A simple method for measuring the relative force exerted by myosin on actin filaments in the *in vitro* motility assay: evidence that tropomyosin and troponin increase force in single thin filaments

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We have studied the effect of an internal load on the movement of actin filaments over a bed of heavy meromyosin (HMM) in the *in vitro* motility assay. Immobilized α-actinin can bind to actin filaments reversibly and ultimately stop the filaments from moving. Above a critical concentration of α-actinin, thin filament velocity rapidly diminished to zero. The fraction of thin motile filaments decreased linearly to zero with increasing α-actinin concentration. The concentration of α-actinin needed to stop all filaments from moving (0.8 μg/ml with actin) was very consistent both within and between experiments. In the present study we have defined the 'index of retardation' as the concentration of α-actinin needed to stop all filament movement, and we propose that this index is a measure of the isometric force exerted by HMM on actin filaments. When we measured the effect of immobilized α-actinin on motility in the presence of 10 mM P_i we found that the index of retardation was 0.62 ± 0.07 ($n = 3$) times that in the absence of P_i. This observation is in agreement with the reduction of isometric tension in chemically-skinned muscle due to P_i. In a series of comparative experiments we observed that tropomyosin and troponin increase the index of retardation and that the degree of increase depends upon the tropomyosin isoform studied. The index of retardation of actin is increased 1.8-fold by skeletal-muscle tropomyosin, and 3-fold by both cardiac-muscle and smooth-muscle tropomyosin. In the presence of troponin the index of retardation is 2.9–3.4-fold greater than that of actin with all tropomyosin isoforms.

Key words: contractility, isometric force, Ca^{2+}-regulation, regulatory proteins, co-operativity.

INTRODUCTION

The investigation of Ca^{2+}-regulation of muscle thin filaments by tropomyosin and troponin has been significantly advanced by the use of the *in vitro* motility assay [1–5]. In this method myosin is immobilized on to a microscope cover glass, and solution containing thin filaments, labelled on actin with highly fluorescent rhodamine-phalloidin (ϕ), is infused past. When ATP is added the thin filaments move over the myosin.

In the normal *in vitro* motility assay the thin filaments are unloaded, therefore the interpretation of the results obtained from such systems is limited to comparisons with unloaded shortening in intact muscle. The function of muscle is to exert force and to do mechanical work. It would therefore be highly desirable to be able to measure how troponin–tropomyosin regulates force exerted upon single thin filaments using the *in vitro* motility assay.

Measures of force have been successfully made using thin filaments attached to glass microneedles [6–9]. Although this method is direct and can be quite precise it also requires great

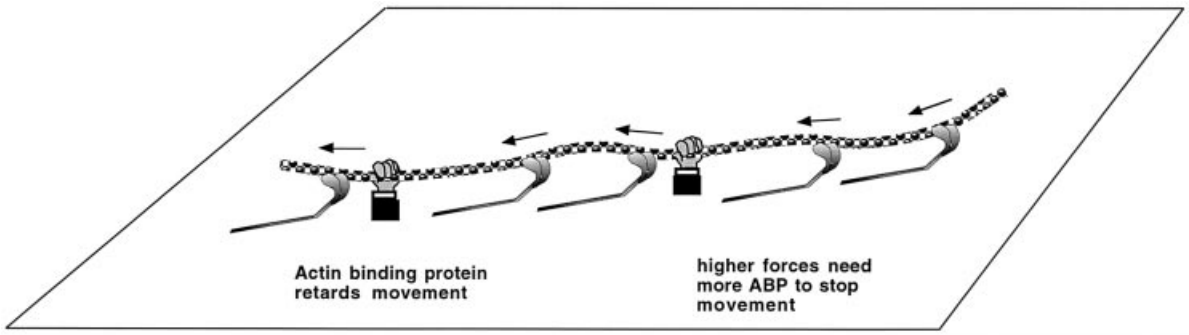

Figure 1 The principle of filament retardation by an actin-binding protein

Actin-ϕ is observed moving over skeletal-muscle HMM attached to a silicone-coated glass surface in the presence of ATP. Immobilized actin-binding protein retards this movement and at high concentrations stops movement completely.

Abbreviations used: ϕ, rhodamine-phalloidin; actin-ϕ, rhodamine-phalloidin-labelled actin filaments; DTT, dithiothreitol; HMM, heavy meromyosin; NEM, *N*-ethylmaleimide; NEMS-1, NEM-treated subfragment-1; pPDM, *p*-phenylenedimaleimide.

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skill and specialized equipment. This means it is only possible to make a small number of measurements. We have been investigating the mechanism of control of actin-filament motility by human cardiac troponin and tropomyosin, and the effect of [Ca^{2+}] and hypertrophic cardiomyopathy mutations on motility [5,10–12]. In such work it is necessary to have a simple high-throughput method to determine whether changes to the proteins in the thin filament alter isometric force.

In the present study we have investigated a technique which could enable us to compare isometric force of the actomyosin interaction using conventional *in vitro* motility detection and analysis equipment. The principle of this assay is to place an internal load upon the thin filament to retard filament movement due to the myosin motor. This is achieved by using an actin-binding protein attached to the cover glass along with the immobilized myosin motor protein (Figure 1). Warshaw et al. [13] first demonstrated this principle using *N*-ethylmaleimeide (NEM)-treated heavy meromyosin (HMM) and *p*-phenylenedimaleimide (pPDM)-treated HMM, while Janson et al. [14] showed that α-actinin and filamin can also stop filament movement. The greater the force on an actin filament, the higher the concentration of actin-binding protein needed to stop movement [15].

In the present study we have investigated the effect of immobilized α-actinin on filament motility. We have found that the effect of α-actinin on the fraction of filaments moving is a consistent and sensitive method that can detect changes in isometric force of single actin filaments interacting with immobilized HMM. We have used this technique to study how tropomyosin and troponin may control force.

METHODS

Proteins

Rabbit fast skeletal-muscle HMM, F-actin, tropomyosin and troponin were prepared using previously described methods [1,16–19]. Human cardiac tropomyosin and troponin were isolated as described by Purcell et al. [5]. Smooth muscle tropomyosin was prepared as described in [20] and the recombinant Ala-Ser-α-striated-tropomyosin isoform was expressed in *Escherichia coli* as described previously [10]. α-Actinin was prepared from chicken gizzard by the method of Craig et al. [21]. All protein concentrations were determined by the Lowry method. F-actin was stored at 4 °C and used within 4 weeks of preparation. HMM was used within 4 days of preparation and was treated to remove inactive heads every day. Tropomyosin and α-actinin stock solutions (2–3 mg/ml) were divided into small portions and frozen at −20 °C. Frozen α-actinin was satisfactory for at least 6 months.

In vitro motility assay

The *in vitro* motility assay was performed as described by Fraser and Marston [1] using 100 μg/ml skeletal-muscle HMM on cover glasses, coated with silicone by soaking in 0.2 % (v/v) dichloromethylsilane in chloroform. F-actin was labelled with ϕ as described by Kron et al. [16]. Actin-ϕ–tropomyosin and actin-ϕ–tropomyosin–troponin complexes were formed at 10 × assay concentration: 100 nM actin-ϕ, 150–400 nM tropomyosin and 0–600 nM troponin were mixed in 50 mM KCl, 25 mM imidazole/HCl, 4 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 0.5 mg/ml BSA, pH 7.4 (buffer B) and incubated for 30–60 min. The complexes were diluted 10-fold immediately prior to infusion into the motility cell.

A flow cell was prepared from a freshly siliconized coverslip and a microscope slide [16]. Assay components and buffers were infused into the flow cell at 30–60 s intervals. First, two 50 μl aliquots of HMM at 100 μg/ml were infused in buffer A (50 mM KCl, 25 mM imidazole/HCl, 4 mM $MgCl_2$, 1 mM EDTA and 5 mM DTT, pH 7.4) to provide a coating of immobilized HMM on the coverslip. In experiments using immobilized α-actinin or NEM-treated subfragment-1 (NEMS-1), these proteins were mixed with 100 μg/ml HMM and the mixture was applied to the motility cell. This was followed by 2 × 50 μl of buffer B (i.e. buffer A + 0.5 mg/ml BSA) then 2 × 50 μl of 10 nM actin-ϕ ± associated tropomyosin–troponin in buffer B. Buffer C [50 μl; buffer B + 0.1 mg/ml glucose oxidase, 0.02 mg/ml catalase, 3 mg/ml glucose, 0.5 % (w/v) methylcellulose ± troponin at assay concentration] and 50 μl of buffer D (buffer C + 1 mM ATP) were then infused. The motility cell was mounted on to a brass block maintained at 27 °C by circulating water from a thermostatic bath.

The movement of actin-ϕ–tropomyosin filaments over the immobilized skeletal-muscle HMM was observed under a Zeiss Axiolab Microscope with epifluorescence illumination (63 × /1.4 objective) with a DAGE-SIT-68 camera and recorded on video tape. Videos were digitized and the movement was analysed by manual tracking [1] or by using the automatic tracking programme described by Marston et al. [22].

RESULTS

Immobilized α-actinin stops filament movement

α-Actinin (1 μg/ml) was infused into the motility cell followed by the blocking buffer (buffer B). When 10 nM actin-ϕ with 0.5 % methylcellulose was infused, actin filaments bound to the cover glass. This indicated that α-actinin could cross-link actin-ϕ to the silica surface. When the cover glass was coated with 100 μg/ml skeletal-muscle HMM and actin-ϕ was infused, actin filaments also bound to the surface, and upon the addition of ATP up to 90 % of the filaments moved at a uniform speed. In previous work [2] we demonstrated that actin filament velocity over our silica-coated surface was independent of HMM concentration down to less than 20 μg/ml, indicating that the surface offered little resistance to actin filament movement, unlike nitrocellulose surfaces.

When a low concentration of α-actinin was included with HMM on the cover glass, the motility of actin-ϕ filaments (10 nM) was substantially reduced, such that at an average of 0.8 μg/ml α-actinin no movement of filaments could be seen. A similar concentration of α-actinin mixed with the actin-ϕ filaments but not attached to the surface had no effect on motility, thus indicating that the α-actinin effect was due to cross-linking of immobilized α-actinin to actin-ϕ.

We examined the quality of actin-ϕ filament movement under conditions (0.3 μg/ml α-actinin) where the inhibition of motility was incomplete (Figure 2). When we tracked 18 randomly selected filaments from a single motility cell over a 20 s period we observed some filaments moving, some stationary and a considerable proportion of filaments that stopped and/or started moving during the observation period. This differs from actin filaments 'switched off' by troponin and tropomyosin where such 'erratic' filaments make up less than 5 % of the total [4,22]. This observation shows that the opposing effects of the HMM motor and the α-actinin retardation were reversible equilibrium processes. If this is the case it is legitimate to analyse filament movement in terms of the properties of the whole population

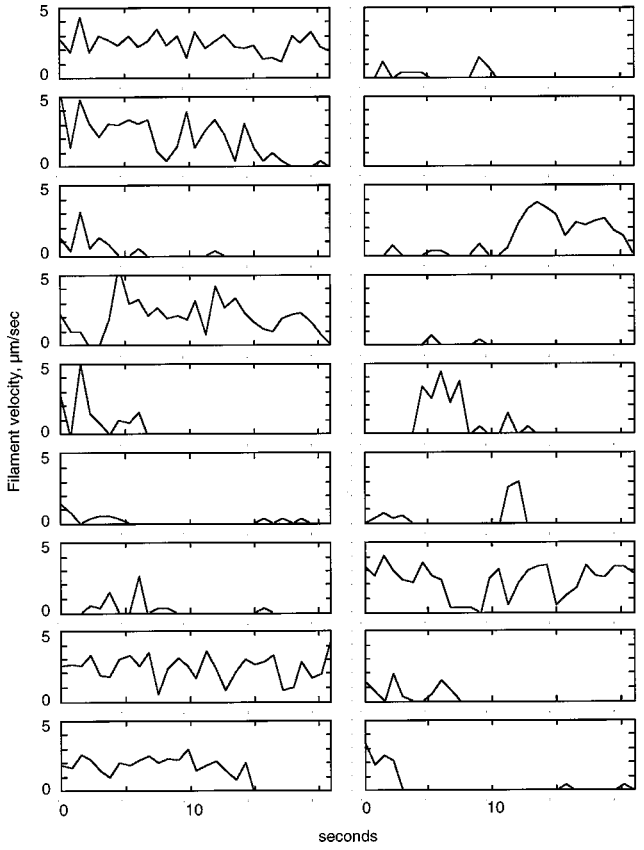

Figure 2 Actin-ϕ filament movement over HMM in the presence of immobilized α-actinin

The cover glass was coated with 100 μg/ml HMM mixed with 0.3 μg/ml α-actinin. A time-series of 30 images was collected at 0.75 s intervals. There were approx. 80 actin-ϕ filaments within the field of view (100 μm $\times$ 100 μm). Filaments (18) were chosen at random from the first frame and each one was manually tracked over a period of 21 s. For each filament we have plotted velocity (distance moved between frames/0.75 s) against time. Filaments moving at a constant velocity, filaments not moving and filaments which stopped or started during the measurement period were observed.

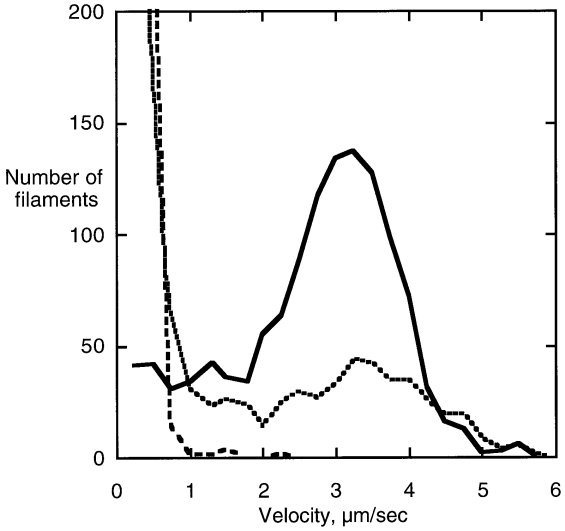

Figure 3 Effect of α-actinin on actin-ϕ filament movement

The movement of fluorescent actin-ϕ filaments over a microscope slide coated with 100 μg/ml skeletal-muscle HMM in 1 mM MgATP during a 0.6 s time interval was recorded and analysed. Filament vectors (1000) were obtained and plotted as a frequency histogram. In the absence of α-actinin, over 80% of filaments moved as a single population, mean velocity 2.9 μm/s (continuous line). With the addition of 0.4 μg/ml α-actinin to the surface half of the filaments stopped moving (dotted line). With the addition of 0.8 μg/ml α-actinin all of the filaments stopped moving (dashed line).

rather than properties of individual filaments. We therefore analysed filament movement using our automatic tracking protocol [22].

All the filaments in 10–15 frames recorded at 0.6 s/frame were tracked (up to 1000 individual vectors) and plotted as frequency histograms (Figure 3). These indicate that the effect of α-actinin is to give a wider spread of velocities when the fraction of motile filaments is reduced by about half, but without any consistent change in the mean velocity of the population of filaments; this reflects the frequent stopping and starting seen in individual filaments (Figure 2). We investigated whether this distribution was related to filament length by manually tracking short filaments (< 1 μm) and long filaments (> 6 μm) separately and found that the spread of velocities was the same in both cases (3.35 μm/s $\pm$ 1.3 and 3.26 μm/s $\pm$ 1.5 respectively).

We analysed motility in terms of the parameters, fraction of motile filaments and the average velocity of the remaining motile filaments, as described by Marston et al. [22] (Figure 4). The relationship between α-actinin concentration and the average velocity of motile filaments was erratic: sometimes it behaved in an almost all-or-nothing way with velocity remaining nearly constant with increasing α-actinin and then suddenly dropping to zero; at other times it approximated to a linear relationship. There was a consistent linear decrease in the fraction of motile filaments as α-actinin concentration increased. We found that this relationship was observed for a single α-actinin preparation tested with different HMM and actin-ϕ preparations over a period of several weeks. The mean correlation coefficient for linear regression fits obtained from nine experiments was 0.99 $\pm$ 0.1 (mean $\pm$ S.E.M.) and the mean x-axis intercept was 0.87 μg/ml α-actinin $\pm$ 0.01 (mean $\pm$ S.E.M., $n = 14$). We concluded that the most consistent way of determining the concentration of α-actinin needed to stop filament movement was from the relationship of the fraction of motile filaments to α-actinin concentration. We have therefore adopted an empirical 'index of retardation', defined as the x-axis intercept of linear regression fits of the data. However it should be noted that a value for the index of retardation could equally be obtained by visual inspection of the velocity versus α-actinin or the motile fraction versus α-actinin plots and would yield similar values. We propose that the index of retardation is an indirect measure of the isometric force produced by immobilized HMM interacting with thin filaments. In the present study we used three α-actinin preparations which gave slightly different index of retardation values for pure actin: 0.5, 0.8 and 1.2 μg/ml.

The ability of an actin-binding protein to stop filament movement is a general phenomenon and not related to the nature of the actin-binding protein. Thus we were able to confirm previously published results with two other species: NEMS-1 (Figure 4) and pPDM-treated HMM (results not shown). Although the numerical value of the index of retardation varied greatly between the three protein species, the relative effect of adding skeletal-muscle tropomyosin to the actin-ϕ filaments was the same in all cases: an approximately 2-fold increase.

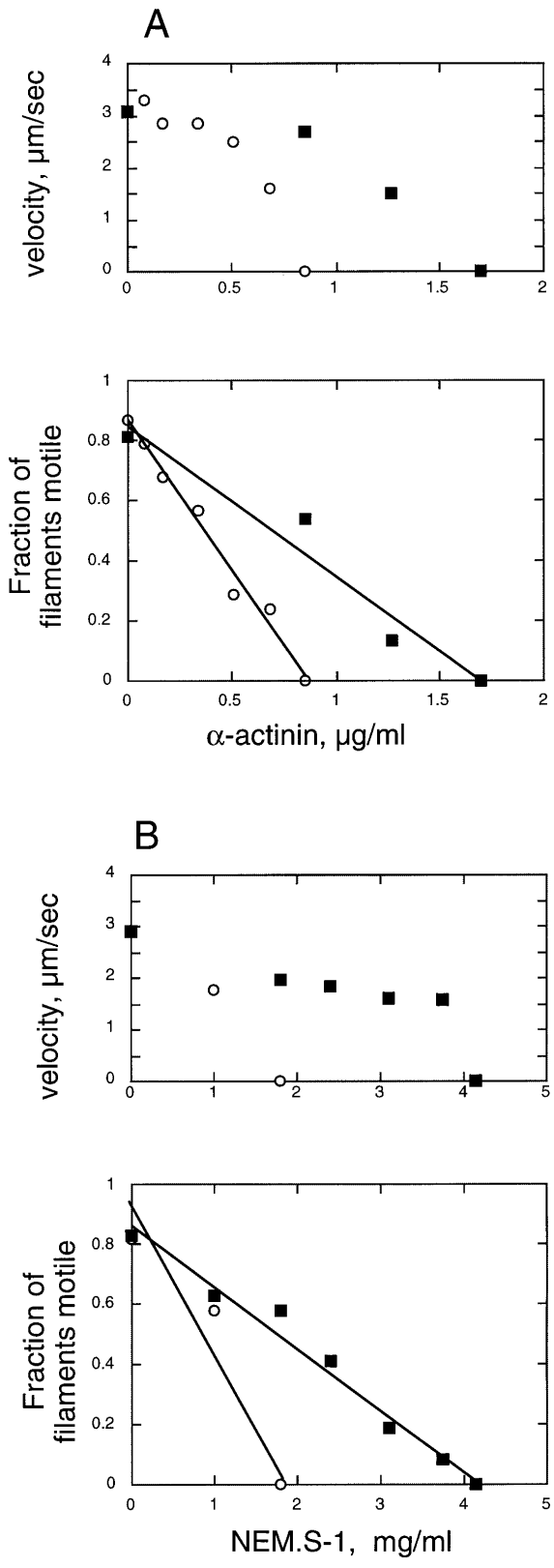

Figure 4 Effects of α-actinin and NEMS-1 on filament velocity and the fraction of motile filaments

(**A**) The effect of immobilized α-actinin on thin filament velocity (top panel) and fraction of motile filaments (bottom panel). (**B**) The effect of immobilized NEMS-1 on filament motility. (○) Actin-φ filaments (10 nM); (■) actin-φ (10 nM)–skeletal-muscle-tropomyosin filaments (30 nM). The lines are least squares regression fits used to determine the index of retardation.

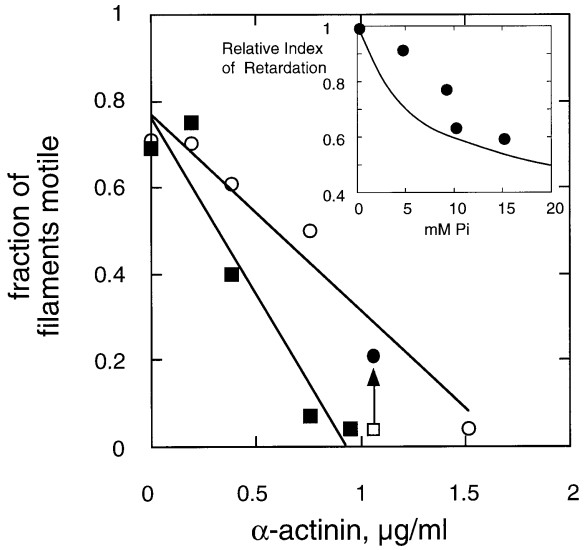

Figure 5 Effect of P_i on the fraction of filaments moving in the presence of immobilized α-actinin

(○) Actin-φ filaments and (■) actin-φ filaments in buffer containing 10 mM P_i (KCl concentration was reduced to maintain constant ionic strength). P_i reduced the amount of α-actinin needed to stop filament movement. The fraction of motile filaments measured in 10 mM P_i (□) is increased when P_i-free buffer is infused into the motility cell (●). The inset shows the decrease in the index of retardation as a function of P_i concentration at constant ionic strength. ●, Experimentally measured index of retardation relative to P_i-free buffer measured in dual cells. The line corresponds to the calculated decrease in isometric force with increasing P_i concentration from [27].

Effect of P_i on the index of retardation

High concentrations of P_i reduce isometric tension in chemically skinned muscle fibres and myofibrils [23,24], therefore examining the effect of P_i on the index of retardation could establish the proposed relationship between the index and isometric force. When we measured the effect of immobilized α-actinin on motility in the presence of 10 mM P_i we found that the concentration of α-actinin needed to stop all movement was 0.62 ± 0.07 ($n = 3$) times the concentration needed in the absence of P_i. The index of retardation decreased with increasing P_i concentration up to 15 mM, the maximum possible without changing ionic strength. Thus the index of retardation had decreased under conditions where isometric force was expected to decrease. Moreover the effect was reversible: if P_i-free buffer was infused into a motility cell with a concentration of immobilized α-actinin that just stopped all movement in the presence of P_i, the filaments started moving again (Figure 5).

Effect of tropomyosin and troponin on the index of retardation

We examined the effects of different isoforms of tropomyosin on the index of retardation. Tropomyosin always increased the index of retardation (Figure 4), however, there were large differences between the tropomyosin isoforms (Table 1). Since different α-actinin preparations had slightly differing index of retardation values when tested with the control actin-φ filaments, we did paired experiments with a dual cell and normalized the index values of regulated filaments to that of actin-φ. Actin–rabbit-skeletal-muscle-tropomyosin (predominantly αβ-heterodimers) needed 1.8 ± 0.1 ($n = 5$) times more α-actinin to stop

Table 1 Effect of tropomyosin and troponin on the retardation index, and upon sliding velocity, weak affinity for subfragment-1 · ADP · P_i, K_T and co-operative unit size

α-Actinin retardation measurements were made in paired experiments with actin as control. Results are expressed as *x*-axis intercept relative to actin control (see Figure 4). Means ± S.E.M. are shown for *n* separate experiments. Saturating concentrations of tropomyosin and troponin were used and these were determined by titration immediately before the force measurements, as described in our previous papers [2,5]. Weak binding was assayed as described by Marston and Redwood [40]. Other references: *[39], †[37], ‡M. El-Mezgueldi, personal communication, §[31], ‖[38]. N.A., not applicable; N.D., not determined.

	Actin	Actin–skeletal-tropomyosin	Actin–cardiac-tropomyosin	Actin–smooth-tropomyosin	Actin–Ala-Ser-α-tropomyosin	Actin–skeletal-tropomyosin + troponin (pCa 5)	Actin–skeletal-tropomyosin + NEMS-1
Retardation index relative to actin	1	1.8 ± 0.1 ($n = 5$)	3.0 ± 0.1 ($n = 3$)	3.0 ± 0.2 ($n = 3$)	1.9 ± 0.1 ($n = 4$)	3.0	3.0
Relative weak binding affinity	1	1.02	N.D.	1.25	1.5–2.4	N.D.	N.D.
Maximum velocity relative to actin§	1	1.0 ± 0.01	1.2 ± 0.03	1.28 ± 0.02	1.25 ± 0.03	1.25 ± 0.02	1.21 ± 0.08
K_T‖	N.A.	0.155				0.143	
Co-operative unit size	1	5–6*	N.D.	8–15†	3–5‡	10–15*	N.A.

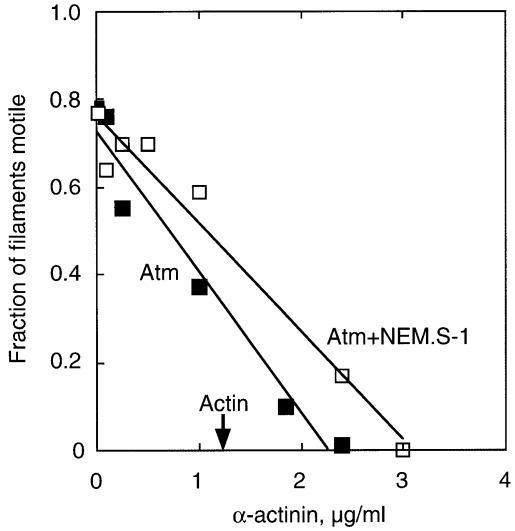

Figure 6 Effect of NEMS-1 on the index of retardation of actin and actin–tropomyosin filaments

The fraction of actin-ϕ filaments moving over skeletal-muscle HMM plus increasing amounts of α-actinin was measured. Lines are linear regression fits of the data. Actin-Ala-Ser-α-tropomyosin (Atm) filaments in the presence (□) and absence (■) of 10 nM NEMS-1. The arrow indicates the index of retardation of pure actin in this experiment.

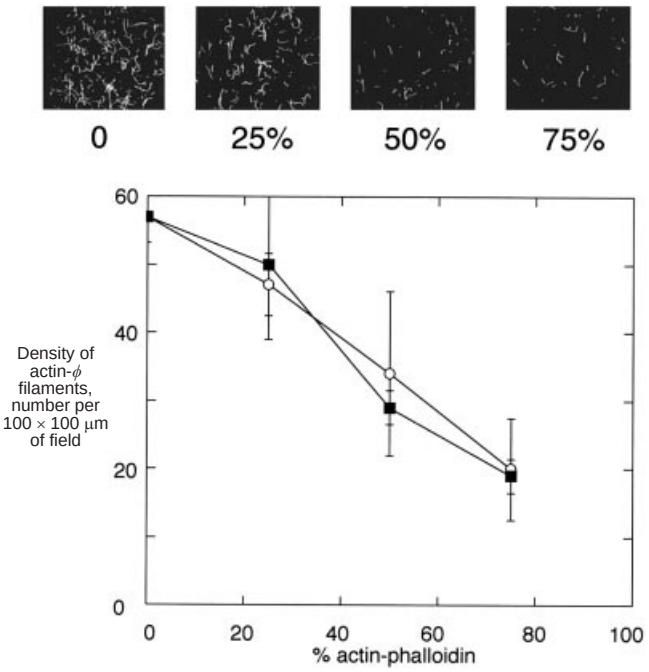

Figure 7 Competition between actin-ϕ and phalloidin-actin

α-Actinin (1 μg/ml) was infused into motility cells followed by 10 nM actin-ϕ + phalloidin-actin in various ratios. The top panel shows the appearance of the motility cell with increasing proportions of phalloidin-actin. The density of actin-ϕ filaments decreased as it was displaced by unlabelled actin. Density was measured using our automatic detection system. Displacement of actin-ϕ by actin-phalloidin (○) and actin-phalloidin–smooth-muscle-tropomyosin (■) was indistinguishable, indicating they have the same affinity for α-actinin.

movement compared with actin-ϕ. Actin-ϕ–striated-muscle-α-tropomyosin (from human cardiac-muscle) and actin-ϕ–smooth-muscle-tropomyosin (from sheep aorta, mainly $\alpha\beta$-heterodimers) filaments gave index of retardation values of 3.0 ± 0.1 ($n = 3$) and 3.0 ± 0.2 ($n = 3$) times that of actin-ϕ filaments respectively. Somewhat surprisingly filaments containing the recombinant human striated-muscle α-tropomyosin expressed in *E. coli*, which had an Ala-Ser N-terminal extension instead of the acetyl group present in native tropomyosin, had a lower index of retardation than the α-tropomyosin extracted from human cardiac tissue [1.9 ± 0.1 ($n = 4$) times actin-ϕ, compared with 3.0].

The effect of troponin on the index of retardation depended upon the tropomyosin isoform. When skeletal-muscle troponin was added to actin-ϕ–rabbit-skeletal-muscle tropomyosin the index of retardation increased to 2.9 ± 0.2 ($n = 3$) times actin. However, when the troponin was added to actin-ϕ–human-cardiac-muscle-tropomyosin or actin-ϕ–smooth-muscle-tropomyosin filaments there was no significant increase in the index of retardation. The index of retardation relative to actin was changed from 3.0 ± 0.1 ($n = 3$) to 3.4 ± 0.6 ($n = 2$) when troponin was added to actin-ϕ–human-cardiac-muscle-tropomyosin and from 3.0 ± 0.2 ($n = 3$) to 3.1 when troponin was added to actin-ϕ–smooth-muscle-tropomyosin filaments.

NEMS-1 can 'switch on' actin–tropomyosin filaments by binding strongly to actin in the presence of ATP. The effect has been demonstrated in ATPase assays [25], force assays in glycerinated muscle fibres [26] and in unloaded filaments in the *in vitro* motility assay [2]. We investigated the effect of mixing thin filaments with NEMS-1 on the index of retardation. Adding 10 nM NEMS-1 to actin filaments had no effect on the index of

retardation, which was expected since NEMS-1 acts via tropomyosin. NEMS-1 also had no effect on the index of retardation of actin–human-cardiac-tropomyosin filaments, presumably because they were already fully switched on. NEMS-1 did, however, increase the index of retardation of actin–Ala-Ser-α-tropomyosin filaments and actin–skeletal-muscle-tropomyosin filaments (Figure 6 and Table 1). When 10 nM NEMS-1 was added to actin–Ala-Ser-α-tropomyosin filaments that had been stopped by the addition of 1.2 μg/ml α-actinin, the filaments started moving again. Titration of α-actinin in the presence of 10 nM NEMS-1 showed that it increased the index of retardation to the maximum level obtained with native cardiac α-tropomyosin (Figure 6).

We determined whether regulatory proteins could affect the affinity of α-actinin for actin by a competition experiment. Microscope cover glasses were coated with a 1 μg/ml α-actinin solution and then a mixture of actin-ϕ and non-fluorescent phalloidin-labelled actin filaments were infused. The ability of non-fluorescent phalloidin-actin and non-fluorescent phalloidin-actin–smooth-muscle-tropomyosin to displace actin-ϕ was found to be identical (Figure 7).

DISCUSSION

Validation of the relative force assay

We have demonstrated that immobilized α-actinin can bind to actin filaments and impose a load which retards filament movement due to HMM motor protein in the *in vitro* motility assay and ultimately stops the filaments from moving. We have defined the index of retardation as the concentration of α-actinin needed to stop all filament movement and we propose that this index is a measure of the isometric force exerted by HMM on actin filaments. In a series of comparative experiments we have observed that tropomyosin and troponin increase the index of retardation and that the degree of increase depends upon the tropomyosin isoform studied.

Measuring the effect of P_i on the index of retardation may provide a critical test of our hypothesis. P_i specifically reduces isometric tension in chemically skinned muscle fibres [23,24]. The free energy of ATP hydrolysis available for tension generation by actomyosin, ΔG^{ATP}, is equal to $\Delta G° + RT \ln\{[ATP]/([ADP][P_i])\}$, therefore a high P_i concentration reduces ΔG^{ATP} and this leads to a fall in isometric tension. Pate et al. [27], found both by experimentation and by calculation, that P_i in the 5–15 mM range reduced isometric force in skinned muscle fibres to approx. half that observed in the absence of P_i. This reduction in force, due to P_i, was faithfully recorded by the α-actinin-based motility assay as a decrease in the index of retardation, which was quantitatively similar to that predicted by the thermodynamic equation; moreover the effect of P_i could be reversed when P_i was removed (Figure 5).

Our method for obtaining a measure of isometric force is based on the same principle as that used by Haeberle [28,29], using NEM-treated myosin as the actin-binding species. Thus 'F_{NEM}' and 'index of retardation' are equivalent parameters [28,29]; however F_{NEM} has not been extensively characterized or validated. A direct comparison of the effect of incorporation of skeletal-muscle tropomyosin into thin filaments upon F_{NEM} and force measured by microneedle displacement showed a similar increase in both cases [9,28]. We also observed a 2-fold increase in the index of retardation due to tropomyosin, using both α-actinin and NEMS-1 as the actin-binding protein. Both the effect of P_i and comparison of the effect of tropomyosin with direct assays support our hypothesis that the index of retardation is a measure of isometric force, although we cannot precisely specify the nature of the relationship. This interpretation is restricted to conditions where at least 80 % of filaments are motile before α-actinin is added. Therefore we can only study the effect of regulatory proteins that do not reduce the fraction of motile filaments.

The use of α-actinin to measure the index of retardation has several advantages over using NEM-treated myosin, NEMS-1 or pPDM-treated HMM [29,30]. Firstly, it is effective at low concentrations: less than 1 μg/ml α-actinin mixed with 100 μg/ml HMM stopped actin filaments from moving. Secondly, α-actinin does not cause filaments to fragment [29]. This enabled us to obtain a value for the concentration needed to stop movement, while using our well-established techniques of movement analysis [1,22]. Although the technique cannot give an absolute value for isometric force it can distinguish differences in the index of retardation of 10 % when two different types of reconstituted thin filaments are compared in a split cell moving over the same bed of myosin and α-actinin. It is possible to perform and analyse several such experiments in one day, which far exceeds the rate of data acquisition by microneedle measurements. We have successfully used this assay to determine the effects of hypertrophic cardiomyopathy mutations in tropomyosin and troponin T on force exerted on thin filaments [11,31].

Modulation of force by tropomyosin and troponin

Increases in force exerted by actin filaments when tropomyosin is bound have been reported [9,28], but no comparative study has previously been made. In the present study we observed that cardiac-muscle tropomyosin and smooth-muscle tropomyosin increased the index of retardation by more than skeletal-muscle tropomyosin, and that troponin in activating conditions (pCa 5) further increased the index of retardation of actin–skeletal-muscle-tropomyosin filaments. This clearly indicates that troponin and tropomyosin control force production at the level of single filaments, as well as velocity and the fraction of motile filaments [1–4]. The mechanism for changing force by tropomyosin and troponin presumably reflects the co-operative/allosteric system of regulation in thin filaments [32,33]. In this respect, the observation (Table 1) of an apparent maximum index of retardation, equivalent to 3 times the index of retardation of actin alone, is significant. The maximum is attained by smooth-muscle and cardiac-muscle tropomyosin alone or by skeletal-muscle tropomyosin plus troponin or NEMS-1 and by recombinant Ala-Ser-α-tropomyosin plus NEMS-1. Since both troponin and NEMS-1 act on actin–tropomyosin to switch the system fully on, an index of retardation of three times that of actin filaments probably represents the force exerted on fully switched on thin filaments.

In principle, force exerted by myosin on thin filaments may be increased if the 'unitary force' (the size of a single force event) or 'duty cycle' (fraction of time during each crossbridge cycle that force is produced) increases. However, all the measurements so far made indicate that unitary force is the same for all myosin II motor proteins [34,35]. The duty cycle can vary widely between myosin isoforms, being greatest for the slowest myosin type. It seems unlikely that troponin and tropomyosin could affect the duty cycle, since increases in force are associated with higher filament velocity (Table 1). Another way of varying force exerted on a single filament is by recruitment of more force-generating crossbridges. This is possible, since under the conditions where filament movement is studied in the *in vitro* motility assay the number of force-generating crossbridges associated with each actin monomer in the steady-state is probably less than 1 %. This follows from the observation that the force per actin monomer

exerted on thin filaments is low (15 pN/μm [6], equivalent to 0.04 pN/actin) whereas the force produce by a single crossbridge is reported as approx. 6 pN [36].

In Lehrer and Geeves' model of thin filament regulation [33,37] the recruitment and turnover of crossbridges is defined in terms of four parameters: the equilbrium between on and off states, K_T, the co-operativity of this equilibrium, n, the weak myosin binding affinity, K_1, and the rate-limiting tension generating step, k_{+2} [33,37]. In Table 1 we have shown the effects of tropomyosin and troponin upon the index of retardation and these parameters. It can be observed that the maximum speed of filament movement (probably dependent upon k_{+2}), the on/off equilibrium and the weak binding affinity are not correlated with the index of retardation, whereas co-operativity, does correlate. This suggests that the co-operativity of thin filaments may be the main factor which determines crossbridge recruitment in our system, since the greatest force is obtained with thin filaments containing smooth muscle tropomyosin (co-operativity = 8–15) or troponin–tropomyosin (co-operativity = 10–15).

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