Cross-Bridge Binding to Actin and Force Generation in Skinned Fibers of the Rabbit Psoas Muscle in the Presence of Antibody Fragments Against the N-Terminus of Actin

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ABSTRACT To assess the significance of the NH_2-terminus of actin for cross-bridge action in muscle, skinned fibers of rabbit psoas muscle were equilibrated with Fab fragments of antibodies directed against the first seven N-terminal residues of actin. With the antibody fragment, active force is more inhibited than relaxed fiber stiffness, or stiffness in rigor or in the presence of magnesium pyrophosphate. Inhibition of stiffness in rigor or with magnesium pyrophosphate does not necessarily indicate involvement of the NH_2-terminus of actin in strong cross-bridge binding to actin but may simply result from the large size of the Fab. At high Fab concentrations, active force is essentially abolished, whereas stiffness is still detectible under all conditions. Thus, complete inhibition of active force apparently is not due to interference with cross-bridge binding to actin but may result from the Fab-mimicking inhibition of the thin filament by Troponin-I binding to the NH_2-terminus of actin at low Ca^{2+}. However, although Troponin-I is released from the NH_2-terminus at high Ca^{2+}, the Fab is not, thus disallowing force generation upon increase in Ca^{2+}. These data are consistent with involvement of the NH_2-terminus of actin in both weak cross-bridge binding to actin and Ca^{2+} regulation of the thin filament.

INTRODUCTION

It is believed that during muscle contraction myosin heads cycle between two main groups of states as ATP is hydrolyzed. The states of the first group are characterized by the low affinity of myosin heads for actin and their limited ability to activate thin filaments. They are commonly termed the "weakly binding" cross-bridge states. Myosin cross-bridges in the second group of states, commonly termed the "strongly binding" states, have much higher actin affinity under comparable experimental conditions and, in contrast to the weakly binding states, can activate thin filaments. It has been hypothesized that the transition from the weakly bound to the strongly bound cross-bridge configuration is a major step in force generation in muscle (Eisenberg and Greene, 1980; Eisenberg and Hill, 1985; Brenner, 1990; Brenner et al., 1995; Kraft et al., 1995a).

Several lines of experimental evidence indicate structural differences between the weakly and strongly bound actomyosin complexes (Craig et al., 1985; Applegate and Flicker, 1987; Yu and Brenner, 1989; Barnett and Thomas, 1989; Duong and Reisler, 1989; Brenner and Yu, 1993a; Berger and Thomas, 1994; Thomas et al., 1995; Walker et al., 1994, 1995; however, also see Pollard et al., 1993), suggesting that different sites on actin and myosin may be involved in the two types of interaction. This idea is incorporated into the structural model of actomyosin complexes (Rayment et al., 1993), which predicts mainly electrostatic interactions between myosin and actin in the weakly bound states (in the presence of ATP), and the combined electrostatic and hydrophobic interactions for the strongly bound complexes. On the basis of biochemical data (Reisler, 1993) and structural considerations, the electrostatic interactions are proposed to involve primarily the basic residues in loop 626–647 on the myosin head and the cluster of acidic residues in the N-terminal segment of actin and its 21–29 and 92–103 loops (Rayment et al., 1993).

The binding of the first 10 N-terminal residues of actin to myosin was originally documented and analyzed in chemical cross-linking studies (Mornet et al., 1981; Sutoh, 1991; Yamamoto, 1991). From more recent work it seems that this binding is particularly important in the presence of ATP (Chaussepied and Morales, 1989; DasGupta and Reisler, 1989; Bertrand et al., 1989) in the weakly bound actomyosin states. However, not only the myosin head but also the regulatory proteins Troponin-I (TnI) (Grabarek and Gergely, 1987; Levine et al., 1988) and caldesmon (Patchell et al., 1989; Bartegi et al., 1990; Levine et al., 1990; Adams et al., 1990) were proposed to interact with the N-terminal residues of actin.

Further clarification of these binding interactions was obtained from the work with N-terminal actin mutants (Sutoh et al., 1991; Cook et al., 1993; Crosbie et al., 1994). These studies revealed that the electrostatic interactions of myosin and caldesmon with the N-terminus of actin are functionally important even if they contribute little to the overall binding of these proteins. Similar conclusions were also reached from previous experiments with peptide antibodies. It was shown in these studies that Fab(1–7), antibody fragments against the first seven N-terminal residues on actin, inhibited the binding of myosin subfragment-1 (S-1) to actin in both the presence and absence of nucleotides (DasGupta and Reisler, 1991, 1992). In the presence of ATP, acto-S-1 ATPase was inhibited by Fab(1–7) to a

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larger extent than expected from the inhibition of S-1 binding to actin. It was concluded that Fab(1–7) could inhibit both the binding of S-1 to actin in the presence of ATP and a catalytic step in the ATP hydrolysis cycle (DasGupta and Reisler, 1992).

In this study, we examined the effect of Fab(1–7) antibody fragments on cross-bridge binding to actin and force generation in skinned rabbit psoas muscle fibers. A preliminary account of this work was presented earlier (Brenner et al., 1993).

MATERIALS AND METHODS

Fiber preparation

Skinned fibers of rabbit psoas muscle were isolated and chemically skinned with Triton X-100 as previously described (Brenner, 1983; Brenner and Yu, 1993b; Yu and Brenner, 1989) but with the recent modification of adding several protease inhibitors (for details see Kraft et al., 1995a). Single fibers were isolated as previously described (Yu and Brenner, 1989) but within a few hours after dissection of fiber bundles from the rabbit. Isolated single fibers were kept for up to 5 days without any detectable loss of sarcomeric proteins but with the advantage of much improved diffusion of substrate and products.

Solutions

All solutions were adjusted to pH 7.0 at the experimental temperature (5°C). Chemicals were obtained from Sigma Chemie (München, Germany), except when noted otherwise. Skinning solution contained (in mM): 5.0 KH_2PO_4; 3.0 magnesium acetate; 5.0 EGTA; 1.0 Na_2ATP (Merck, St. Louis, MO); 50 sodium creatine phosphate; 5.0 NaN_3; 10 glutathione; 2.0 DTT, 0.1 AEBSF (Calbiochem, La Jolla, CA); 0.01 each of leupeptin, antipain, E64, and pepstatin; and 1 μg/ml aprotinin (cf. Kraft et al., 1995a). Standard experimental solutions contained (in mM): 10 imidazole, 2.0 $MgCl_2$, 1.0 MgATP, and 1.0–3.0 EGTA (or CaEGTA). Preactivation and activation solutions contained an additional 10 mM caffeine and 500 U/ml (Sigma units) of creatine kinase; ionic strength was adjusted by adding the appropriate amount of sodium creatine phosphate, assuming a contribution to ionic strength of 3 mM/mM of sodium creatine phosphate. Rigor solution contained (in mM): 10 imidazole, 2.5 EGTA, and 2.5 EDTA. Magnesium pyrophosphate ($MgPP_i$) solution contained (in mM): 10 imidazole, 4.0 $MgPP_i$, 2.0 $MgCl_2$, 1.0 CaEGTA, and 0.2 Ap_5A. MgATPγS-solution contained (in mM): 10 imidazole, 10 MgATPγS, 2.0 $MgCl_2$, 3.0 EGTA, 200 glucose, 0.5 U/ml hexokinase, and 0.2 Ap_5A. ATPγS was purified as previously described (Kraft et al., 1992). For all solutions except preactivating and activating solution, ionic strength was adjusted by adding appropriate amounts of potassium propionate.

Fab fragments

Affinity purified Fab(1–7) antibody fragments were prepared as described previously (Miller et al., 1987). The rhodamine labeling of the antibody fragment was carried out in 0.1 $MNaCO_3$, pH 9.0, at 50:1 molar ratio of tetramethyl rhodamineisothiocyanate (TRITC) to Fab(1–7) (1.0 mg/ml). The reaction was allowed to proceed for 8 h in the dark at 4°C. The labeled Fab was separated from excess TRITC on a Sephadex G-50 spin column and by subsequent dialysis versus PBS. The labeling stoichiometry (0.2 TRITC/Fab) was determined spectrophotometrically.

Mechanical apparatus and x-ray diffraction

Equipment for recording of force, fiber stiffness, and equatorial x-ray diffraction patterns was described in detail elsewhere (Brenner, 1980; Brenner et al., 1984; Brenner and Eisenberg, 1986).

Stiffness measurements

Fiber stiffness was measured by imposing ramp-shaped stretches on the skinned fibers. Speed of stretch was between 10^3-10^4 (nm/half-sarcomere)s^{-1}, unless stated otherwise. Stiffness (chord stiffness) was defined as the ratio of force increment over that length increment when amplitude of stretch had reached 2 nm/half-sarcomere.

Confocal microscopy

Equilibration of skinned fibers with rhodamine-labeled Fab (1–7) was followed by confocal microscopy to characterize the labeling pattern within the sarcomere and to estimate the time required for reaching equilibrium of the antibody across the fiber.

The system for confocal microscopy was described previously (Kraft et al., 1995b) and consisted of a fluorescence microscope (Axiophot, Zeiss, Oberkochen, Germany), a confocal scanner (MRC-600, Biorad, Zurich, Switzerland) and a work station (Silicon Graphics Personal Iris 4D/25). The system was equipped with an argon/krypton mixed gas laser and allowed simultaneous recording of both rhodamine and fluorescein signals. The images were recorded using a Zeiss Planapochromat 40×, N.A. 1.0, oil immersion objective. Image processing was carried out on the Silicon Graphics work station using "Imaris" (Messerli et al., 1993), a 3D-multichannel image-processing software specialized for confocal microscopic images (Bitplane AG, Technopark, Zürich, Switzerland).

To follow equilibration by confocal microscopy, fibers were mounted in a shallow slot-shaped chamber built on a microscope slide and closed with a coverslip (cf. Kraft et al., 1995b).

Equilibration of skinned muscle fibers with Fab(1–7)

Skinned fibers were equilibrated with Fab(1–7) in a relaxing solution. The ionic strength of the relaxing solution was set at 50 mM so that potential effects of Fab(1–7) on relaxed fiber stiffness could be monitored continuously. The incubation medium was composed of standard relaxing buffer to which 25 mM glutathione and protease inhibitors were added as described for the skinning solution. Without these additional components, mechanical stability of the fibers decayed, presumably because of protease activity contaminating the Fab(1–7) preparation, such that after several hours of incubation, fibers could no longer sustain transfer through an air-solution interface.

RESULTS

Time course of inhibition of active force and relaxed fiber stiffness

To examine the equilibration of skinned rabbit psoas fibers with Fab(1–7) molecules, we first followed the effect of Fab(1–7) on relaxed fiber stiffness and active force during several hours of incubation. A representative example of such measurements is shown in Fig. 1. After between 8 and 10 h of incubation, the active force, both at high (170 mM) and low (50 mM) ionic strength, was essentially fully abolished, whereas relaxed fiber stiffness was reduced by only ~40–50%. The inhibition of relaxed fiber stiffness was also virtually unchanged by ionic strength conditions (between 50 and 170 mM), suggesting that the effect of Fab(1–7) was not significantly altered when actin affinity of weakly bound cross-bridges was increased by lowering the ionic strength.

When protease inhibitors were not included in the incubation medium, relaxed fiber stiffness continued to decrease

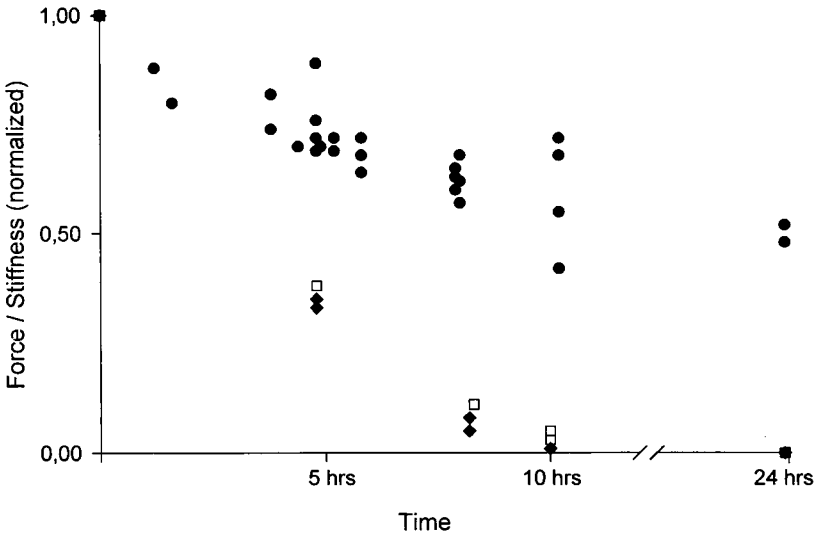

FIGURE 1 Inhibition of active force and relaxed fiber stiffness by 0.05 mg/ml Fab(1–7). Time course of inhibition of relaxed fiber stiffness at μ = 50 mM (●) and of active force at μ = 50 mM (□) and 170 mM (◆). Note that active force is almost completely inhibited (<2–3%), whereas relaxed fiber stiffness is reduced to only ~50–60%.

with time of incubation to values lower than those shown in Fig. 1. This decrease, however, was accompanied by a decay in fiber stability such that they eventually could not sustain the transfer through an air-solution interface. In the presence of protease inhibitors, relaxed fiber stiffness reached a steady level after between 10 and 12 h, suggesting that ~12 h of incubation were required for the equilibration of single skinned fibers with Fab(1–7).

To probe whether the more pronounced inhibition of active force could result from a decrease in Ca^{2+}-sensitivity of the contractile system in the presence of Fab(1–7), active force measurements were also carried out at increased Ca^{2+}-concentrations. An increase of free Ca^{2+}-concentration, at least up to 2 mM, did not result in a detectable recovery of active force (data not shown).

Equilibration of muscle fibers with Fab(1–7) and resulting labeling pattern within the sarcomere

Time course

To better establish the time course of equilibration of skinned muscle fibers with Fab(1–7), and to identify the structural element(s) that bind the antibody fragment, the equilibration of skinned muscle fibers with rhodamine-labeled Fab(1–7) (0.05 mg/ml) was followed by confocal microscopy. As shown in Fig. 2, after ~12 h of incubation the fluorescence reached a uniform intensity all the way to the core of the fiber. In contrast to that, only superficial labeling can be seen after 40 min of incubation with Fab(1–7). Fig. 2, *B* and *C*, shows homogenous fluorescence within each sarcomere, except for the H-zone in which fluorescence intensity remains low. This pattern is consistent with homogenous and selective labeling along the whole length of actin filaments.

Concentration dependence

After 12 h of incubation at 0.05 mg/ml Fab(1–7), the relaxed fiber stiffness was inhibited by 42 ± 15% (mean ± SD, n = 6). At this antibody concentration the active force was essentially abolished, at both high and low ionic strength (Fig. 1). At higher concentrations of Fab(1–7), the relaxed fiber stiffness was inhibited further to ~25% of the original value. However, inhibition of relaxed fiber stiffness may not have reached saturation even at the highest Fab(1–7) concentration (0.5 mg/ml) studied.

Structural examination of fibers incubated with Fab(1–7)

We previously observed massive structural disorder (e.g., due to actin filament bundling) that resulted in complete

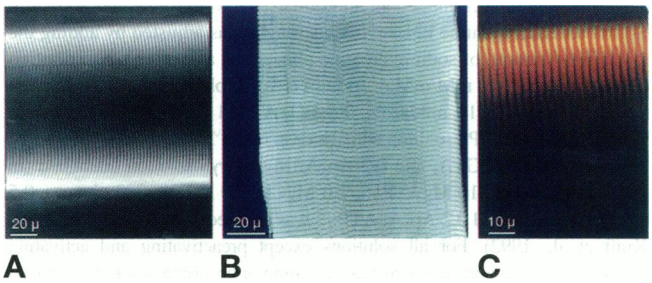

FIGURE 2 Equilibration of skinned fibers with Rhodamine-labeled Fab(1–7). The incubations of fibers with 0.05 mg/ml rhodamine-labeled Fab(1–7) were carried out in relaxing solution (μ = 50 mM) for 40 min (*A*) and 13 h (*B*). Note that in *B* the homogeneously labeled areas of the sarcomeres correspond to actin filaments; i.e., the nonfluorescently labeled gaps represent the H-zone. This is more clearly identified in *C* where the sarcomeric M-line was labeled with an antibody directed against the M-line-specific protein myomesin (Eppenberger et al., 1981; Grove et al., 1984). This antibody, in turn, was stained with a secondary FITC-labeled rabbit anti-goat antibody (Cappel Dynatech, Zürich, Switzerland). Before incubation with the rhodamine-labeled Fab(1–7), the fiber was preincubated for 4 h with both the antimyomesin antibody (0.02 mg/ml) and the FITC-labeled rabbit anti-goat antibody (diluted 1:100). Even after 4 h of incubation no equilibration was reached for staining the M-line-specific protein myomesin, i.e., only the superficial layers of the fiber were stained. Approximately 30 min after addition of 0.05 mg/ml rhodamine-labeled Fab(1–7) to the incubation medium (relaxing solution, μ = 50 mM), both rhodamine and fluorescein signals were recorded simultaneously. The core region of the fiber is near the bottom edge of the figure.

loss of even the strongest equatorial reflections *1,1,* and *1,0,* when single fibers were equilibrated with some S-1-derived peptides at concentrations (micromolar) that affected active force and relaxed fiber stiffness (Brenner, unpublished data). To rule out the possibility that such massive structural disorder was the main cause for the observed inhibition of active force and relaxed fiber stiffness in the presence of Fab(1–7), equatorial diffraction patterns of fibers were recorded in the presence and absence of antibodies (Fig. 3).

We found a decrease in the intensity of the *1.0* reflection by ~18%, associated with a corresponding increase in the *1.1* reflection (~22%) upon addition of Fab(1–7). This is consistent with binding of proteins or protein fragments to actin with some inhibition of cross-bridge attachment. The increase in background counts by 12% reflects an increase in diffuse scatter within the fibers (nonmuscle background is subtracted), which is possibly due to the presence of additional mass in the beam (e.g., presence of the Fab) or to some small amount of structural disorder or both. Because the total integrated intensities within the reflections is also increased (by 8%), the main factor seems to be increase in mass rather than structural disorder. Thus, the diffraction patterns shown in Fig. 3 rule out that the nearly complete loss of active force results from a massive structural disorder in the fibers incubated with Fab(1–7).

Inhibition of fiber stiffness by Fab(1–7) in rigor and in the presence of $MgPP_i$

Several measurements were carried out to test whether the complete inhibition of active force by Fab, in contrast to a smaller loss of relaxed fiber stiffness, was due to greater inhibition of strong than of weak cross-bridge interactions with actin. In these experiments, the effects of Fab(1–7) on active force and fiber stiffness under relaxing and strong binding (rigor or in the presence of 4 mM $MgPP_i$) conditions were compared. Fig. 4 shows that Fab(1–7) apparently is able to inhibit fiber stiffness in rigor or in the presence of MgPPi to a larger extent than in relaxed fibers (*open columns*). In the presence of Fab, however, fiber stiffness in rigor and in the presence of MgPPi was still higher than in relaxed fibers under the same ionic conditions (*shaded columns*). Thus, the difference in the inhibition of active force versus relaxed fiber stiffness may be due in part to additional interference of Fab(1–7) with the transition to and/or with strong cross-bridge interactions.

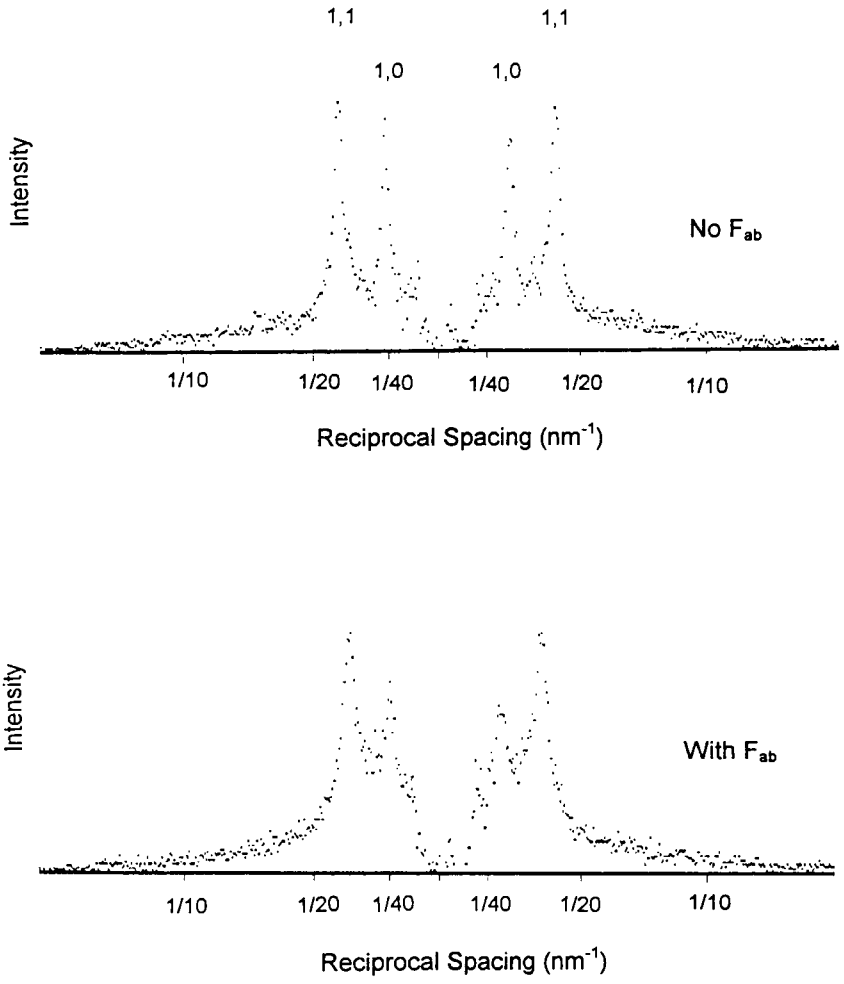

FIGURE 3 Equatorial diffraction patterns of relaxed fibers (μ = 50 mM) in the presence and absence of Fab(1–7). The fibers were incubated for 12 h in relaxing solution (μ50 mM) with 0.1 mg/ml Fab before recording diffraction patterns in the presence of Fab(1–7).

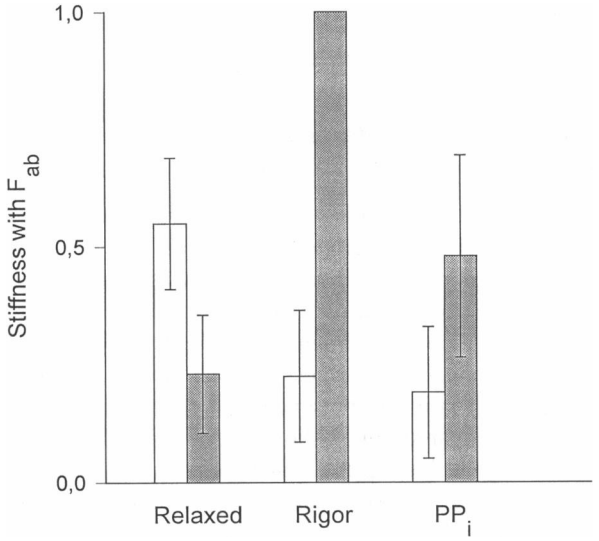

FIGURE 4 Inhibition of relaxed fiber stiffness (μ = 50 mM), stiffness in rigor (μ = 170 mM), and stiffness in the presence of 4 mM MgPPi (μ = 170 mM). Stiffness values in the presence of Fab were normalized for each condition to the stiffness in the absence of Fab(1–7) (*open columns*) or to the rigor stiffness in presence of Fab (*shaded columns*). The concentration of Fab was 0.05 mg/ml.

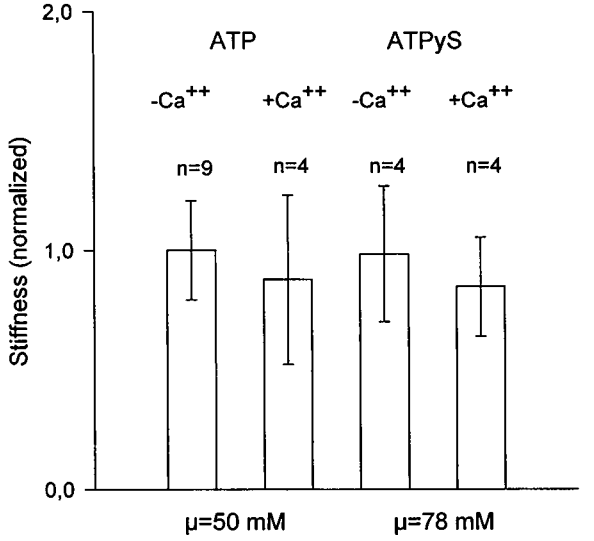

FIGURE 5 Effect of Ca^{2+} on the inhibition of fiber stiffness by Fab(1–7) in the presence of MgATP (1.0 mM) or MgATPγS (10 mM) (cf. Kraft et al., 1992). Speed of stretch was $\sim 10^4$ (nm/half-sarcomere)s $-$ 1. Note that at this speed of stretch stiffness in the presence of Ca^{2+}, on average, is comparable with that at the low Ca^{2+} concentration. The Fab(1–7) concentration was 0.05 mg/ml.

Effect of Ca^{2+} on the inhibition of cross-bridge binding to actin by Fab(1–7)

Another possible reason for the much more effective inhibition of active force compared with relaxed fiber stiffness could be a more potent inhibition of weak cross-bridge binding to actin by Fab(1–7) in the presence of Ca^{2+}, similar to our observations with caldesmon and its actin-binding fragments (Kraft et al., 1995a). To address this possibility, under conditions at which active force was essentially abolished, we examined 1) the inhibition of fiber stiffness by Fab(1–7) in the presence of ATP and ATPγS at high (pCa 4.5) and low Ca^{2+} (pCa > 8) and 2) the effect of Ca^{2+} on fiber equatorial diffraction patterns. We used ATPγS as a slowly hydrolyzed ATP-analog (Goody and Eckstein, 1971; Bagshaw et al., 1973; Dantzig et al., 1988; Kraft et al., 1992) that allows accumulation of cross-bridges in weakly binding states even in the presence of Ca^{2+}. Thus, even if some active turnover were still possible in the presence of the Fab, with ATPγS both at high and low Ca^{2+} essentially all cross-bridges accumulate in weak binding states. If the complete inhibition of active force by Fab (1–7) were related to a greater Fab effect on weak cross-bridge binding to actin in the presence of Ca^{2+} then 1) fiber stiffness observed with ATP or ATPγS should be much smaller in the presence of Ca^{2+} than in its absence (relaxing conditions), and 2) equatorial diffraction patterns recorded under the same conditions should show much less cross-bridge attachment in the presence of Ca^{2+}.

Fig. 5 shows that fiber stiffness is not significantly more inhibited at high Ca^{2+} than at low Ca^{2+} in either the presence of MgATP or of MgATPγS. Also (not shown), Ca^{2+} has no detectable effect on equatorial x-ray diffraction patterns of fibers incubated with Fab(1–7) at both 50 and 170 mM ionic strength. Thus, both fiber stiffness and equatorial diffraction patterns indicate that the inhibition of weak cross-bridge binding to actin by Fab(1–7) is not significantly changed by the presence of Ca^{2+}.

Effect of Ca^{2+} on stiffness-speed profiles recorded in the presence of MgATPγS or MgATP

We recently demonstrated (Kraft et al., 1992) that 1) Ca^{2+} has a characteristic effect on stiffness speed of stretch profiles in the presence of ATPγS and that 2) this effect was apparent even when the [ATPγS] was raised to counter its lower affinity for myosin in the presence of Ca^{2+}. At high Ca^{2+}, the stiffness-speed profiles were shifted by almost two orders of magnitude to slower stretch velocities. Because actin affinity of cross-bridges in the presence of ATPγS was affected by Ca^{2+} only marginally, our data suggests that actin binding kinetics of ATPγS cross-bridges were slowed by ~20- to 100-fold at high Ca^{2+} (Kraft et al., 1992).

The more effective inhibition of active force generation by Fab(1–7) compared with inhibition of weak cross-bridge attachment to actin could be due to the interference of Fab(1–7) with the Ca^{2+}-activation of fibers. This raises the question of whether the Ca^{2+}-effect on actin binding kinetics of ATPγS cross-bridges is also abolished when active force is completely inhibited by Fab(1–7). As shown in Fig. 6 *A*, when force is fully inhibited by Fab(1–7), Ca^{2+} still has a similar effect on the stiffness-speed relationship in the presence of 10 mM MgATPγS, as observed by Kraft et al.

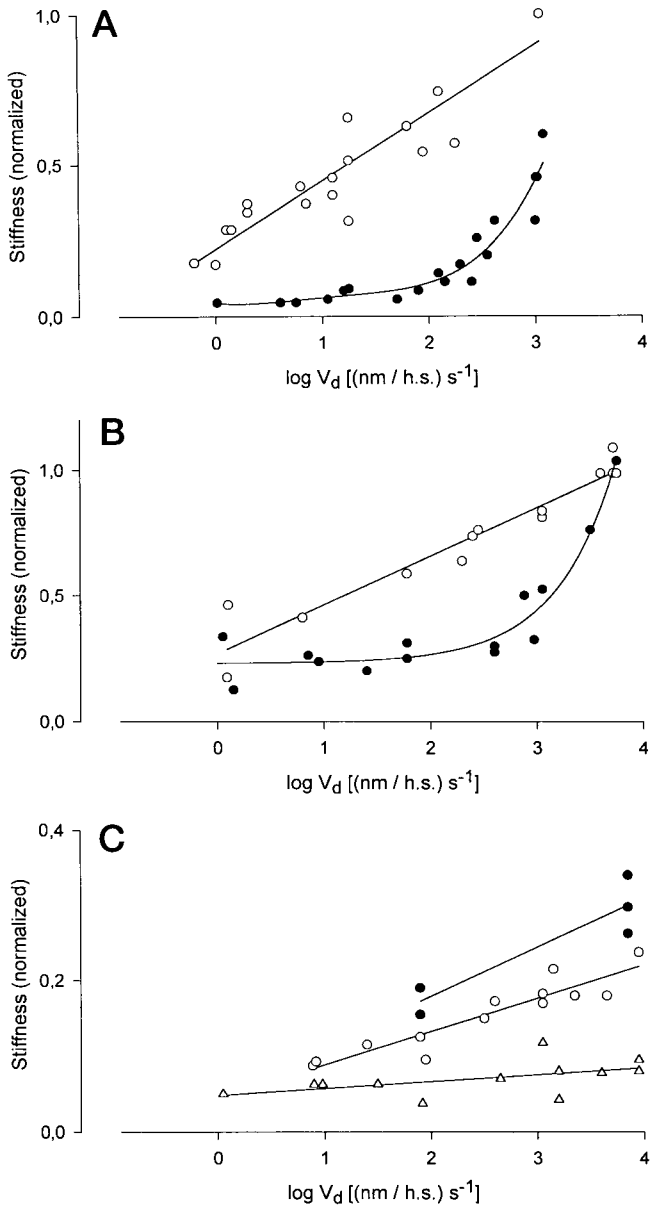

FIGURE 6 Effect of Fab(1–7) on stiffness-speed relationship in the presence and absence of Ca^{2+}. (*A*) 10 mM MgATPγS and 0.05 mg/ml Fab(1–7), μ = 75 mM; ● $-Ca^{2+}$, ○ $+Ca^{2+}$; (*B*) 1 mM MgATP, μ = 50 mM; ● $-Ca^{2+}$, ○ Ca^{2+}; (*C*) 1 mM MgATP, μ = 170 mM; Δ $-Ca^{2+}$, ○ $+Ca^{2+}$. All data normalized to stiffness observed with the fastest stretches in the presence of Ca^{2+} at μ = 50 mM. The concentration of Fab in *B* and *C* was 0.48 mg/ml. The higher Fab concentration was used to ensure that active force was completely abolished. In the presence of both ATPγS and ATP, stiffness-speed relationships are similarly affected by Ca^{2+}. Presumably, no strong interactions due to active cross-bridge cycling were present even with MgATP (no active force generated at this Fab concentration). Also, note the speed dependence of the much inhibited fiber stiffness in the absence of nucleotide (●, in *C*).

(1992) in the absence of the Fab. A similar behavior was observed in the presence of MgATP, after the active force in fibers was completely abolished with 0.48 mg/ml Fab(1–7), i.e., under conditions at which possible Ca^{2+} effects on weak interactions in the presence of MgATP could be studied (Fig. 6, *B* and *C*). The characteristic effect of Ca^{2+} upon stiffness-speed relationship recorded in the presence of MgATPγS, both with and without the Fab fragment, can also be detected in the presence of MgATP.

Fig. 6 *C* also includes data on rigor fiber stiffness (in the absence of nucleotide) under the same ionic strength conditions (170 mM). The rigor stiffness is strongly inhibited and sensitive to the speed of stretch applied to the fiber. This contrasts with fiber stiffness measurements in the absence of both nucleotide and Fab(1–7). Without the Fab-fragment, rigor fiber stiffness is essentially independent of speed of stretch, at least over the range of velocities shown in Fig. 6. Additional studies, however, will be necessary to further characterize this speed-sensitive rigor stiffness, e.g., for comparison with other conditions like the moderately strong cross-bridge binding in the presence of $MgPP_i$.

Activation of the contractile system by strong cross-bridge binding to actin

Because Fab(1–7) could completely abolish active force while weak cross-bridge binding both at high and low Ca^{2+} is still possible, the Fab apparently does not selectively interfere with cross-bridge attachment in the presence of Ca^{2+}. It is possible, however, that active force inhibition by Fab reflects an inhibition/blocking of a kinetic step in the cross-bridge cycle subsequent to weak cross-bridge binding to actin such that active cross-bridge cycling is impaired. To probe for such Fab effects, we examined whether active force can be recovered upon activation of actin filaments by strong cross-bridge attachment, e.g., by decreasing MgATP concentration in the fiber. Fig. 7 shows that in the presence of 0.05 mg/ml Fab(1–7), reduction of MgATP-concentra-

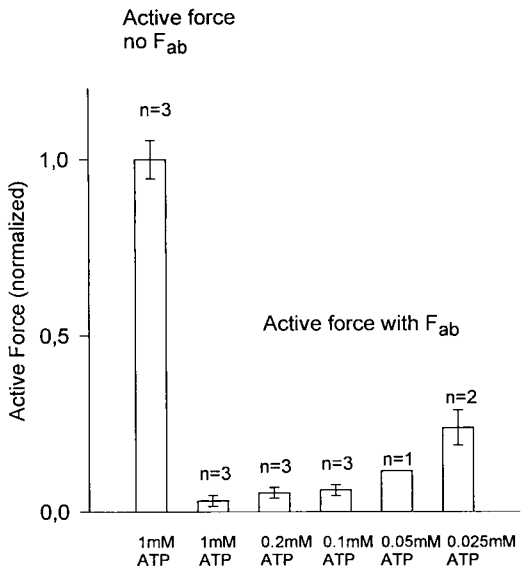

FIGURE 7 Effect of a decrease in MgATP concentration on the inhibited active force. Fab concentration was 0.05 mg/ml; μ = 170 mM. (At higher Fab concentrations, active force did not recover, even at low ATP concentrations; data not shown).

tion allows active tension to recover to some degree. However, the extent of recovery seems to be limited to the level at which fiber stiffness is inhibited by Fab(1–7) in the presence of MgPPi or in rigor. At high concentrations of Fab(1–7), e.g., 0.48 mg/ml, at which stiffness in rigor and in the presence of MgPPi are much more inhibited (to ~5% of their initial stiffness in the absence of Fab), no recovery of active force is seen with decreasing amounts of MgATP. In this case there may be too few strong cross-bridge interactions, even in the absence of nucleotide, to achieve significant activation of the thin filament.

DISCUSSION

The major findings of the present study are summarized below.

Under conditions at which Fab(1–7) inhibits relaxed fiber stiffness only partially, active force generation can be completely abolished. The active force cannot be recovered by an increase in free [Ca^{2+}]. Also, the inhibition of active force larger than that of relaxed fiber stiffness cannot be accounted for by a more effective inhibition of weak cross-bridge binding to actin in the presence of Ca^{2+} (tested in the presence of ATPγS as nucleotide analog), as was shown previously in the case of caldesmon interference with weak cross-bridge binding to actin (Kraft et al., 1995a). However, some recovery of active force is achieved by decreasing [MgATP], i.e., by activation of the contractile system through strong cross-bridge binding.

Fiber stiffness in the absence of nucleotides (rigor) or in the presence of MgPPi (i.e., when cross-bridges are strongly bound to actin) is also inhibited, even to a larger proportion than relaxed fiber stiffness. However, although more effectively inhibited, stiffness observed in the absence of nucleotide or in the presence of MgPPi is still larger than the relaxed fiber stiffness under similar ionic conditions. Upon inhibition by Fab(1–7), rigor fiber stiffness seems more sensitive to speed of stretch than it is in the absence of Fab.

Ca^{2+} still affects weak cross-bridge binding to actin in the presence of MgATPγS, even when active force is essentially absent and weak interactions are partially inhibited by Fab.

Inhibition of fiber stiffness in the absence of nucleotide and in the presence of MgPPi is somewhat unexpected if the N-terminus of actin, against which the Fab is targeted, is involved only in weak cross-bridges binding to actin or contributes little to actin affinity of the strongly bound myosin. However, considering the size of a Fab molecule and the flexibility of the N-terminus of actin, it would not be surprising if some area on the surface of actin, in addition to its N-terminus, is shielded from the interaction with myosin heads. Such a shielding, although not apparent in a recent electron microscopy study of actin-Fab(1–7) complexes (Orlova et al., 1994), may strongly depend on the density of actin decoration by Fab. That strong cross-bridge binding is not completely blocked has been demonstrated by the existence of ternary complexes of actin-Fab-S1 in solution (DasGupta and Reisler, 1991). The steric interference with strong cross-bridge binding to actin, however, cannot account for the much more effective inhibition of active force. The force is essentially abolished under conditions at which fiber stiffness in the absence of nucleotide or with MgPPi is still between 20 and 50% of its value in the absence of Fab.

One way to account for the much more effective inhibition of active force by Fab is to consider the antibody binding to the N-terminus of actin as analogous to the interaction of TnI with the N-terminus of actin in the absence of Ca^{2+}. The TnI binding to actin in conjunction with changes in tropomyosin results in the inhibition of active cross-bridge cycling within a regulated unit of actin, but not only at that actin monomer to which the TnI molecule is bound. However, although TnI dissociates from the N-terminus of actin upon Ca^{2+}-binding to TnC, the Fab is not expected to do so. Thus, the bound Fab may interfere with the activation of the regulated actin unit when Ca^{2+} concentration is raised. Such a possibility has been indicated by solution studies with actin, S-1, and Fab(1–7) (DasGupta and Reisler, 1991). Accordingly, partial occupancy of actin filaments by Fab(1–7) may result in a complete inhibition of activation (i.e., active force generation) and only partial inhibition of relaxed and rigor fiber stiffness.

At the molecular level, complete inhibition of active force and the more effective inhibition of strong cross-bridge binding compared with weak binding could be accounted for if Fab(1–7) interferes with the movement of tropomyosin to its activated state on actin, i.e., the state that allows strong cross-bridge interaction with actin. This view is supported by the observation that Fab(1–7) alters cross-bridge binding to actin in the absence of nucleotide, as indicated by changes in the fiber stiffness speed relationship. In the absence of Fab(1–7), rigor fiber stiffness is little dependent on the speed of stretch (Brenner et al., 1986). At high [Fab(1–7)], however, the stiffness speed relationship is rather similar to that of weakly bound cross-bridges (Fig. 7 *C*), as if more rigid bonds were blocked. Most likely, the effect of Fab(1–7) on tropomyosin is indirect, via TnI. Fab(1–7) should alter the interactions of TnI with actin (because of the competition for the N-terminus of actin; DasGupta and Reisler, unpublished data) and perhaps with other Tn subunits and with tropomyosin. Such altered subunit interactions within the regulatory complex could impair tropomyosin movement on actin.

The detectable effect of Ca^{2+} on weak cross-bridge interactions with actin in the presence of Fab(1–7) may seem somewhat surprising in the context of the above explanation. However, Fab(1–7) may interfere only with more extended tropomyosin movements such that active cross-bridge cycling and strong cross-bridge binding are affected, whereas more subtle Ca^{2+} effects on weakly bound cross-bridges may still be possible (Fig. 6).

Another molecular scenario, which could explain both the strong inhibition of active force and the inhibition of strong cross-bridge binding to actin by Fab(1–7), is that the

antibody inhibits or blocks a kinetic step after the weak binding of cross-bridges to actin. Clearly, if a transition from weakly to strongly bound states of actomyosin is blocked, no active force would be developed. However, even rigor stiffness would be strongly inhibited in such a case because all fiber incubations with Fab(1–7) were carried out under relaxing conditions. Thus, the subsequent transfer of these fibers to a rigor solvent may not eliminate completely the inhibition of the transition between weakly and strongly bound cross-bridge states. It is not difficult to provide a structural rationale for such a molecular explanation of Fab action. The binding of the N-terminus of actin to the 626–647 loop on myosin may provide an appropriate "ordering template" for this random coil segment of S-1. The disorder-order transition of this loop (cf. Holmes, 1995), which is strategically positioned over strongly binding segments of actin, could increase the stereospecificity of the weak cross-bridge binding to actin and thus facilitate formation of stereospecific, strong actomyosin binding and a shift from less ordered (nonstereospecific) to ordered (stereospecific) cross-bridge attachment (Matsuda and Podolsky, 1984; Craig et al., 1985; Applegate and Flicker, 1987; Yu and Brenner, 1989; Brenner and Yu, 1993a; Berger and Thomas, 1994; Walker et al., 1994, 1995; Brenner et al., 1995; Thomas et al., 1995). The binding of Fab(1–7) to the N-terminus of actin would then interfere with such ordering of the 626–647 loop on S1 and the transition to the strongly bound cross-bridge states. This explanation could also account for the loss of in vitro motility and actomyosin ATPase activity in N-terminal actin mutants (Sutoh et al., 1991; Cook et al., 1993). These ideas could be tested and additional insight into the mechanisms by which Fab and perhaps actin mutants interfere with the activation of the thin filaments could be gained from studies using fluorescently labeled regulatory proteins incorporated into the skinned fibers.

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