributing to the decreased velocity. Under standard conditions we see clear evidence for cross-linking of actin filaments and oligomerization of caldesmon (Haeberle et al., 1992). Although we have not specifically addressed this question experimentally, it seems possible that a mixture of caldesmon oligomers, which would potentially include actin-actin cross-linkers, actin-myosin cross-linkers, and myosin-myosin cross-linkers, might impede filament motion (chicken gizzard has two cysteine residues, one located at the C-terminal actin-binding end of the molecule and one at the N-terminal myosin-binding end). A mixture of dimers would include molecules having at their two extreme ends either two myosin binding sites, two actin-binding sites or one actin-binding and one myosin-binding site. For example, cross-linked antiparallel actin filaments should have reduced motility because of the 180° opposed driving forces acting on the individual filaments. In contrast, we have shown that tethering by monomeric caldesmon under reducing conditions does not impede filament motion or reduce filament velocity (Haeberle et al., 1992).

EFFECTS OF CALPONIN ON FILAMENT VELOCITY

Under reducing conditions, calponin slows the velocity of filaments with both dephosphorylated and phosphorylated myosins, but under standard conditions calponin increases the velocity of filaments with dephosphorylated myosin (i.e., nonmoving filaments move). Under reducing conditions, filament velocity with phosphorylated myosin is decreased by calponin from 2.0 μm/s to 0.7 μm/s, and is decreased from 0.4 to 0.1 with dephosphorylated myosin (Haeberle, 1994). Based on the observed increase in isometric force in the presence of calponin, the simplest explanation is that calponin slows the off-rate of cross-bridges. This is consistent with the dramatic increase in filament binding seen in the presence of calponin (Haeberle, 1994). At 80 mM KCl, continuous filament motility can be measured with 10 μg/ml myosin, whereas even 100 μg/ml myosin gives only very intermittent motility in the absence of calponin. The apparently contradictory effects of calponin on dephosphorylated myosin under standard and reducing conditions can be explained by the presence of a mechanical load under standard conditions.

SUMMARY AND CONCLUSIONS

In the course of investigating the regulatory actions of several smooth-muscle regulatory proteins, we have consistently found evidence that photoillumination of motility flow cells results in myosin oxidation and subsequent myosin-dependent mechanical loading. Essentially all of our comparative findings using standard and reducing conditions are consistent with this suggestion. The extent of mechanical loading by this mechanism will be dependent on a number of experimental conditions including: 1) oxygen content of

buffers, 2) intensity of illumination, 3) concentration of reducing agent in motility buffers, 4) concentration of other proteins in motility buffers, 5) duration of illumination, 6) flow cell temperature, 7) oxidation state of proteins when added to buffers, and 8) concentration of the individual components of any oxygen scavenger systems and the type of oxygen scavenger used. We can approximate the load in our system, using standard conditions, to be in the range of 1–10% of maximal force produced by unregulated actin and smooth muscle myosin. Although a load of this magnitude would have only minor effects for fully activated actomyosin, it would have major effects at reduced levels of activation as demonstrated by the absence of motility with dephosphorylated myosin. This may also provide an explanation for some of the controversy in the literature concerning the effects of light-chain phosphorylation, tropomyosin, caldesmon, and calponin on motility with smooth-muscle myosin (Shirinsky et al., 1992; Okagaki et al., 1991; Haeberle, 1993, 1994; Haeberle et al., 1992). At low salt concentrations (25 mM KCl) there are loading effects with smooth-muscle myosin that have been attributed to the weak-binding cross-bridge state (Warshaw et al., 1990) that are not present at higher salt concentrations (80 mM KCl), and are probably unrelated to the effects seen under nonreducing conditions. This is entirely consistent with the established ionic-strength dependence of the weak-binding state.

Our studies have also suggested ways to evaluate the presence of mechanical loading. The addition of smooth-muscle tropomyosin to the motility buffer provides an ideal probe, because it increases isometric force but not velocity under unloaded conditions. A tropomyosin-dependent increase in velocity is a good indication of mechanical loading. Obviously any maneuver that increases only the on-rate for cross-bridges should be equally effective.

Finally, we have found that a much-simplified motility buffer containing no oxygen scavenger system, high concentrations of DTT (10–100 mM), and a carrier protein (1–5 mM BSA), when used in conjunction with reduced illumination and digital image processing, essentially eliminates photobleaching, illumination-dependent protein oxidation, and myosin-dependent mechanical loading.

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DISCUSSION

Session Chairperson: Kenneth A. Johnson
Scribe: Debra Silver

SAMUEL CHACKO: Joe, a lot of laboratories which work with caldesmon find that if you keep the DTT concentration within range of 1–10 mM, they are reduced. My question to you is the caldesmon that you use. At the time you use it, have you tested to see whether you are working with a native caldesmon in the reduced form or were they oxidized to start with?

JOE HAEBERLE: We've looked at it pretty extensively using native gels. As I said, just putting caldesmon with 1 mM DTT in that motility buffer, with the oxygen scavenger system, and even using degassed buffers, will result in a partial oxidation of caldesmon. It's relatively difficult to get the exact proteins out of the flow cell that were used in the motility assay, but it's almost a foregone conclusion. If you have photobleaching, you've got the potential for photochemical modification of proteins. So, if you're on the verge of having oxidized proteins before you put them in the flow cell, in my mind, it is sort of a foregone conclusion. There will be additional oxidation of these proteins in the flow cell. And, as I pointed out, there is substantial evidence now that myosin itself is being oxidized and a certain fraction of the myosin we put in is loading the filament motion. This can be eliminated by the use of higher concentrations of reducing agents. So, I think it is a very reasonable concern. I've seen a lot of data that suggests that it sort of exists widely or broadly in a lot of motility data.

JERRY POLLACK: Just a followup to your statement. For those of us who are using in vitro motility assays, we obviously are concerned with these effects very much. I wonder if you have made some quantitative estimate of the dif-