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Myosin regulatory light chain phosphorylation inhibits shortening velocities of skeletal muscle fibers in the presence of the myosin inhibitor blebbistatin

Melanie Stewart · Kathy Franks-Skiba · Roger Cooke

Abstract Phosphorylation of skeletal myosin regulatory light chain (RLC) occurs in fatigue and may play a role in the inhibition of shortening velocities observed in vivo. Forces and shortening velocities were measured in permeabilized rabbit psoas fibers with either phosphorylated or dephosphorylated RLCs and in the presence or absence of the myosin inhibitor blebbistatin. Addition of 20 μM blebbistatin decreased tensions by $\sim 80\%$ in fibers, independent of phosphorylation. In blebbistatin maximal shortening velocities (V_{max}) at 30°C, were decreased by 45% (3.2 ± 0.34 vs. 5.8 ± 0.18 lengths/s) in phosphorylated fibers but were not inhibited in dephosphorylated fibers (6.0 ± 0.30 vs. 5.4 ± 0.30). In the presence of 20 μM blebbistatin, K_m for V_{max} as a function of [ATP] was lower for phosphorylated fibers than for dephosphorylated fibers (50 ± 20 vs. 330 ± 84 μM) indicating that the apparent binding of ATP is stronger in these fibers. Phosphorylation of RLC in situ during fiber preparation or by addition of myosin light chain kinase yielded similar data. RLC phosphorylation inhibited velocity in blebbistatin at both 30 and 10°C, unlike previous reports where RLC phosphorylation only affected shortening velocities at higher temperatures.

Keywords Myosin phosphorylation · Skeletal muscle · Blebbistatin · Motor protein

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Introduction

Muscle fatigue is characterized by decreases in a number of muscle parameters including force and shortening velocity. Numerous studies have investigated the mechanism of inhibition but the precise mechanism is unknown (Fitts 1994; Westerblad and Allen 2003; Cooke 2007). Fatigue affects every system involved in muscle mechanics. It is known that the inhibition in function involves, in part, mechanisms that directly affect the interaction of actin and myosin, and these include product inhibition due to the accumulation of metabolites. Although replication of these metabolite levels in vitro results in a decrease in shortening velocities, the observed inhibition does not account for the extent seen in vivo (Fitts 1994; Westerblad et al. 1997, 2002; Myburgh 2004; Cooke 2007).

The role of phosphorylation in skeletal muscle is not as clear as it is in smooth muscle and is the subject of research by a number of groups. In smooth muscle it is well documented that the phosphorylation of the myosin regulatory light chain (RLC) is important in the control of activation of smooth muscle (Butler and Siegman 1998; Stull et al. 1990). In skeletal muscle RLC is phosphorylated by a dedicated calcium-calmodulin kinase, myosin light chain kinase (MLCK) (Pires et al. 1974) and dephosphorylated by a phosphatase (Morgan et al. 1976). Calcium activation of the kinase during heavy use and fatigue, leads to increased RLC phosphorylation, which provides a likely mechanism for the regulation of muscle mechanics under these conditions. A correlation between RLC phosphorylation and the force achieved in a twitch following a lengthy tetanus or a train of twitches first suggested a role for phosphorylation in twitch potentiation. (For review see (Sweeney et al. 1993). More conclusive evidence, that RLC phosphorylation was related to increased twitch tension, was found, in vitro, by

Persechini and Cooke (Persechini et al. 1985), where it was shown that RLC phosphorylation increased tension in partially activated permeablized fibers. More definitively, twitch potentiation was absent, or greatly attenuated, in transgenic mice lacking MLCK (Zhi et al. 2005).

The effect of myosin RLC phosphorylation on shortening velocity has been studied extensively, is an active site of investigation and still the subject of debate. Crow and Kushmerick initially suggested that shortening velocities were inhibited by myosin RLC phosphorylation, from a correlation observed in mouse skeletal muscle during a sustained tetanus (Crow and Kushmerick 1982a, b). However, this hypothesis was disputed by Barsotti and Butler (Butler et al. 1983; Barsotti and Butler 1984) who found a dissociation between shortening velocities or ATPase activities and myosin RLC phosphorylation. No effect of myosin phosphorylation on tension or velocity was found in fully activated permeabilized fibers (Persechini et al. 1985; Sweeney and Kushmerick 1985; Sweeney and Stull 1990).

A number of small molecules are known to inhibit muscle tension, including phosphate, phosphate analogs, butane dione monoxime and blebbistatin. In the great majority of conditions where tension is inhibited, velocity is also inhibited (Dantzig and Goldman 1985; Chase et al. 1993; Seow et al. 1997; Regnier et al. 1999; Franks-Skiba et al. 2007). The inhibition of velocity has been hypothesized to be the result of an internal load imposed by non-working myosin heads. The limited conditions in which velocity is not inhibited include vanadate (Pate et al. 1994), but only at high temperatures and in dephosphorylated fibers, and phosphate under most conditions. The present paper adds blebbistatin to the list of myosin inhibitors that inhibit tension but do not inhibit velocity, and shows that this property is controlled by phosphorylation.

Recent work from our laboratory, has found that under conditions where fiber tensions are partially inhibited, RLC phosphorylation inhibits the inhibition of shortening velocities, (Franks-Skiba et al. 2007; Karatzaferi et al. 2007). Analogs of Pi and the transition state with ADP.P., such as BeF_3, AlF_4 and VO_4, bind to myosin at the nucleotide binding site, but populate different pre-power stroke states, and are thought to form structures similar to myosin in complex with ATP or ADP.Pi (Fisher et al. 1995; Smith and Rayment 1996). Addition of these analogs inhibits fiber tension but inhibits the maximum extrapolated velocity (V_{max}) V_{max} differently. In the presence of vanadate, for example, RLC phosphorylation inhibits shortening velocity compared with fibers with dephosphorylated RLC; although isometric tensions were not affected (Franks-Skiba et al. 2007). A similar effect was found at low pH (6.2) and at high concentration of phosphate (30 mM), (such found in fatigue in vivo), where RLC phosphorylation inhibited V_{max} relative to

dephosphorylated RLC in fibers (Karatzaferi et al. 2007). However, little or no effect of phosphorylation on shortening velocity, was observed for the analogs BeF_3, or AlF_4 at pH 7 (Franks-Skiba et al. 2007). In the above studies, the effect of RLC phosphorylation on shortening velocity was found only at elevated temperatures, i.e. 30°C but not 10°C. At the lower temperature most agents that inhibited tension, e.g. phosphate analogs or lower pH, also inhibited shortening velocity and phosphorylation of the fibers had little or no effect.

Blebbistatin is a novel compound and a useful pharmacological tool for investigations of the molecular mechanisms of the actomyosin cycle. Blebbistatin was recently identified as a specific inhibitor of non-muscle myosin II, (Straight et al. 2003) which inhibited the contraction of the cleavage furrow without disrupting mitosis or the contractile ring assembly (Limouze et al. 2004). It has been subsequently shown to be an uncompetitive inhibitor that binds to a hydrophobic pocket at the apex of the 50 kDa cleft, close to the γ-phosphate binding pocket (Allingham et al. 2005). Blebbistatin preferentially binds to the closed state (based on the position of switch II) in which ATP is hydrolyzed and results in the state Myosin•ADP•P_i (Kovacs et al. 2004). Binding to the actin binding cleft would prevent this cleft from closing. We have used blebbistatin because it induces a known structure in myosin, with kinetics that have been thoroughly analyzed and it stabilizes the structure of the thick filament (Xu et al. 2008); a structure known to be disordered by myosin phosphorylation (Levine et al. 1996). In addition Blebbistatin does not bind to the nucleotide site, as do the phosphate analogs used in previous studies. Currently, a number of research groups are utilizing blebbistatin because of its specificity for myosin and the fact that it has little effect on the rest of the contractile apparatus.

Reported here are measurements of certain parameters of the mechanics of permeabilized rabbit psoas muscle fibers with phosphorylated or dephosphorylated myosin in the presence or absence of blebbistatin at different concentrations of ATP and different temperatures. Myosin phosphorylation inhibits velocity in the presence of blebbistatin, and lowers the K_m for V_{max} at a range of ATP concentrations. For the first time an effect of phosphorylation has been found at lower temperatures. This last observation has been attributed to the ability of blebbistatin to stabilize the binding of myosin heads to the core of the thick filament.

Materials and methods

Chemicals were purchased from Sigma (USA) Chemical Co, USA, except (±) blebbistatin [(±)-phenyl-1, 2, 3,

4-tetrahydro-4-hydroxypyrrolo [2, 3-B]-7-methylquinolin-4-one] or (+) blebbistatin which were obtained from Toronto Research Chemicals, Inc., 2 Brisbane Road, North York, ON M3J 2J8 Canada. The racemic mixture was used as reports with regard to activity of each enantiomer have been mixed. Blebbistatin was dissolved in dimethylformamide (DMF) and stored at $-20°C$ before diluting with buffer shortly before use. Care was taken to limit its exposure to light as there is evidence that, exposed to certain wavelengths in the visible, it loses its effectiveness. Buffers were prepared as follows: relaxing buffer— 120 mM K-acetate, 5 mM $MgCl_2$, 1 mM EGTA, 5 mM K-phosphate, 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) and 4 mM ATP, unless otherwise stated, at pH 7.0, ionic strength = ~ 170 mM. Activating buffer was similarly prepared but with the addition of 1 mg/ml creatine kinase, 20 mM creatine phosphate and $CaCl_2$ added to pCa of 4.3, ionic strength ~ 190 mM. Lysis buffer: 26% glycerol, pH 4–6 ampholines, 30% Triton X 100, 8 M urea, 92 mM dithiothreitol (DTT). Pro-Q for staining isoelectric focusing gel was obtained from Molecular Probes, Eugene, OR.

Preparation of muscle fibers

Rabbits were sacrificed according to the protocols approved by the Institutional Animal Care and Use Committee. Muscle fibers were permeabilized by the method described in (Karatzaferi et al. 2007). Bundles of fibers were tied to supports, placed in glycerol solution (120 mM K-acetate, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM MOPS set at pH 7.0 and 50% glycerol (v/v)) and gently mixed overnight at 4°C. Bundles were then placed in fresh solution (120 mM K-acetate, 5 mM $MgCl_2$, 5 mM EGTA, 50 mM MOPS set at pH 7.0 and 50% glycerol (v/v)) pH 7.0 incubated at 4°C for 4 h before transferring to $-20°C$ for storage. Permeabilized muscle fibers can be stored for up to several months without loss of integrity.

### Myosin phosphorylation. Preparation of fibers

In most experiments phosphorylated myosin was prepared by the following method. Permeabilised rabbit psoas muscle fibers were incubated in glycerol solution as above that also contained phosphatase inhibitors and ATP (i.e. 5 mM ATP, 20 mM NaF and 20 mM K-phosphate, pH 7.0). The time course was approximately a week at 4°C with mixing.

In other experiments individual fibers with dephosphorylated myosin RLC were mounted onto the tensiometer and partially purified exogenous MLCK (see "Preparation of muscle fiber myosin light chain kinase" below) with calmodulin were added, and shortening velocities, tensions,

etc. recorded. Specifically muscle fibers were incubated with relaxed rigor buffer with ~ 300 μg/ml MLCK, 5 μg/ml calmodulin, 5 mM Pi, 2 mM $CaCl_2$, 4 mM ATPγS for 30 min at 22°C. Fibers were transferred to activating solution set at 5°C before measurement of shortening velocities at either 30 or 10°C as described below in "Measurement of the mechanics of muscle contraction".

Myosin phosphorylation. Isolectric focusing gel

Phosphorylation of muscle fiber RLC was determined using isoelectric focusing gels (pH 4.0–6.0) as described previously (Franks-Skiba et al. 2007). Gels were stained with Pro-Q, which stains more strongly (approximately 4 times per μg of light chain loaded) phosphorylated serines and threonines; it also stains dephosphorylated light chains of myosin, but to a much lesser extent (see Fig. 1).
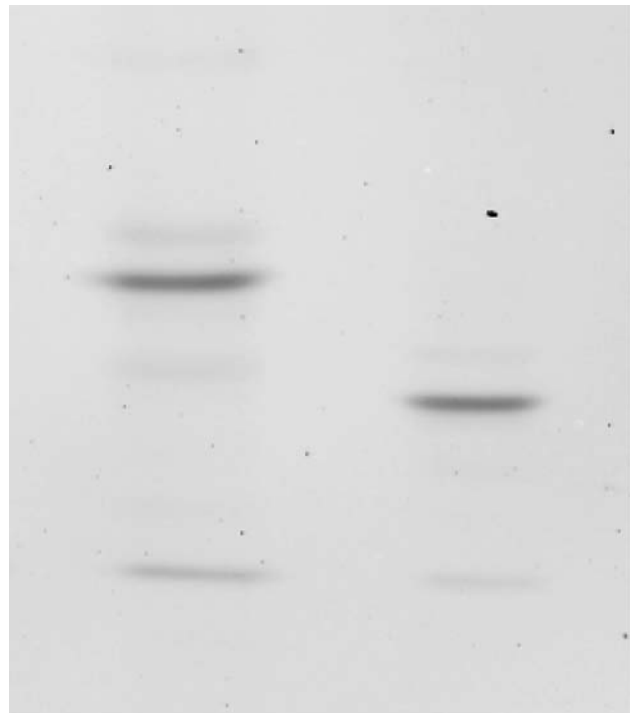
Fig. 1 Isoelectric focusing gel of two fiber preparations. Muscle fibers were phosphorylated in situ during preparation as described in Materials and Methods. Light chains were separated by an isoelectric gel, pH 4–6, and stained with Pro-Q. Pro-Q has greater specificity for phosphorylated serines and threonines but also stains the dephosphorylated RLC. Pro-Q stains phosphorylated RLC 4 times more strongly than dephosphorylated RLC. The *uppermost band* is dephosphorylated RLC the *second band* is phosphorylated RLC and the *lowest band* is light chain 3. *Lane* 1 shows fibers where RLC is almost entirely dephosphorylated and *lane* 2 shows fibers where RLC is almost entirely phosphorylated. The amount of protein loaded in *lane* 1 is greater than that in *lane* 2 in order to observe the dephosphorylated band

Preparation of myosin regulatory light chain kinase

Freshly excised muscle was washed in ice cold 2mMEDTA, extracted, minced, placed ice cold solution of 0.1 M KH_2PO_4, 0.05 M K_2HPO_4, 0.3 M KCl, set at pH 6.5 with stirring for 15 min, centrifuged ($10,000g$, 10 min, 4°C) and the precipitate resuspended and centrifuged again as before. Precipitate was dissolved in a solution of 0.5 M KCl, 20 mM K-phosphate, 12.5 mM $MgCl_2$ and 0.1 M $CaCl_2$, pH 8.0 at room temperature. ATP was added to a final concentration of 5 mM and the sample allowed to phosphorylate at room temperature for 30 min. Following a series of washes MLCK is separated from myosin by centrifugation. This preparation follows protocols provided by Danuta Szczesna-Cordary, personal communication.

Measurement of the mechanics of muscle contraction

Mechanical measurements were obtained using the temperature-jump method (Karatzaferi et al. 2007). Single fibers, ~0.5 cm in length by ~60–70 μm in diameter, or pairs, were mounted and fixed with vinyl adhesive between a solid state force transducer (Akers 801; Aksjelskapet Microelectronic, Hovtea, Norway) and an arm connected to a rapid motor (General Scanning, Inc., Watertown, MA, USA). The motor arm is adjusted to the point where the muscle fiber(s) are just no longer slack and then stretched 10%. Sarcomere lengths were periodically measured with a laser at wavelength 638.2 nm, and remained within acceptably intact limits of ~2.2–2.4. Typically fibers were first placed in cold (5°C) relaxing buffer, before being transferred to cold (5°C) activating buffer. Following a 2 min equilibration period fibers were periodically and rapidly transferred to and from a third solution containing activating buffer set at a temperature of 30 or 10°C according to the experiment and load clamps recorded. It has been found that measurements made during brief periods at the elevated temperature allows for a greater number of measurements to be made before fiber tension deteriorates beyond 10% of the original and some can be attributed to sarcomere heterogeneity. Transfers to 30°C solution and measurements recorded were ~1 s. Blebbistatin is light sensitive and precautions were taken to ensure that solutions containing blebbistatin had minimum exposure to light (Sakamoto et al. 2005). Shortening velocities were measured as described previously (Karatzaferi et al. 2007).

Force–velocity data from isotonic releases were analyzed by a least squares, nonlinear fit of the data to the Hill equation given below

$$V = V_{max} \cdot (\alpha/P_0)(1 - P/P_0)/(\alpha/P_0 + P/P_0)$$

where P is tension at velocity V, P_0 is isometric tension, V_{max} is the maximum velocity extrapolated to zero tension and α/P_0 is a variable related to the curvature of the relationship, the smaller this variable the more curved is the relationship. The fit, was performed using Kaleidagraph 3.6, which provides 95% confidence limits for the variables, which are given in the text. Force velocity curves were composed of 20–40 data points that were obtained from 5 to 10 different fibers.

Results

Force inhibition with blebbistatin

Blebbistatin decreases isometric tension in fibers to the same extent irrespective of the phosphorylation status of myosin, Fig. 2. Control fibers were incubated in medium containing equivalent volumes of DMF and showed no such decrease in tension. Figure 2 also shows that the concentration of blebbistatin necessary to produce a decrease of 80% in tension is ~15 μM. Subsequent experiments were conducted at 20 μM blebbistatin to ensure that at least 75% inhibition of force was maintained.
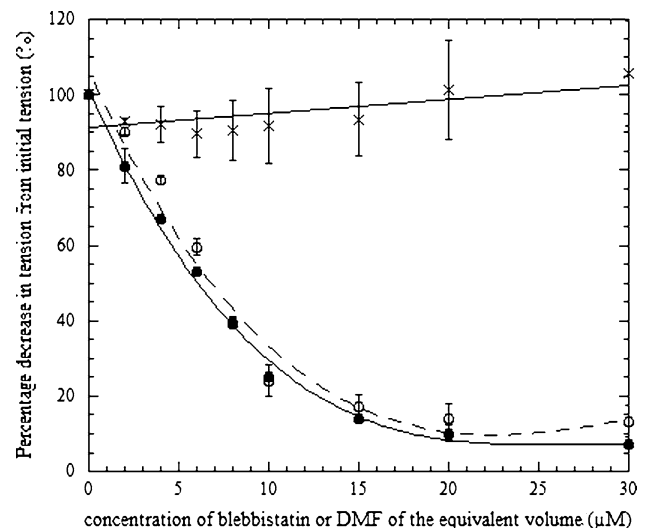
Fig. 2 Tension is shown as a function of the concentration of blebbistatin. Individual muscle fibers were mounted on to the tensiometer and placed in relaxing buffer plus the specified concentrations of blebbistatin and set at 5°C. Control fibers were incubated in the concentration of DMF equivalent to that used in the experiments with blebbistatin. Following activation fibers were then transferred to activating solution with/without blebbistatin or DMF set at 30°C for ~2 s and tensions recorded. *Empty circles* represent data for fibers with dephosphorylated RLC in the presence of blebbistatin, *filled circles* that for phosphorylated RLC in the presence of blebbistatin and X's represent fibers with phosphorylated RLC incubated in solutions with a volume of DMF added that was equivalent to the corresponding blebbistatin concentration. Data represent means ± SEMs for at least 3 fibers

Effect of myosin phosphorylation and the myosin inhibitor blebbistatin on shortening velocities at 30°C

Fiber shortening velocities were measured in the presence and absence of 20 μM blebbistatin. In the presence of 20 μM blebbistatin, maximal shortening velocities for fibers with phosphorylated RLC, 3.2 ± 0.34, were decreased by 47% compared with fibers with dephosphorylated RLC, 6.0 ± 0.30, see Figs. 3 and 4. The inhibition of shortening velocity by RLC phosphorylation is only seen in the presence of blebbistatin; in its absence phosphorylation had no effect on shortening velocity, see Fig. 5a, b. Blebbistatin has no effect on shortening velocities in fibers with dephosphorylated RLC even though forces were decreased by at least 75%. Shortening velocity inhibition by blebbistatin is only seen in phosphorylated fibers, shown in Fig. 5a. There was no systematic variation in the curvature correlated with myosin phosphorylation, in agreement with previous observations (Sweeney and Kushmerick 1985; Franks-Skiba et al. 2007; Karatzaferi et al. 2007). Similar differences in shortening velocities were also found using incubations that contained 10 μM blebbistatin.

Effect of the myosin inhibitor blebbistatin and myosin phosphorylation on shortening velocities in permeabilized rabbit psoas muscle fibers at 10°C

Our previous work had found that the inhibition of fiber shortening velocity by phosphorylation occurred only at higher temperatures and not at 10°C, a temperature where many studies are carried out due to greater fiber stability. Thus we repeated our studies of blebbistatin at the lower temperature. The data obtained at 10°C was qualitatively similar to that obtained at 30°C, see Fig. 6. Addition of 20 μM blebbistatin inhibited tension equally in phosphorylated and dephosphorylated fibers and to the same extent as it did at 30°C. In the absence of blebbistatin, phosphorylation had no effect on either V_{max} or α/Po, as observed previously under other conditions (Sweeney and Kushmerick 1985; Franks-Skiba et al. 2007; Karatzaferi et al. 2007). The addition of 20 μM blebbistatin did not affect the force velocity curve in dephosphorylated fibers but inhibited maximal shortening velocity for fibers with phosphorylated RLC by 57%, Fig. 6. This inhibition is a little greater than that seen at 30°C, 47%.

Effect of different isomers of blebbistatin

Reports of the stereospecificity of blebbistatin have been mixed. (+) Blebbistatin has been found to be inactive for ATPase in human platelet non muscle myosin II (Straight et al. 2003) whereas Kovacs et al. (Kovacs et al. 2004)
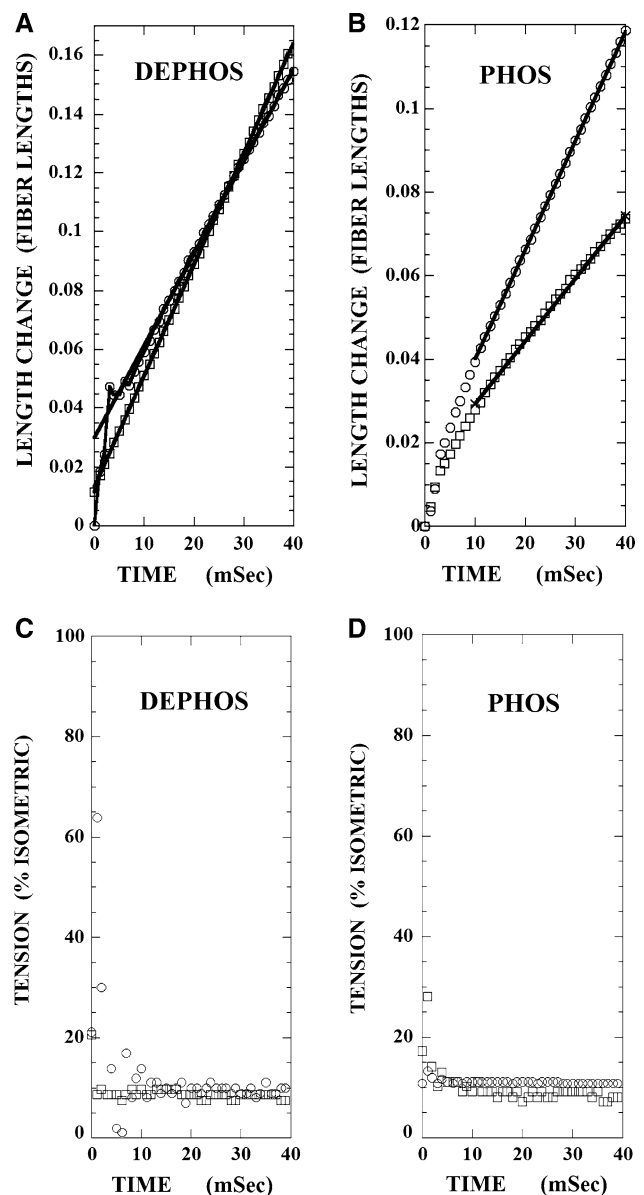

Fig. 3 Isotonic release data for rabbit psoas muscle fibers under the four conditions studied. **a** and **b** show the position of the motor arm to which the fibers were attached as a function of time during the clamp. **c** and **d** show release tensions as a function of time following initiation of a tension clamp to ~10% of isometric tension. *Open circles*, data obtained in the absence of blebbistatin, *open squares*, data obtained in the presence of blebbistatin. The slope of a least-squares fit to the position versus time data over the period 10–40 ms following release is also shown (*solid lines*) and was taken as the shortening velocity. Note that in the absence of blebbistatin there is a greater elastic component early in the position trace due to the greater tensions generated. Dephosphorylated fibers plus blebbistatin isotonic tension = 12% velocity = 3.15 lengths/s; in the absence of blebbistatin isotonic tension = 11% velocity = 3.36. Phosphorylated fibers plus blebbistatin isotonic tension = 10% velocity = 1.97 lengths/s; in the absence of blebbistatin isotonic tension = 11% velocity = 2.8 lengths/s

have found similar data for both enantiomers in rabbit skeletal muscle myosin. The effect of (+) blebbistatin on tensions was determined. Pairs of fibers were mounted on
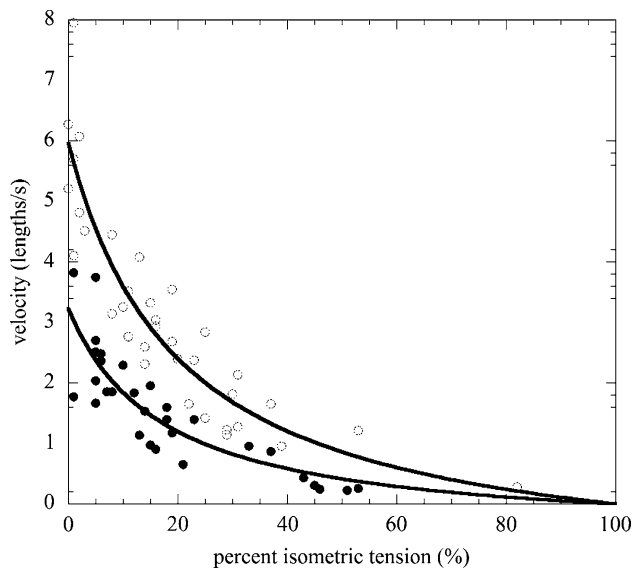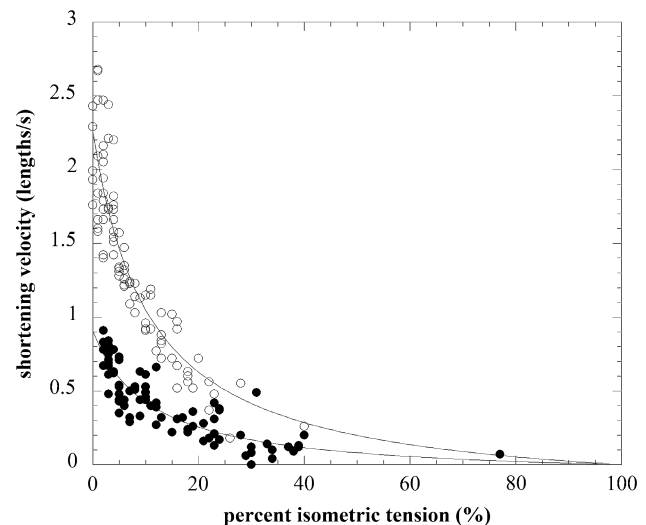
Fig. 4 The effect of myosin phosphorylation on shortening velocities of muscle fibers in the presence of 20 μM blebbistatin at 30°C. *Filled circles* represent data from fibers with phosphorylated RLC, V_{max} 3.2 ± 0.34, α/Po 0.2 ± 0.06; *empty circles* represent that from fibers with dephosphorylated RLC, V_{max} 6.0 ± 0.30, α/Po 0.2 ± 0.04. In this and following figures the *lines* show fits to the data using the Hill equation. The fit, was performed using Kaleidagraph 3.6, which provides 95% confidence limits for the variables. Force velocity curves were composed of 20–40 data points that were obtained from 5 to 10 different fibers
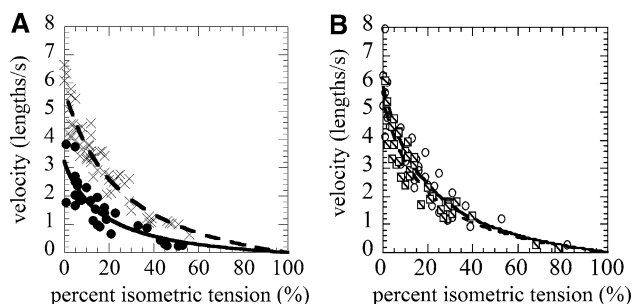
Fig. 5 The effect of 20 μM blebbistatin on shortening velocities of muscle fibers at 30°C with phosphorylated RLC (**a**) or dephosphorylated RLC (**b**). **a** *Filled circles* and *solid line* represent data from fibers with phosphorylated RLC incubated in the presence of 20 μM blebbistatin; V_{max} 3.2 ± 0.34, α/Po 0.17 ± 0.06; X's and *dashed line* represent that for phosphorylated RLC in the absence of blebbistatin; V_{max} 5.8 ± 0.18, α/Po 0.26 ± 0.03. **b** *Empty circles* and *solid line* represent dephosphorylated fibers in the presence of 20 μM blebbistatin; V_{max} 6.0 ± 0.30 α/Po 0.2 ± 0.03; and *crossed boxes* and *dashed line* show data in the absence of blebbistatin; V_{max} 5.4 ± 0.3, α/Po 0.2 ± 0.03. Data were fit using the Hill equation, *solid lines*, dephosphorylated RLC, *dashed lines* phosphorylated RLC

the tensiometer and placed in relaxing buffer set at 5°C, then transferred to active solution at 5°C for 2 min, lastly moved to activating solution set at 30°C for ∼2 s, forces measured and the fiber returned relaxing buffer. This procedure was repeated with the same fiber placed in 20 μM

Fig. 6 The effect of myosin phosphorylation on shortening velocities in the presence of 20 μM blebbistatin at 10°C. This is the same experiment as shown in Fig. 4 but with a lower temperature. *Filled circles* represent data for fibers with phosphorylated RLC; V_{max} 0.9 ± 0.06, α/Po 0.11 ± 0.02; *empty circles* that for dephosphorylated RLC, V_{max} 2.3 ± 0.2, α/Po 0.07 ± 0.02. In the absence of blebbistatin fiber shortening velocities were not affected by phosphorylation: phosphorylated RLC, V_{max} 2.12 ± 0.04 α/Po 0.10 ± 0.01; dephosphorylated RLC, V_{max} 2.26 ± 0.07, α/Po 0.11 ± 0.01. Data were fit using the Hill equation

(+) blebbistatin. On average a 29% decrease in tension was found which does not compare with a 80% decrease found with 20 μM (±) blebbistatin. Therefore, we find that the (+) isomer has partial activity.

Measurement of K_m [ATP]

Previous work has shown that in the presence of vanadate, RLC phosphorylation also lowers the K_m for V_{max} as a function of ATP. The maximum velocity of shortening is shown as a function of the ATP concentration for fibers inhibited by 20 μM blebbistatin, Fig. 7. In the presence of blebbistatin the fibers have a greater apparent affinity for ATP, shown by the lower value of K_m in phosphorylated fibers, $K_m = 50 ± 20$ μM, than in dephosphorylated fibers, $K_m = 330 ± 84$ μM. This effect is similar to that seen in vanadate (Franks-Skiba et al. 2007). In the absence of inhibitors phosphorylation has no effect on K_m (Franks-Skiba et al. 2007).

Effect of phosphorylation of individual fibers using purified MLCK

In our previous work we had achieved phosphorylated fibers by in situ phosphorylation during the period of storage in the rigor/glycerol solution. In the presence of ATP and the phosphatase inhibitors, phosphate and KF,
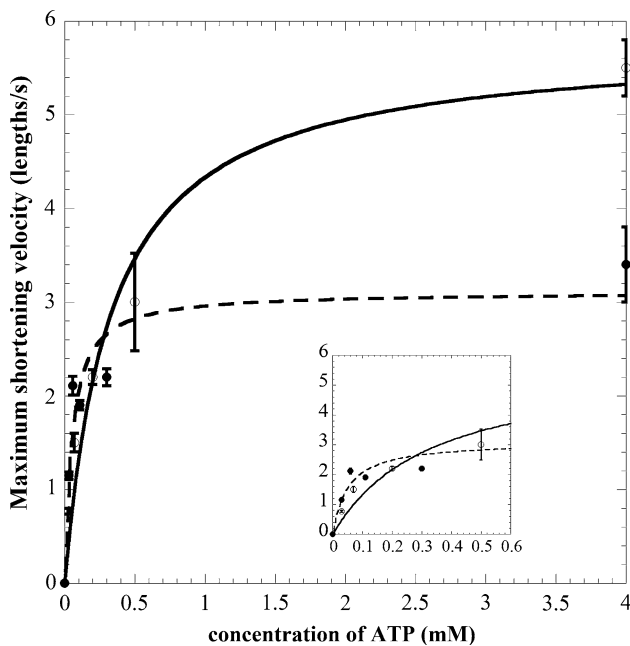
Fig. 7 The effect of myosin phosphorylation on maximal shortening velocity as a function of ATP concentration in the presence of 20 μM blebbistatin at 30°C: fibers with phosphorylated RLC, *filled circles* and fibers with dephosphorylated RLC, *empty circles*. Error bars on the points represent the standard error from 3 to 4 different force velocity curves. The maximum shortening velocity was determined by fitting force–velocity data using the Hill plot and extrapolated maximal shortening velocities were plotted here against [ATP]. A fit to the data define $K_m = 300$ μM ± 84 μM, $V_{max} = 5.7 \pm 0.5$ lengths/s for dephosphorylated fibers compared with $K_m = 50$ μM ± 20 μM, $V_{max} = 3.1 \pm 0.3$ lengths/s, for phosphorylated fibers. Inset shows more detail in the range of [ATP] 0–0.6 μM. Errors on the parameters represent 95% confidence limits determined by the fit to the data

Table 1 Force-velocity parameters of fibers phosphorylated by added myosin light chain kinase

Incubation[a]	Blebbistatin[b]	V_{max}^{c}	α/P_o	%[d]
−MLCK	0	5.3 ± 0.2	0.18 ± 0.02	0
−MLCK	20 μM	4.9 ± 0.4	0.26 ± 0.02	−8
+MLCK	0	4.6 ± 0.2	0.17 ± 0.02	−14
+MLCK	20 μM	3.5 ± 0.2	0.27 ± 0.06	−35

[a] Fibers were incubated in buffer containing 4mMATPγS, MLCK, calmodulin and 20μMblebbistatin, in rigor buffer at 22°C, for 30 min, see "Materials and methods" for details. The fiber was then transferred to activating solution for measurement of forces and shortening velocities

[b] Concentration of blebbistatin in measurement of force–velocity

[c] Following incubation, shortening velocities were measured at 30°C and data fitted to the Hill equation (Hill 1938), data represent 95% confidence limits on the fit to the data

[d] Percentage change from control, i.e. row 1 fibers with dephosphorylated myosin incubated without MLCK, calmodulin or blebbistatin

fibers became phosphorylated over about a week, while in the absence of these reagents they became dephosphorylated. As this procedure may have changed other properties of the fibers, it was important to show that the effects of RLC phosphorylation could be reproduced by direct phosphorylation of the RLC using MLCK.

Fibers were mounted on the tensiometer and incubated in a partially purified MCLK, calmodulin and ATPγS. The ATP analog, ATPγS, was used because it is a good substrate for MLCK but a poor substrate for activating tension in the fibers. In addition the thiophosphate attached to serine on the RLC is resistant to the phosphatase. Individual muscle fibers were mounted on the tensiometer and phosphorylated by addition of MLCK and calmodulin at 22°C for 30 min, as described in "Materials and methods". This temperature was chosen as higher temperatures resulted in destabilization of the fiber sarcomere lattice structure. Following incubation, shortening velocities were recorded at 30°C, using the temperature jumps as described above. The results presented in Table 1 show that RLC phosphorylated using this procedure produced the same

inhibition of shortening velocity in the presence of blebbistatin as found for the fibers phosphorylated during storage in glycerol. Figure 8 shows that the level of phosphorylation achieved using this procedure was high.

Discussion

Relation to previous work

This work is an extension of our efforts to understand the mechanism of the inhibition of shortening velocity during muscle fatigue, and in particular the role of myosin phosphorylation in this process. Our previous work has found that, in conditions where sizable populations of myosin heads in states not generating force, myosin phosphorylation inhibits velocity. These conditions have included the addition of vanadate, which is known to trap myosin in a pre-power stroke state, and a combination of higher phosphate (30 mM) and lower pH (6.2). However, not all phosphate analogs produce this effect, for example AlF_4 or BeF_3 at pH 7.0 have no such effect and it was not observed previously except at high temperatures, 30°C. We find no effect of myosin phosphorylation on either velocity or tension in the absence of inhibitors, in fibers in which all myosin heads are involved in active cycles with actin.

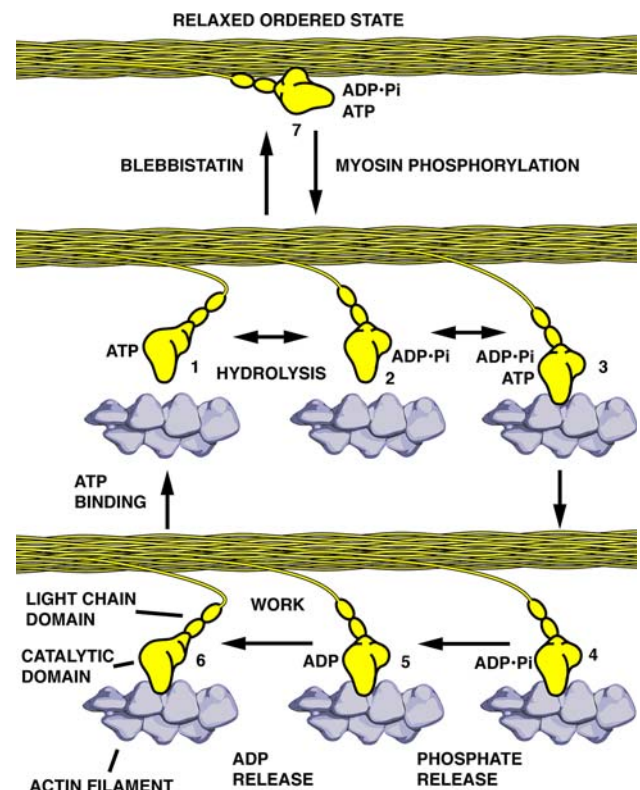### The mechanism of phosphorylation induced inhibition of velocity

The mechanism by which phosphorylation induces inhibition of shortening velocity can be explained by its effect on the array of myosin heads bound to the thick filament.
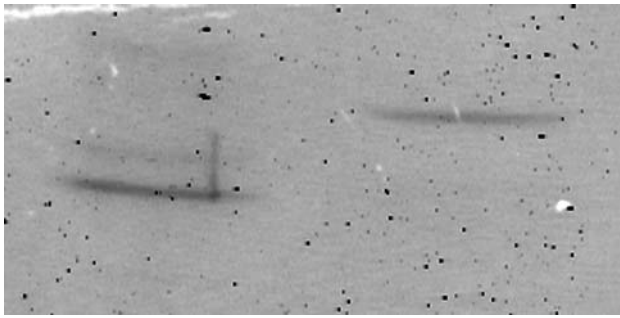
Fig. 8 Isoelectric focusing gel of muscle fibers following incubation with or without 4 mM ATPγS, exogenous MLCK and calmodulin in the presence of 20 μM blebbistatin, at 22°C, 30 min. Each *lane* represents protein from a single muscle fiber. The *left* lane is the result of the above incubation and shows that the myosin was indeed phosphorylated. The *right* lane is from a fiber incubated in precisely the same manner but without MLCK and calmodulin and shows no evidence of myosin phosphorylation

Fig. 9 The actomyosin cycle. State 1 represents myosin dissociated from actin following the binding of ATP. In state 2 ATP is hydrolyzed which alters the myosin structure to the pre-power stroke conformation. In state 3 myosin binds weakly to actin and does not generate force. Following isomerization to produce state 4, force is generated. In state 5 phosphate is released, actin forms a stronger bond with myosin and force is produced. Release of ADP brings the cycle to state 6 where the rigor bond is formed—the tight binding of actin and myosin. State 7 shows a myosin head bound to the thick filament. The presence of blebbistatin stabilizes this ordered state (Xu et al. 2008); whereas myosin phosphorylation disorders it (Levine et al. 1996)

When myosin heads bind to the thick filament they form an ordered array. It is the disruption of this order that is believed to be the mechanism of inhibition of shortening velocity. The array itself was first visualized in resting frog muscles by electron microscopy and X-ray diffraction (reviewed in (Huxley and Faruqi 1983) and has since been shown in a variety of muscle types (Levine 1993). The array is labile, and an example of disruption to the array can be found in mammalian muscles, where order is only seen at higher temperatures (Wray 1987; Xu et al. 2003). In normal physiological conditions such as that in relaxed fibers, and probably also in fibers with large populations of non-force generating heads, these heads can be sequestered by binding to the thick filament where they are sterically unable to reach and interact with actin filaments. This effect will only operate in fibers with dephosphorylated myosin. In contrast phosphorylation of myosin causes disruption of the ordered array, increasing the fraction of myosin heads in a disordered configuration in the space around the thick filament, see Fig. 9 (Levine et al. 1996), which means that these heads can more readily bind with actin in non-force generating states, State 3 in Fig. 9.

We have previously observed the close correlation induced between the release of myosin heads from the thick filament array and the inhibition of velocity. In normal conditions all myosin heads are undergoing active cycles, and few heads, if any, are bound to the core of the thick filament. Here myosin phosphorylation has no apparent effect on velocity. Under conditions where the cycle is inhibited by a reagent(s), we find that the velocity is inhibited along with the tension under conditions where the myosin heads are not bound to the core of the thick filament. These conditions include lower temperatures, or phosphorylated fibers, both of which disrupt the array. If the array is stable then velocity does not tend to be

inhibited as for dephosphorylated fibers in vanadate. Vanadate stabilizes the array to a greater extent than BeF_3 (Wray 1987; Xu et al. 2003), and shortening velocities of dephosphorylated fibers are inhibited in BeF_3, but not in vanadate. At lower temperatures, the array is unstable, and all of the phosphate analogs inhibit both tension and velocity. We also observed that in dephosphorylated fibers inhibited by vanadate, there was an abrupt change in velocity, which was inhibited below 22°C but not above (Pate et al. 1994). Such an abrupt change suggests involvement of a highly cooperative structure, and the thick filament provides a structure capable of such a degree of cooperativity.

A major difference between the new observations and the previous ones is that dephosphorylated fibers inhibited by blebbistatin have fast shortening velocities, equal to control fibers, and that myosin phosphorylation has the same ability to inhibit velocity at 10°C as it does at 30°C.

The observations that dephosphorylated fibers inhibited at 10°C by blebbistatin, are fast, but that phosphorylated fibers are slow, strongly support the stability of the ordered thick filament array as playing a role in the inhibition of velocity. Thus the inhibition of velocity by phosphorylation can be connected to disruption of this array, which is its only established structural effect.

The hypothesis discussed above suggests that velocity is inhibited when there are large populations of myosin heads in non force states that are capable of interacting with the thin filament. The inhibition of velocity depends on the properties of the non-cycling heads. Why would the presence of these non-force generating myosin heads interacting with the thin filament inhibit velocity? One hypothesis is that they bind transiently to the filament and exert a drag. Inhibition of sliding filament has been found with such binding (Cuda et al. 1997).

However, the observations of changes in K_m complicate the above explanation. The value of K_m is not affected by phosphorylation, in fully activated fibers in the absence of some inhibitory agent such as blebbistatin, or vanadate. In the presence of two of these inhibitors, vanadate and now blebbistatin, phosphorylated fibers have a much lower value of K_m, indicating apparent stronger binding of ATP, and possibly other nucleotides as well. Thus the effect of phosphorylation on K_m requires the presence of myosin heads, presumably bound weakly to actin in non-force states. Such heads could be on the same myosin molecule, or on adjacent ones. Other possibilities include direct interaction of the regulatory light chain with the active site, affecting binding of nucleotides. Communication between the active site and a phosphorylated serine on the regulatory light chain has been observed previously (Mazhari et al. 2004). However this mechanism does not explain the lack of an effect of phosphorylation on fiber velocity in the absence of a large population of non-force cross-bridges. A preliminary report, which described the inhibition of velocity by myosin phosphorylation using an in vitro assay, in which actin filaments move over a surface coated with myosin, also suggests that there is some direct effect of myosin phosphorylation on the binding of nucleotides (Greenberg et al. 2008). There was no thick filament structure in these assays, however there may have been a population of myosin heads that could bind weakly to actin but were sterically unable to execute power strokes.

Each of the two hypotheses discussed above have data that supports them. At this point there are no data that rule out either hypothesis. It is also possible that the velocities are influenced by some completely different mechanism involving non-crossbridge elements such as titin or C-protein, however there are no data that support such mechanisms.

Properties of the myosin-blebbistatin complex

Blebbistatin binds to a cleft, which begins at the nucleotide binding site and ends at the actin binding site. The cleft divides the molecule into two regions known as the upper 50 kDa and lower 50 kDa domains (Allingham et al. 2005). This cleft must close before myosin can form a strong bond with actin and blebbistatin is thought to bind in such a way as to prevent closure. Blebbistatin binds to the myosin.ADP.Pi complex with high affinity and interferes with phosphate release; the step that is generally accepted as involved in the transition to the force production steps. Blebbistatin arrests the myosin cycle in states that are either detached from actin, or bind only weakly to it. (Kovacs et al. 2004). Thus with blebbistatin we are investigating the mechanical behavior of a myosin state that is well defined structurally. The state that is populated in the presence of blebbistatin is an analog of a state that is also highly populated during fatigue.

Of particular importance for our studies is the observation that Blebbistatin stabilizes the binding of myosin heads to the core of the thick filament (Xu et al. 2008). Thus our observation that shortening velocities are not inhibited by blebbistatin in dephosphorylated fibers, but inhibited in phosphorylated fibers, at both 10 and 30°C, provides additional evidence that the array is involved in the mechanism of inhibition as discussed above.

Partial inhibition of fibers by reagents such as vanadate or blebbistatin results in two populations of myosin heads. Because the binding and release of the inhibitors is slow compared to the cycle time, a fiber whose tension has been inhibited by 80%, has ∼20% of its heads undergoing active cycles, and 80% in non-force states, with slow exchange between the two populations. Because myosin phosphorylation does not appear to inhibit the cycle of the normally cycling cross bridges in the uninhibited fibers, the different effects of phosphorylation seen with different inhibitors must be due to the interactions between the cycling heads and the non-cycling heads, as only the non-cycling heads are different.

Summary

The inhibition of shortening velocities in fatigue in skeletal muscle has not been completely explained by known mechanisms. Alterations in concentrations of metabolites account for some but not all of this inhibition and evidence from this laboratory and others has clearly demonstrated that myosin phosphorylation plays an important role.

The recently discovered myosin inhibitor blebbistatin, like that of other such inhibitors, e.g. vanadate, have revealed important information regarding the role of myosin phosphorylation in the inhibition of shortening

velocities in skeletal muscle. The mechanism of action for blebbistatin in myosin, is now well established and its specificity makes it a unique and a very effective pharmacological tool. It populates a state, Myosin•ADP•P$_i$, which mimics a state populated during fatigue. In conditions where this non-force generating state is populated, myosin phosphorylation significantly inhibits shortening velocities. In the absence of an elevated population of non-force states, myosin phosphorylation has no effect on velocity. Thus our results provide strong support for the hypothesis that myosin phosphorylation is involved in the inhibition of shortening velocities during fatigue in vivo.

One mechanism proposed to explain the results presented here is that phosphorylation of myosin prevents the binding of myosin heads to the core of the thick filament, forming an ordered array (Levine et al. 1996). The inability of myosin heads to lie in this configuration means that heads are more able to interact with actin thus generating a population of heads in non-force generating states. Conversely dephosphorylated myosin heads are sequestered and are sterically unable to reach and interact with actin filaments. The data, however, do not rule out the possibility that myosin phosphorylation inhibits velocity by directly affecting the binding of nucleotides.

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