Kinetics of Thin Filament Activation Probed by Fluorescence of *N*-((2-(Iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole-Labeled Troponin I Incorporated into Skinned Fibers of Rabbit Psoas Muscle: Implications for Regulation of Muscle Contraction

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ABSTRACT Making use of troponin with fluorescently labeled troponin I subunit (*N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole-troponin I, IANBD-TnI) that had previously been described in solution studies as a probe for thin filament activation (Trybus and Taylor, 1980. *Proc. Natl. Acad. Sci.* 77:7209–7213), we present a new approach that allows the kinetics of thin filament activation to be studied in skinned muscle fibers. After the exchange of native troponin for fluorescently labeled troponin, the fluorescence intensity is sensitive to both changes in calcium concentration and actin attachment of cross-bridges in their strong binding states (Brenner et al., 1999. *Biophys. J.* 77:000–000). Imposing rapid changes in the fraction of strongly attached cross-bridges, e.g., by switching from isometric contraction to high-speed shortening, causes changes in thin filament activation at fixed Ca^{2+} concentrations that can be followed by recording fluorescence intensity. Upon changing to high-speed shortening we observed small (<20%) changes in fluorescence that became faster at higher Ca^{2+} concentrations. At all Ca^{2+} concentrations, these changes are more than 10-fold faster than force redevelopment subsequent to the period of unloaded shortening. We interpret this as an indication that equilibration among different states of the thin filament is rapid and becomes faster as Ca^{2+} is raised. Fast equilibration suggests that the rate constant of force redevelopment is not limited by changes in the activation level of thin filaments induced by the isotonic contraction before force redevelopment. Instead, our modeling shows that, in agreement with our previous proposal for the regulation of muscle contraction, a rapid and Ca^{2+}-dependent equilibration among different states of the thin filament can fully account for the Ca^{2+} dependence of force redevelopment and the fluorescence changes described in this study.

INTRODUCTION

One major challenge in understanding the molecular mechanisms of muscle contraction is elucidation of the mechanisms by which muscle contraction is regulated at the cross-bridge level, e.g., to determine which reaction steps are affected by the Ca^{2+}-controlled movement of the tropomyosin strands on the surface of the actin filaments. Two alternative concepts were proposed for the general principle of regulation of active force by tropomyosin. In one concept, the number of cross-bridges in force-generating states is assumed to be controlled by modulation of the number of cross-bridges that can interact with actin, i.e., it is assumed that with increasing Ca^{2+} concentration the number of actively cycling cross-bridges increases, while cycling kinetics of active cross-bridges, e.g., the probabilities of cycling between nonactivating (weak binding) and activating (strong binding) states of the myosin head, do not change with Ca^{2+} (recruitment concept; Podolsky and Teichholz, 1970). Alternatively, it was proposed (Julian, 1969) that Ca^{2+} increases the probability that cross-bridges enter their force-generating state(s) without altering the number of actively cycling cross-bridges (rate modulation principle).

These opposing concepts result in different interpretations of changes in commonly used measurements such as the force-pCa relation. We demonstrated (Brenner, 1988, 1993) that for the rate modulation principle, changes in cross-bridge cycling kinetics at constant Ca^{2+} binding to the thin filament will have profound effects on the force-pCa relation by affecting both apparent Ca^{2+} sensitivity (the midpoint of the force-pCa relation) and, for asymmetrical force-pCa relations like those in skeletal muscle, the apparent cooperativity (steepness of the force-pCa relation). That is, in the rate modulation concept no fixed coupling exists between the force-pCa relation and Ca^{2+} binding to thin filaments. In contrast, the recruitment concept predicts that active force developed at different Ca^{2+} concentrations is directly proportional to Ca^{2+} binding to the regulatory proteins within the thin filament, irrespective of cross-bridge cycling kinetics. The effect of phosphorylation of the regulatory light chain on force-pCa relations in skeletal muscle (Persechini et al., 1985) was proposed to be one possible example that changes in force-pCa relations can indeed result from changes in cross-bridge cycling kinetics while Ca^{2+} binding to troponin C (TnC) remains unchanged (Brenner and Morano, 1990; Sweeney and Stull, 1990).

To investigate cross-bridge cycling kinetics and a possible Ca^{2+} dependence, we previously introduced an experimental protocol in which the time course of force redevel-

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opment after a period of unloaded or lightly loaded shortening was used to obtain information about the apparent rate constants that determine the transition of cross-bridges from the non-force-generating states to the force-generating states, and the return from the force-generating states back to the non-force-generating states via release of ADP and binding of a new ATP molecule (Brenner, 1985; Brenner and Eisenberg, 1986). We also showed that this force redevelopment is sensitive to the level of Ca^{2+} activation in that increasing Ca^{2+} results in faster redevelopment of force (Brenner, 1988). Based on correlation of the rate constant of force redevelopment with measurements of both fiber ATPase and active force at different Ca^{2+} concentrations, we proposed that in agreement with the earlier proposal of Julian (1969), a major mechanism in the regulation of muscle contraction is an increase in the probability of the transition of cross-bridges into their force-generating states, resulting in increasing accumulation of cross-bridges in these states (Brenner, 1988). We further pointed out that the Ca^{2+} dependence of the probability of proceeding from the nonactivating (weak binding) states of the myosin head to the activating (strong binding), force-generating states (f_{app} in our notation) can be accounted for on the basis of commonly accepted kinetic schemes for ATP hydrolysis if actin exists in at least two states, e.g., an inactive and an active state, and equilibration between these states of the actin filament is Ca^{2+} dependent and rapid on the time scale of active turnover. If instead equilibration is assumed to be slow relative to active cross-bridge cycling (e.g., f_{app} or ATPase activity), such kinetic schemes will result in no Ca^{2+} dependence of force redevelopment; i.e., they will predict a recruitment-type behavior (cf. Brenner, 1988). Simplified versions of this concept have since been used by others to account for the Ca^{2+} dependence of force redevelopment (e.g., Landesberg and Sideman, 1994; Hancock et al., 1997; Regnier et al., 1998).

So far, however, it remained unclear whether equilibration between different states of the thin filament is indeed fast and Ca^{2+} sensitive. Moreover, interpretation of the time course of force redevelopment in terms of cross-bridge cycling kinetics did not remain unquestioned. It was argued that reactivation of thin filaments after inactivation during unloaded shortening (Millar and Homsher, 1990) or during the restretch period (Swartz and Moss, 1992) limits the time course of force redevelopment and may be responsible for the slowing of force redevelopment when Ca^{2+} is reduced. This is because during unloaded shortening the number of strongly attached cross-bridges is thought to be very much reduced (reduced fiber stiffness; Julian and Sollins, 1975; Ford et al., 1985; Stehle et al., 1993). Strong cross-bridge attachment, however, was shown to enhance activation of the thin filaments (e.g., Bremel and Weber, 1972).

We had shown earlier that the time course of force redevelopment after lightly loaded shortening is similar to that after shortening under loads where the increased fraction of strongly attached cross-bridges lessens the possibility of deactivation of the thin filaments (Brenner, 1988). We also showed that the rate constant of force redevelopment after unloaded shortening is rather similar to the rate constant of phase 4 of force transients in response to stepwise length changes (Huxley and Simmons, 1971). Both observations suggested that a possible change in activation of the thin filaments, induced by the period of unloaded shortening, is not a dominating factor limiting the rate constant of force redevelopment. However, some uncertainty remained because 1) force redevelopment could only be followed after shortening under loads up to some 50% of full isometric force. At higher loads the force transient during the restretch period largely obscured force redevelopment. 2) It was also argued (Swartz and Moss, 1992) that deactivation may actually occur during the restretch period when force-generating cross-bridges become forcefully dissociated during large-amplitude filament sliding. Thus the question remained whether deactivation/reactivation processes of the thin filaments, induced by unloaded shortening, cause the observed Ca^{2+} dependence of k_{redev} or, alternatively, whether the Ca^{2+} dependence of k_{redev} results from the Ca^{2+} dependence of the probability that a cross-bridge enters its activating (strong binding) states as we had previously proposed (Brenner, 1988).

With our newly developed method for exchanging native troponin for troponin with fluorescently labeled subunits, e.g., *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labeled troponin I (TnI), we are now able 1) to test whether large changes in thin filament activation occur during unloaded shortening and subsequent force redevelopment, and 2) to evaluate the kinetics for changes in thin filament activation, e.g., in response to sudden changes in strong cross-bridge attachment. This method makes it possible to distinguish whether the rate constant of force redevelopment is limited by slow reactivation of the thin filaments after shortening-induced deactivation (e.g., Millar and Homsher, 1990; Swartz and Moss, 1992) or, rather, reflects the Ca^{2+} dependence of cross-bridge cycling kinetics (e.g., Ca^{2+} dependence of f_{app}) due to fast and Ca^{2+}-dependent equilibration between different states of the thin filament, as we had previously proposed (Brenner, 1988).

Using IANBD-labeled troponin I to monitor the state of activation of thin filaments (cf. Trybus and Taylor, 1980; Brenner et al., 1999), we find that during unloaded shortening the fluorescence intensity changes but only partially toward the relaxed value. The maximum effect is seen at intermediate activation levels and is limited to $<20\%$ of the difference seen between relaxed and fully activated conditions. Most importantly, however, the kinetics of this change are rather rapid, i.e., more than 10 times faster than the rate constant of force redevelopment. Modeling shows that our previously proposed concept of rapid, Ca^{2+}-dependent equilibration among different states of the thin filament can fully account for the Ca^{2+} dependence of the rate constant of force redevelopment and the fluorescence changes observed in the present study. A preliminary account of this work was previously communicated to the

Biophysical Society (Brenner and Chalovich, 1995; Chalovich and Brenner, 1995; Brenner et al., 1997, 1998).

MATERIALS AND METHODS

Preparation of proteins

Tropomyosin, troponin, and troponin with TnI modified at Cys^{133} were prepared as previously described (Brenner et al., 1999). The sulfhydryl group of TnI was modified, while TnI was incorporated into tropomyosin-troponin by incubation of tropomyosin-troponin with a fivefold molar excess of IANBD (Molecular Probes) in *N*,*N*-dimethylformamide. To avoid artifacts from slight labeling of tropomyosin, the troponin complex was isolated by hydroxyapatite chromatography such that no labeled tropomyosin was present in the final product.

Fiber preparation

Skinned fibers of rabbit psoas muscle were isolated and chemically skinned with Triton X-100 including our recently described modifications (for details see Kraft et al., 1995a). Single fibers were isolated according to the method of Yu and Brenner (1989) within a few hours after dissection of fiber bundles from the rabbit and were kept in skinning solution for up to 5 days.

Solutions

All solutions were adjusted to pH 7.0 at the experimental temperature (5°C). Chemicals were obtained from Sigma Chemie München, FRG, except when noted otherwise.

The skinning solution contained (in mM) 5.0 KH_2PO_4; 3.0 Mg-acetate; 5.0 EGTA; 1.0 Na_2ATP (Merck) 50 Na-creatine-phosphate; 5.0 NaN_3; 10 glutathione; 2.0 dithiothreitol; 0.1 4-(2-aminoethyl)-benzene sulfonyl fluoride (Calbiochem); 0.01 each of leupeptin, antipain, E64, and pepstatin; and 1 mg/ml aprotinin (cf. Kraft et al., 1995a).

Preactivation and activation solutions contained (in mM) 10 imidazole, 2.0 $MgCl_2$, 1.0 MgATP, 1.0–3.0 EGTA (or CaEGTA), 10 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.

The exchange buffer contained (in mM) 20 3-(*N*-morpholino)propanesulfonic acid, 5 $MgCl_2$, 5 EGTA, 240 KCl, 5 dithiothreitol, and 7 μg/ml pepstatin (pH 6.5) at 20°C or 5°C.

Experimental setup

The experimental apparatus for recording of mechanical and fluorescent signals from segments of skinned fibers was the same as previously described (Brenner et al., 1999). In short, for recording of fluorescence signals our standard setup was fitted with a mercury arc lamp and interference filters (Scientific Instruments, Heidelberg, Germany). For excitation of IANBD fluorescence a interference filter (486.1 nm, 11 nm bandwidth; Melles Griot) was placed in front of a mercury arc lamp, and exciting light was focused on the skinned fiber by means of two cylindrical lenses. The emitted light was passed through a OG530 filter with 50% transmission at 530 nm. For further details see Brenner et al. (1999).

Mechanical measurements

Force, force redevelopment, and fiber stiffness were recorded as previously described (Brenner et al., 1986a; Brenner and Eisenberg, 1986). To measure fiber stiffness, rapid (10^4 nm/(half-sarcomere)s^{-1}) ramp-shaped stretches were imposed on the fibers. Apparent fiber stiffness was defined as the force increase when the change in sarcomere length, recorded by laser light diffraction, had reached 2 nm/half-sarcomere (cf. Brenner et al., 1986a). A previously developed routine (Brenner, 1998) was used to account for the domain organization of skinned fibers. In short, the imposed length changes were repeated at different incidence angles between laser beam and fiber; subsequent incidence angels were usually 0.5° apart and covered a range over which intensity in the recorded first-order diffraction maximum was ≥10% of maximum intensity. All individual sarcomere length records were averaged, and stiffness was determined at the point where the length change of the averaged record had reached 2 nm/half-sarcomere.

Experimental protocol

After mounting of a skinned fiber to the force transducer and lever system by use of a cyanoacrylate glue (Histoacryl, Braun Melsungen, Germany), the ends of the fiber were fixed with glutaraldehyde (modified from Chase and Kushmerick, 1988; cf. Kraft et al., 1995a). TnI was exchanged for IANBD-labeled TnI by exchange of the whole troponin complex. For the exchange procedure see Brenner et al. (1999). After exchange, unbound troponin was removed by washing the fibers in several changes of exchange buffer without IANBD-labeled troponin for at least 30 min. During Ca^{2+} activation, fibers were permanently cycled between isometric steady-state contraction and short (150–200 ms) periods of unloaded shortening with subsequent restretch to the initial (isometric) sarcomere length; this maneuver was repeated every 5 s to stabilize the striation pattern during prolonged activation (Brenner, 1983).

Modeling

Mathematical modeling was done with the KINSIM modeling program written by Barshop (Barshop et al., 1983) and placed in microcomputer format by B. Plapp (University of Iowa).

RESULTS

Changes in IANBD fluorescence during unloaded shortening and subsequent force redevelopment

Having previously established that IANBD-labeled TnI is sensitive not only to Ca^{2+} but also to actin attachment of cross-bridges in their strong binding states, we first examined whether unloaded shortening itself or the subsequent restretch to initial filament overlap could deactivate the thin filament such that the rate of reactivation limits the time course of force redevelopment. To do so, we followed IANBD-TnI fluorescence, at different Ca^{2+} concentrations, during isometric steady-state contraction, unloaded/lightly loaded shortening, and the redevelopment of isometric force after restretch of fibers to their original filament overlap at the end of isotonic shortening (Fig. 1). At low (pCa 6.10) and near-saturating (pCa 4.38) activation levels, only a very small change in fluorescence was observed. At intermediate activation levels, some shift of the fluorescence toward the relaxed value occurred, but it was far from the relaxed level at 3.5 V in the example shown in Fig. 1. This shift toward the relaxed level reaches a maximum at intermediate activation levels, and approaches ~20% of the difference observed between relaxing conditions and full Ca^{2+} activation. This increase in fluorescence during the lightly loaded isotonic contraction is reversed during the period of force redevelopment after the switch back to isometric conditions

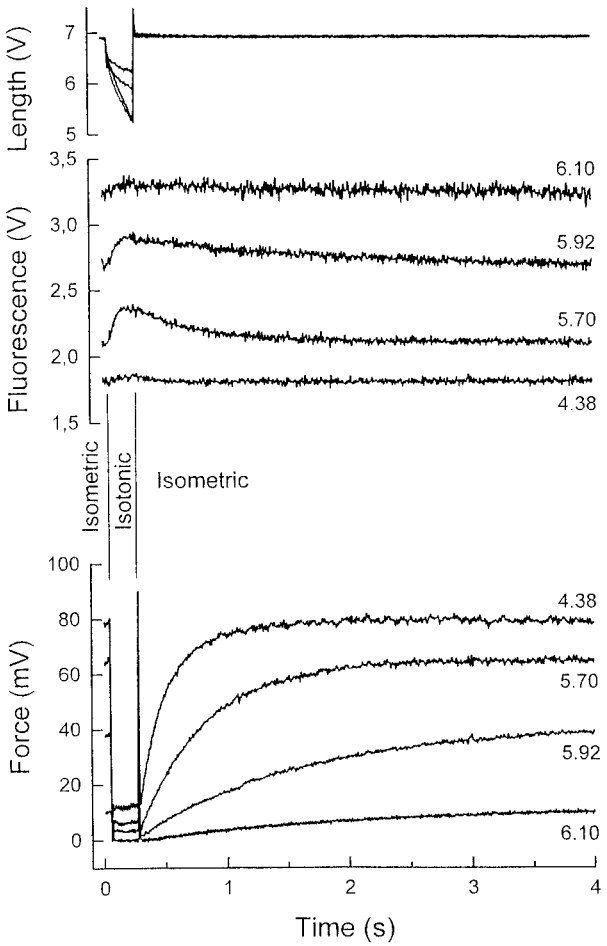


FIGURE 1 IANBD fluorescence during isotonic contraction and subsequent force redevelopment at different activation levels. *Top traces*, length signals; *middle traces*, fluorescence intensity; *bottom traces*, force. Numbers next to traces are pCa values of activating solutions. Note that for intermediate activation levels some change in IANBD-TnI fluorescence occurs during shortening and force redevelopment (maximum change ~20% of difference between relaxed and fully activated level). During isotonic shortening fibers were loaded by some 5–10% of isometric force to avoid slackening of fibers.

(Fig. 1). During force redevelopment, the fluorescence returns to the isometric steady-state level in parallel with the reincrease in isometric force. This observation is consistent with some rather limited "deactivation" of the thin filaments during high-speed shortening that is most prominent at intermediate activation levels. It should be noted that during the period of isotonic shortening fibers were slightly loaded (~5–10% of isometric tension). This small load was applied to avoid slackening of the fiber, by which the fiber might have moved, at least to some extent, out of the exciting beam and/or the object plane of the detection system such that the fluorescence signal would become unreliable. The amplitude of the fluorescence change observed during such lightly loaded shortening is not detectably different from the change in response to unloaded shortening. This is inferred from essentially identical fluorescence changes during the period of force redevelopment, when responses after unloaded shortening are compared with responses after isotonic shortening under loads of 5–10% of isometric tension (data not shown).

Fluorescence during the restretch period

Because it had previously been proposed that during the restretch procedure thin filaments might become deactivated because of forced detachment of force-generating cross-bridges (Swartz and Moss, 1992), we examined the behavior of the IANBD-TnI fluorescence during the restretch maneuver. By recording IANBD fluorescence on a more extended time scale (Fig. 2) and for an intermediate Ca^{2+} activation where the change in IANBD-TnI fluorescence is at maximum, it becomes evident that the increase in fluorescence occurs early in the isotonic period, and no further increase is detected during the restretch period. In a few records a small step in the fluorescence was detectable above the noise level during the restretch maneuver. The direction of this small change, however, was opposite to that of the changes seen upon relaxation and appeared to result from changes in sarcomere length during the restretch back to initial isometric sarcomere length (reduction in the number of fluorophores in the field of view).

Force redevelopment after short periods of unloaded shortening

To further test the proposal that force redevelopment is slowed because of thin filament deactivation induced during

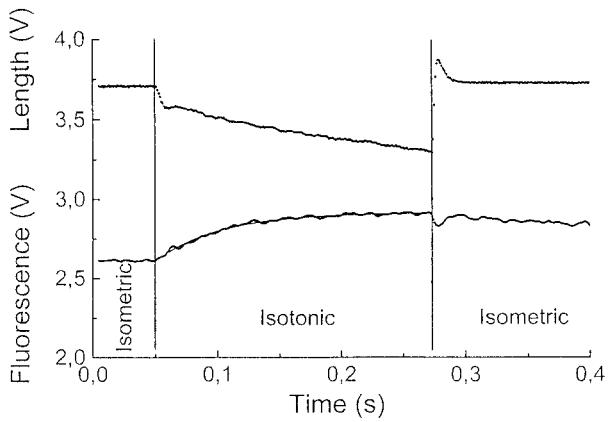


FIGURE 2 IANBD-TnI fluorescence upon switching to isotonic contraction conditions for 220 ms. After this period the fiber is restretched to its initial length and maintained isometrically. Note that the increase in fluorescence occurs during the first 50–100 ms of the isotonic period, and no further increase is seen upon restretch of the fiber. A small stepwise decrease in fluorescence is seen in some records in response to the restretch maneuver. This decrease presumably reflects a decrease in the number of fluorophores in the field of view due to the change in sarcomere length during the restretch. Note, however, that the length changes during isotonic shortening are only on the order of 2% of isometric length. This small value is due to partial activation and the load (~5–10% of isometric force) imposed during isotonic contraction to avoid slackening of the fiber. *Solid line*, fit of a single exponential function with $1/\tau = 19.5\ s^{-1}$.

the period of unloaded shortening, we reduced the duration of the unloaded shortening to as little as 2–5 ms, i.e., to such short periods that IANBD fluorescence changes only very little before the restretch maneuver with subsequent force redevelopment. As shown in Fig. 3, k_{redev} is only slightly affected (<10%), and Ca^{2+} effects on force redevelopment do not disappear, even for the shortest isotonic periods studied.

Kinetics of changes in the thin filaments as reported by IANBD-TnI fluorescence

As is evident from Fig. 1 and in support of our previous work (Brenner et al., 1999), IANBD-TnI fluorescence not only depends on the Ca^{2+} concentration but also responds to changes in strong cross-bridge attachment when we switch to high-speed shortening. Although the response to changes in strong cross-bridge attachment is small and most prominent at intermediate Ca^{2+} concentrations, it nevertheless opens up the possibility of studying the kinetics of changes in the thin filament by following the kinetics of readjustment of IANBD fluorescence to sudden changes in strong cross-bridge attachment. To do so, an intervention was required that allows changes in strong cross-bridge attachment that are sufficiently fast such that changes in IANBD fluorescence are not limited by the time course of the changes in strong cross-bridge attachment.

One way to rapidly change the fraction of strongly attached cross-bridges is to switch from isometric steady-state contraction to unloaded (or nearly unloaded) fiber shortening (Fig. 1 and *inset* in Fig. 4). To see whether changes in strong cross-bridge attachment are sufficiently faster than

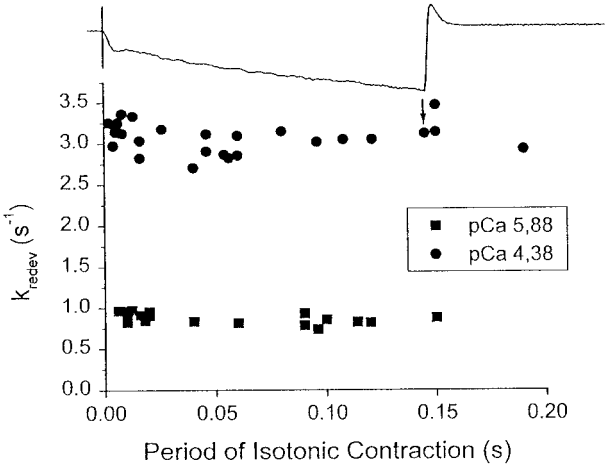


FIGURE 3 Rate constant of force redevelopment (k_{redev}) versus period of isotonic contraction before force redevelopment. *Top trace*, Fiber length to illustrate experimental protocol. Restretch was initiated after different periods of isotonic shortening, ranging from ~5 ms up to 180 ms; the value of k_{redev} that was measured in response to the restretch shown in the length trace is represented by the filled circle indicated by the vertical arrow. Note the small effect of the duration of the isotonic contraction period on the rate constant of subsequent force redevelopment, at both maximum and partial activation.

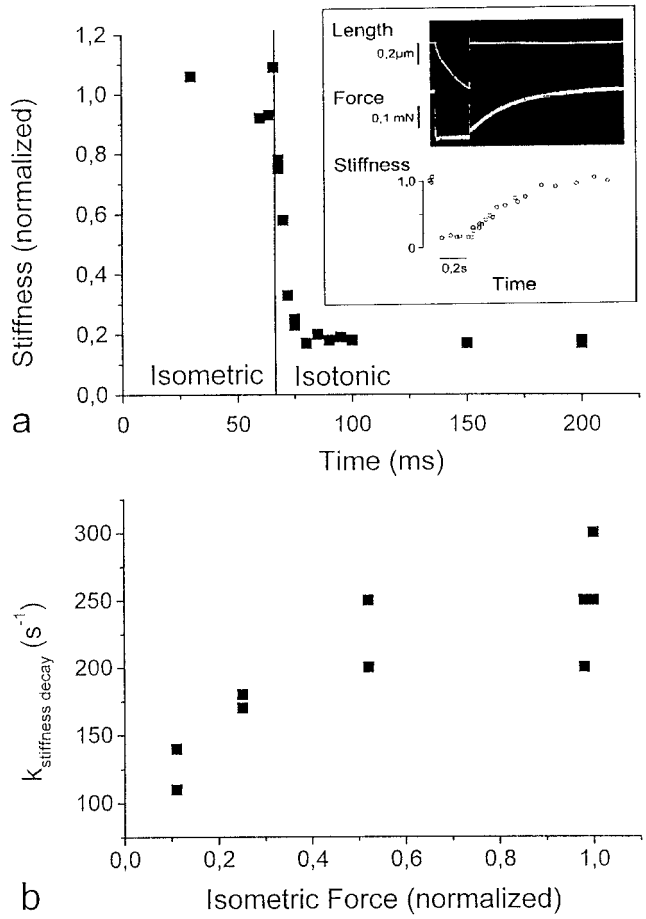


FIGURE 4 Time course of fiber stiffness upon changeover from isometric to isotonic contraction conditions. (*a*) *Inset*: Fiber length, force, and fiber stiffness during isometric steady-state contraction, isotonic shortening, restretch of the fiber to its initial length and subsequent redevelopment of force to the isometric steady-state level (slow time scale). *Main plot*: Time course of stiffness on an expanded time scale covering the changeover from isometric to isotonic conditions (maximum Ca^{2+} activation). (*b*) Apparent rate constant for the drop in fiber stiffness upon changeover from isometric to isotonic conditions at different degrees of Ca^{2+} activation (apparent rate constant plotted versus isometric steady-state force observed at different Ca^{2+} concentrations). Note that at low activation levels the decay in fiber stiffness is slower than at saturating Ca^{2+} concentrations.

the changes in fluorescence when we switch to isotonic conditions, we followed fiber stiffness during and after the changeover from isometric to isotonic contraction conditions. As shown in Fig. 4, the time course of the change in strong cross-bridge attachment, as reported by fiber stiffness, is very rapid. For all activation levels studied, the half-time for the drop in fiber stiffness is <10 ms, corresponding to an apparent rate constant of >100–150 s^{-1} with some dependence on Ca^{2+} concentration (Fig. 4 *b*).

Next we recorded the time course of the changes in the IANBD-TnI fluorescence upon changing from isometric steady-state contraction to lightly loaded isotonic shortening at different degrees of Ca^{2+} activation (Fig. 5). During the period of isotonic shortening, fibers were again lightly

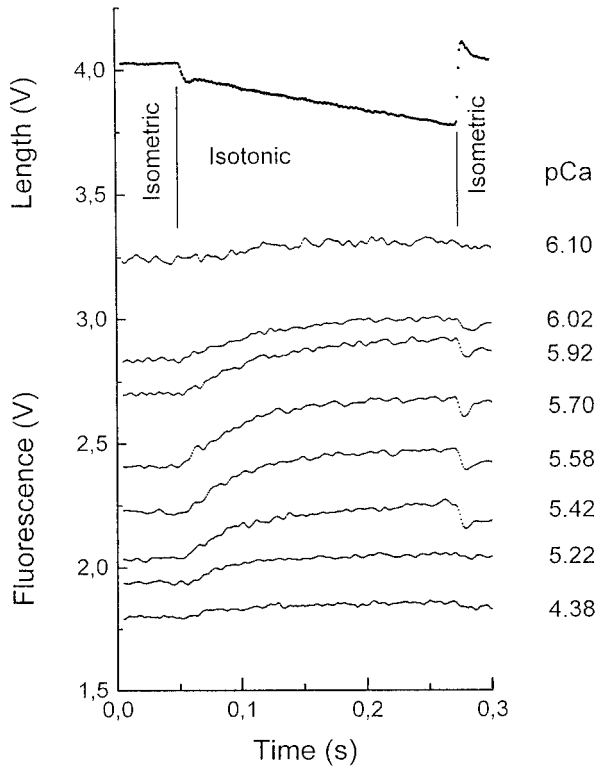


FIGURE 5 Changes in IANBD fluorescence upon initiation of isotonic shortening. Expanded time scale compared to Fig. 1. The numbers next to the traces are the pCa of the corresponding activating solutions. *Top trace*, length signal to illustrate times of change between isometric and isotonic contraction conditions.

loaded to avoid artifacts in the fluorescence signal due to slackening of the fibers when changing to unloaded conditions. Changes in fluorescence due to an increase in the number of fluorophores in the field of view with fiber shortening could easily be separated by a corresponding decrease in fluorescence when fibers were restreched to their initial length at the end of the period of isotonic shortening (cf. Fig. 2).

Fig. 6 *a* shows, for different Ca^{2+} concentrations, the apparent rate constant for the increase in fluorescence seen upon changing from isometric to isotonic steady-state contraction (+), together with the rate constant of force redevelopment (●) and isometric force (■). Note the scatter in the apparent rate constant for the change in fluorescence, especially at low and near-maximum activation levels. This is due to the small magnitude of the observed fluorescence changes that became more clearly detectable only after the signal-to-noise ratio is improved by averaging 10 identical events. The rate constant of the fluorescence change increases with Ca^{2+} from ~10–15 s^{-1} to 50–80 s^{-1}. The amplitude of the fluorescence change versus pCa is also included in Fig. 6 *a* (×).

With the initiation of high-speed shortening, the time course (and apparent rate constant) of the fluorescence change is slower than that for the stiffness change observed in Fig. 4. This suggests that the fluorescence change is not limited by change in the fraction of strongly attached cross-bridges but rather reflects subsequent changes in the state of the thin filaments. In contrast, during force redevelopment, when we change back to isometric conditions, the increase in fiber stiffness is slow (in parallel with tension; cf. Fig. 4 *a*, *inset*) and closely followed by the IANBD-TnI fluorescence (cf. Fig. 1).

Fig. 6 also shows the relations between the rate constant of force redevelopment and isometric force (Fig. 6 *b*) and between fluorescence intensity and isometric force (Fig. 6 *c*).

DISCUSSION

The main findings of the present paper are that 1) at very low and at near-saturating Ca^{2+} concentrations, there is only a small change in IANBD fluorescence during unloaded/lightly loaded fiber shortening and subsequent force redevelopment back to the isometric steady-state level. At intermediate levels of Ca^{2+} activation a somewhat larger yet limited ($\leq$20%) change in fluorescence intensity is seen during this maneuver. 2) The increase in fluorescence toward the level under relaxing conditions occurs early in the period of isotonic contraction, and no further increase is seen as the fibers are restretched to their initial overlap at the end of the isotonic contraction. 3) If the duration of the isotonic period is shortened such that little or no change in IANBD-TnI fluorescence occurs during the isotonic period, the rate constant of force redevelopment is not much affected and the Ca^{2+} effect on k_{redev} does not disappear. 4) The kinetics for reaching the new steady-state fluorescence in response to switching from isometric contraction to lightly loaded shortening become faster when Ca^{2+} is raised and, for all activation levels, are more than 10-fold faster than the rate constant of force redevelopment.

Kinetics of fluorescence changes

IANBD-TnI fluorescence is sensitive to both changes in Ca^{2+} binding to troponin C and changes in strong cross-bridge attachment (Brenner et al., 1999). We therefore used the IANBD fluorescence as a probe to follow the kinetics of changes in the state of the thin filament in response to rapid changes in strongly attached (activating) cross-bridges. Sufficiently rapid changes in the fraction of strongly attached (activating) cross-bridges were accomplished by switching from isometric to isotonic contraction conditions. As shown in Fig. 4, this maneuver allows the fraction of strongly attached cross-bridges to decrease with an apparent rate constant of >150 s^{-1}. Note that fiber stiffness is only used to probe the time course of establishing the new steady-state distribution between nonactivating (weak binding) and activating (strong binding) states of the myosin head and not to estimate the fraction of strongly attached cross-bridges. Possible effects of filament compliance are not critical for this analysis.

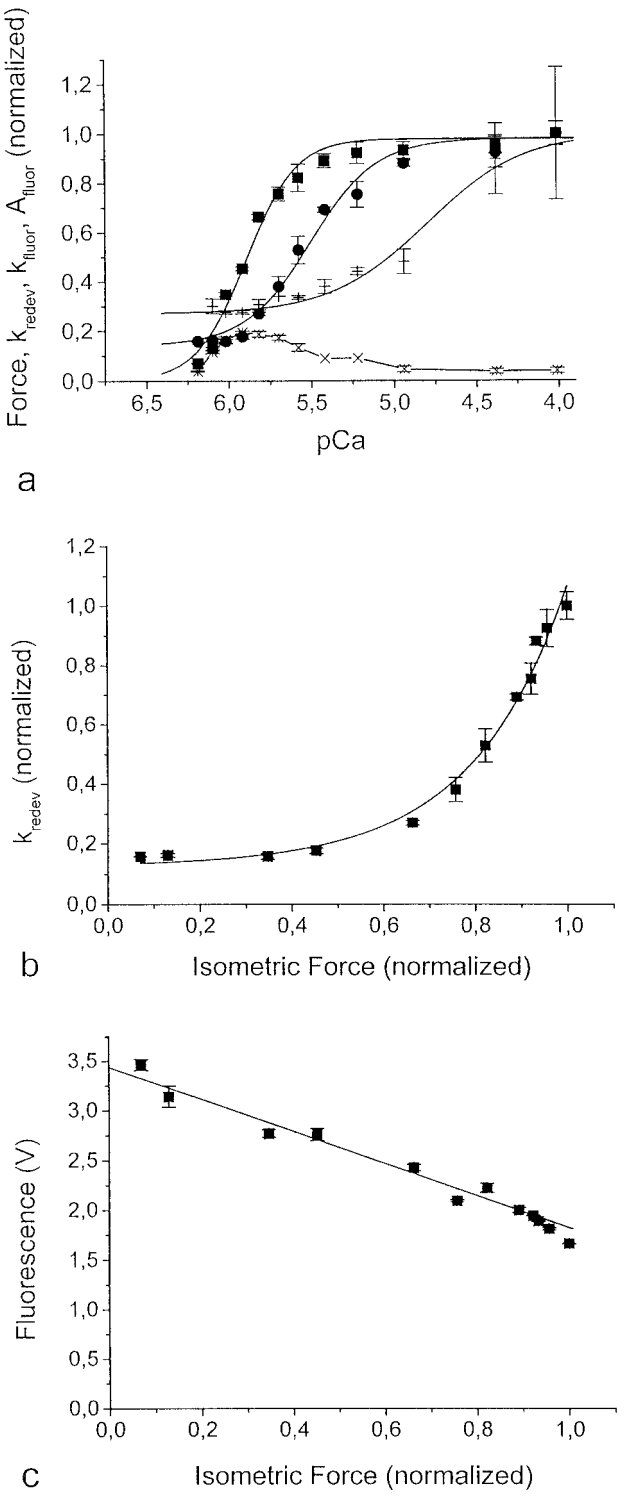


FIGURE 6 Calcium dependence of mechanical and fluorescence changes. (*a*) Isometric force (■), rate constant of force redevelopment (k_{redev}, ●), rate constant of fluorescence change (k_{fluor}, +), and amplitude of fluorescence change (A_{fluor}, ×) at different Ca^{2+} concentrations. Means ± SD. Solid lines are sigmoidal fits to data. Force, k_{redev}, and k_{fluor} are normalized to their mean values observed at maximum Ca^{2+} concentrations. These maximum values were 4.17 s^{-1} and 58 s^{-1} for k_{redev} and k_{fluor}, respectively. The amplitude of fluorescence change (A_{fluor}) is normalized with respect to the difference in fluorescence between relaxation and maximum Ca^{2+} activation. Because several measurements were always made at the maximum activation level, error bars for normalized maxima are included. (*b*) Plot of k_{redev} versus steady-state isometric force at different Ca^{2+} concentrations (replotted from *a*). Note the curved shape of this plot under our conditions (cf. Brenner, 1988). The solid line is a single exponential function fitted to the data. Data points are means ± SD. (*c*) Plot of steady-state fluorescence versus steady-state isometric force at different Ca^{2+} concentrations. Note the indicated trend toward deviation from linearity at high activation levels. Means ± SD.

Figs. 5 and 6 *a* show that the time course of the small fluorescence change is slower than the drop in fiber stiffness upon changeover from isometric to isotonic conditions. Thus the time course of the processes resulting in the observed small fluorescence changes is not limited by the time course of the change in strong cross-bridge attachment when we switch from isometric to isotonic contraction conditions. Instead it appears that the rate constant of the fluorescence change is limited by a process (e.g., readjustment in the extent of thin filament activation) that occurs subsequent to the sudden reduction in strong cross-bridge attachment. As further revealed by Figs. 5 and 6 *a*, the kinetics of this process increases from an apparent rate constant of some 15 s^{-1} to some 60–80 s^{-1} as the Ca^{2+} concentration is raised. It should be noted, however, that our protocol limited us to measuring rate constants smaller than 50–100 s^{-1}. For faster rate constants the drop in the fraction of strongly attached cross-bridges within some 5–10 ms would contribute to the limitation of observed fluorescence changes, specifically by introducing a lag phase before the changes in fluorescence early in the isotonic period. Such a lag became detectable in our modeling at the high Ca^{2+} concentrations (see below). Because in the present study the observed fluorescence changes were not fast enough to produce a detectable lag, it was not necessary to deal with this complication.

Comparison with the rate constant of force redevelopment

As shown in Fig. 6 *a*, changes in the fluorescence of IANBD-labeled troponin upon switching from isometric to isotonic contraction conditions are, at all activation levels, more than 10-fold faster than the time course of force redevelopment. If we interpret the change in IANBD fluorescence as a signal reporting the state of the thin filament activation (Trybus and Taylor, 1980; Brenner et al., 1999), our fluorescence data indicate rapid readjustment of the level of thin filament activation to any changes in strong cross-bridge attachment. Such fast kinetics of readjustment of thin filament activation imply that changes in the activation level of the thin filament during isotonic shortening and force redevelopment will in general have little if any potential for slowing down the rate constant of force redevelopment. This is confirmed by the minimal effects on force redevelopment at both full and partial activation when the duration of the isotonic period is reduced to some 5–10 ms, so that essentially no change in the fluorescence signal

occurs before we switch back to isometric conditions. The lack of an effect from the reduced duration of isotonic contraction is consistent with our earlier finding (Brenner, 1988) that the rate constant of force redevelopment and its Ca^{2+} dependence are essentially identical to the apparent rate constant and its Ca^{2+} dependence of phase 4 of the tension transients after stepwise length releases (Huxley and Simmons, 1971). Such a view also directly accounts for the observation that after a period of isotonic shortening IANBD fluorescence changes back to the isometric steady-state level, essentially in parallel with force redevelopment; the activation level of the thin filaments rapidly readjusts to the continuous but slow recovery of strong cross-bridge attachment toward the isometric steady-state level during the period of force redevelopment. Together with the lack of evidence for deactivation during the restretch maneuver (no detectable further increase in fluorescence toward the relaxed level; cf. Figs. 1, 2), our fluorescence data imply that the rate constant of force redevelopment is not reduced or limited by changes in the state of activation of the thin filament induced by the period of unloaded shortening before force redevelopment. Instead the present fluorescence data strongly support our previous hypothesis that the Ca^{2+} dependence of force redevelopment reflects the Ca^{2+} dependence of cross-bridge cycling kinetics—specifically, a Ca^{2+} dependence of the probability of cross-bridges passing from their nonactivating (weak binding) states to their activating (strong binding) states.

If we interpret the changes in IANBD-TnI fluorescence as a signal for the state of activation of the thin filament (Trybus and Taylor, 1980), the observed fast time course of the fluorescence changes, becoming faster at higher Ca^{2+} concentrations, is consistent with our previously proposed concept that 1) Ca^{2+} controls equilibration between (at least) two states of regulated actin, that 2) this equilibration is fast compared to active turnover, and that 3) the forward rate constant (e.g., from "inactive" to the "active" states of the thin filament) becomes faster when the Ca^{2+} concentration is raised.

It might be argued that unloaded shortening might induce even faster changes in the thin filament than reported by the fluorescence signal reported here, i.e., that these changes are still induced even when the period of unloaded shortening is very much reduced (cf. Fig. 3). This, of course, cannot be ruled out. Such fast processes, however, would have even less potential to slow down the time course of force redevelopment and thus limit the rate constant of force redevelopment.

A kinetic scheme consistent with the observed behavior

In the present work we accumulated further evidence that the time course of force redevelopment after a period of lightly loaded or unloaded shortening reflects cross-bridge cycling kinetics and their Ca^{2+} dependence, as we had previously proposed (Brenner, 1985, 1988, 1990; Brenner and Eisenberg, 1986). This supports the concept that a major factor in the regulation of muscle contraction is the change in cross-bridge cycling kinetics with Ca^{2+} (rate constant f in Julian, 1969; rate modulation concept) and not solely the recruitment of additional active cross-bridges while kinetics of active cross-bridge cycling (e.g., rate constant f in the notation of Huxley, 1957; rate constant f_{app} in the related notation of Brenner, 1988) remain unchanged (cf. Podolsky and Teichholz, 1970; Haselgrove, 1973; Huxley, 1973; Parry and Squire, 1973).

We had previously pointed out that regulation via rate modulation, i.e., the Ca^{2+} dependence of the apparent rate constant for the transition of cross-bridges from the weak binding (nonactivating) states to the strong binding (activating) states, f_{app}, can be accounted for on the basis of the kinetic scheme proposed by Hill et al. (1981), if the following features (1–3) are incorporated (cf. Brenner, 1988):

1. Regulated actin exists in (at least) two forms, an active (A_{on}) and an inactive form (A_{off}).

2. Active and inactive forms of actin are in a rapid equilibrium that is controlled by the Ca^{2+} concentration via Ca^{2+} binding to troponin C. More specifically, the forward rate constant into the active form of actin becomes faster as Ca^{2+} is raised:

$$A_{off} \rightleftharpoons A_{on}$$

with

$$k_{Aoff \rightarrow Aon} = f([Ca^{2+}])$$

At least two states of the actin filament have previously been proposed on the basis of x-ray diffraction studies (e.g., Haselgrove, 1973; Huxley, 1973; Parry and Squire, 1973; Poole et al., 1995). To account for the observed changes in the second actin-based layer lines upon Ca^{2+} activation, it was proposed that the tropomyosin molecules assume at least two different positions on the surface of the actin filament (Haselgrove, 1973; Huxley, 1973; Parry and Squire, 1973; Poole et al., 1995). Two states of regulated actin were also postulated on the basis of biochemical studies to account for the steady-state behavior of myosin subfragment 1 binding to regulated actin (Greene and Eisenberg, 1978; Hill et al., 1980, 1981). More recent biochemical studies led to the proposal of a third state of regulated actin (McKillop and Geeves, 1993; Lehrer, 1994). Recent cryoelectron micrographs are consistent with the presence of at least two and possibly three positions of tropomyosin on actin that are dependent on the state of activation (Lehman et al., 1994, 1995). Thus incorporation of at least two distinctly different states of the thin filament is a well-supported assumption that was already incorporated into the original model of Hill et al. (1981).

We have chosen to consider only two states of the actin filament as in the original model of Hill et al. (1981) rather than more complex models requiring three actin states, as proposed by McKillop and Geeves (1993). This is because

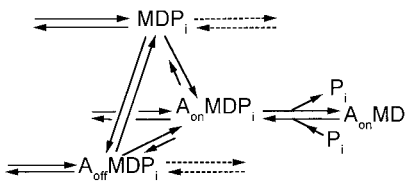
in fibers other states of the actin filament (e.g., the third "open" state of McKillop and Geeves) may become occupied only upon static attachment of rigor-like cross-bridges, while during active contraction this state is little occupied. For instance, the second actin layer line in x-ray diffraction patterns of living muscle changes long before the increase in force in the rising phase of a tetanus and shows little further change during force development (Kress et al., 1986). Furthermore, electron microscopy of native interacting thick and thin filaments (Craig and Lehman, 1999) shows the same position of tropomyosin seen in the presence of Ca^{2+} but not the rigor position; yet active turnover is possible. Finally, using fluorescence probes on troponin C, Martyn et al. (1999) do not see much effect of force-generating cross-bridges much at variance with the static attachment of rigor-like cross-bridges. This again might suggest that active turnover does not require a further change toward state of the actin filament other than that seen upon the addition of Ca^{2+}. Nevertheless, although our simple model is already sufficient to account for the data shown here, it is, of course, not ruled out that additional states of the regulated actin filament are relevant.

3. The transition into activating (strong binding) states via phosphate release reactions is much more likely when myosin heads are attached to the active form of actin than when they are detached or bound to the inactive form of actin (Scheme 1, *dashed arrows*), i.e., when they are essentially "blocked" (cf. Hill et al., 1981).

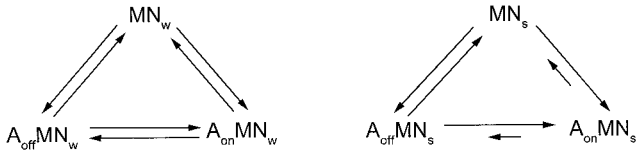
In this model myosin binds to both forms of the actin filament (Scheme 1). A myosin head in a nonactivating (weak binding) state binds with about equally low affinity to both forms of actin (Chalovich et al., 1981; Chalovich and Eisenberg, 1982; Scheme 2). Myosin heads in activating (strong binding) states bind with low affinity to the inactive form of actin, but with high affinity to the active form (cf. Hill et al., 1980; Greene and Eisenberg, 1978; Scheme 2).

Note that as a result of the difference in affinity for the two forms of actin, activating (strong binding) states of the myosin head (MgADP or no nucleotide) stabilize the active form of actin (Hill et al., 1980, 1981).

The reaction sequence shown in Scheme 3 was assumed for a myosin head attached to the active form of regulated actin. Two intermediates with inorganic phosphate are incorporated to account for a two-step P_i release reaction



Scheme I Part of a kinetic scheme illustrating the assumed control of P_i release by the state of the actin filament (cf. Hill et al., 1981). M = myosin; A_{off} = inactive actin; A_{on} = activated actin; D = ADP; P_i = inorganic phosphate; the relative lengths of the arrows indicate qualitatively the change in free energy across this reaction step.

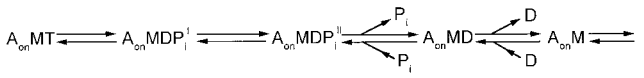


Scheme 2 N = nucleotide; N_w = nucleotide (e.g., MgATP or Mg·ADP·P_i) that yields a weak binding (nonactivating) state of the myosin head (MN_w); N_s = nucleotide (e.g., MgADP or no nucleotide) that yields a strong binding (activating) state of the myosin head (MN_s).

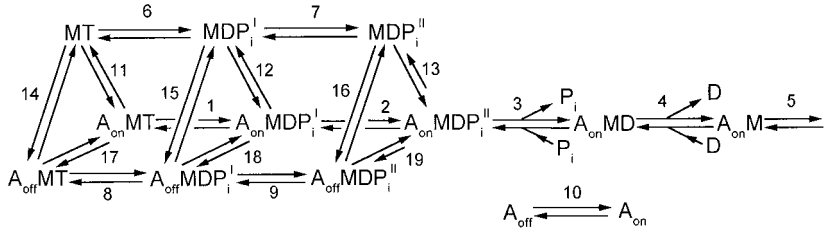
sequence (Millar and Homsher, 1990; Dantzig et al., 1992; Kawai and Halvorson, 1991). Arguments for two such intermediates have also been presented earlier on the basis of solution studies (Stein et al., 1985). Because for the present study we were only looking at processes that are rather slow compared to quick tension recovery in response to rapid stepwise length changes, we did not include, for instance, a second A_{on}MD state that could account for quick tension recovery after such rapid stepwise length changes (Huxley and Simmons, 1971; cf. Brenner, 1990).

Incorporating all of these features results in the following kinetic scheme that has been used for our modeling (Scheme 4). To be able also to compare the amplitude and kinetics of changes in thin filament activation predicted by our kinetic scheme with the amplitude and kinetics of our observed changes in IANBD-TnI fluorescence, we further assumed that the inactive form of regulated actin has high IANBD-TnI fluorescence, while the active form has only half the fluorescence, consistent with the decrease in IANBD-TnI fluorescence upon full Ca^{2+} activation (Brenner et al., 1999).

The rate constants used for the modeling are listed in Table 1; most of these are based on experimental measurements and referenced in Table 1. All simulations were started by first calculating the appropriate initial distribution among the different states. For example, to calculate isometric steady-state conditions, all myosin heads were initially assigned to state MT, and all actins to the A_{off} state (no myosin bound). To account for isometric conditions, the rate constants k_4^+ and k_4^- were set at 0.6 s^{-1} and 500 M^{-1} s^{-1}, respectively. The value for k_4^+ was selected to match the experimentally observed ATPase per head per second for isometric steady-state contraction under the same experimental conditions (Brenner, 1986). The simulation was continued until a steady-state distribution had been reached. The resulting "isometric" steady-state distribution then served as the starting point for modeling the behavior upon changing to isotonic conditions (or, in a few trials, for examining the effects of the release of inorganic phosphate from caged phosphate, by stepping up the concentration of



Scheme 3: T = ATP.



Scheme 4 Note that for the strong binding states of the myosin head, MD and M, the detached states and binding to the inactive form of actin are omitted (because of very low occupancy).

inorganic phosphate). For simulation of the switch from isometric to isotonic conditions, we only changed the values for k_4^+ and k_4^- to 500 s^{-1} and 5 M^{-1} s^{-1}, respectively. The value of 500 s^{-1} was selected to account for the rapid drop in fiber stiffness observed experimentally with the switch from isometric to isotonic steady-state conditions. (Note that with this set of rate constants for k_4^+ and k_4^-, the ATPase activity per head per second for high-speed shortening conditions is sixfold larger than the isometric ATPase activity. This is because ATPase at high-speed shortening becomes mainly limited by the nucleotide cleavage step instead of the ADP dissociation that limits isometric ATPase.) The simulation was again continued until a steady-state distribution had been reached. This was tested by varying the simulation time and comparing the final state distributions. The resulting "isotonic" steady-state distributions were then used as the starting distributions to simulate the processes upon switching from isotonic steady state back to isometric conditions, i.e., to simulate, for example, the time course of force redevelopment. For numerical evaluation of state distributions, time intervals 1/1000 of the total time required to reach steady-state distributions were found to be sufficiently short, i.e., time intervals that were half as long did not detectably change the observed time course of state distributions for the conditions examined in the present study.

Finally, the Ca^{2+} dependence of k_{10}^+ ($= k_{17}^+ = k_{18}^+ = k_{19}^+$) was assumed to be directly proportional to the Ca^{2+} occupancy of TnC. To account for the experimentally observed asymmetrical shape of the force-pCa relation, we assigned values for k_{10} according to $k_{10}^+ = k_{10}^+{}_{max} * [Ca^{2+}]^n/([Ca^{2+}50]^n + [Ca^{2+}]^n)$ with Hill coefficients $n = 4$ and $n = 1.5$ for the lower (up to $[Ca^{2+}]$ yielding 2/3 of maximum force) and the upper parts of the k_{10}^+-pCa relation. $[Ca^{2+}50]$ is the Ca^{2+} concentration for half-maximum saturation of TnC, i.e., half-maximum k_{10}^+. This is undoubtedly a great oversimplification, but it works as a first approximation. Additional experiments with other fluorescent probes, however, will be necessary to evaluate the detailed relations between Ca^{2+} occupancy of TnC and changes in k_{10}^+ with Ca^{2+}.

Fig. 7 *a* (*lower panel*) shows, for different activation levels, the time course of strong cross-bridge attachment ($A_{on}MDP_i^{II} + A_{on}MD + A_{on}M$) normalized to the maximum seen at the highest activation levels. Effects of different activation levels were simulated by only changing the forward rate constants for activation of the thin filament (A_{off} states $\rightarrow A_{on}$ states), i.e., $k_{10}^+ = k_{17}^+ = k_{18}^+ = k_{19}^+$, from 0.1 s^{-1} up to 55 s^{-1}, while the reverse rate constants, $k_{10}^- = k_{17}^- = k_{18}^- = 100 * k_{19}^-$ (note detailed balancing) were kept constant (see Table 1). The maximum value of the forward rate constants (50 s^{-1}) was limited by the fastest observed rate constants for the fluorescence change upon switching to isotonic conditions. The reverse rate constant was determined by matching the rate constant for the fluorescence change seen at the lowest activation levels (around 20 s^{-1}). The number next to each curve is the actual value assumed for $k_{10}^+ = k_{17}^+ = k_{18}^+ = k_{19}^+$. Note that under all conditions studied, the only activating (strong binding) state that is significantly occupied is $A_{on}MD$, while the occupancy of the other attached activating (strong binding) states ($A_{on}MDP_i^{II}$ and $A_{on}M$) is rather small, at most about the size of the symbols. Thus even if cross-bridges in all three states make different contributions to active force, the active force remains approximately proportional to the occupancy of state $A_{on}MD$.

Fig. 7 *a* (*upper panel*) shows the change in the fraction of inactive actin units ($A_{off} + A_{off}MT + A_{off}MDP_i^I + A_{off}MDP_i^{II}$) relative to the total number of actin units upon switching from the "isometric" steady state to "isotonic" conditions for 200 ms and back to "isometric" conditions. Assuming that the fluorescence for $A_{off} = A_{off}MT = A_{off}MDP_i^I = A_{off}AMDP_i^{II}$ equals twice the fluorescence of $A_{on} = A_{on}MT = A_{on}MDP_i^I = A_{on}MDP_i^{II} = A_{on}MD = A_{on}M$, the traces represent the time course of IANBD fluorescence, normalized to the maximum difference between fully relaxed and fully activated conditions (all actin units in A_{off} states versus all in A_{on} states). Fig. 7 *b* is an expanded view of the same traces for the 200-ms period of "isotonic" conditions (labeled "isotonic" in Fig. 7 *a*).

Fig. 8 summarizes the results of our modeling of the changes in cross-bridge distributions at different degrees of activation. Fig. 8 *a* shows the Ca^{2+} dependence of the steady-state fraction of strongly attached cross-bridges (F_{st}, *squares*) compared with 1) the rate constant for the reapproach to the isometric steady-state occupancy of strongly attached cross-bridges (i.e., the equivalent of the rate constant of force redevelopment, k_{redev}, *circles*) and 2) the apparent rate constant (k_{fluor}, +) and amplitude (A_{fluor}, ×) of the change in occupancy of A_{off} states (the equivalent of

TABLE 1 Parameters used for the modeling

$k_1^+ = 7.5\ s^{-1}$	$k_1^- = 2.5\ s^{-1(1)}$
$k_2^+ = 20.0\ s^{-1}$	$k_2^- = 80.0\ s^{-1(2)}$
$k_3^+ = 400.0\ s^{-1}$	$k_3^- = 6.0 \times 10^4\ M^{-1}\ s^{-1(2)}$
$k_4^+ = 0.6\ s^{-1(3)}$	$k_4^- = 500.0\ M^{-1}\ s^{-1}$ (isometric)
$500.0\ s^{-1(4)}$	$5.0\ M^{-1}\ s^{-1}$ (isotonic)
$k_5^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_5^- = 100.0\ s^{-1(5)}$
$k_6^+ = 7.5\ s^{-1}$	$k_6^- = 2.5\ s^{-1(1)}$
$k_7^+ = 0.2\ s^{-1}$	$k_7^- = 800.0\ s^{-1(8)}$
$k_8^+ = 7.5\ s^{-1}$	$k_8^- = 2.5\ s^{-1(1)}$
$k_9^+ = 0.2\ s^{-1}$	$k_9^+ = 800.0\ s^{-1(8)}$
$k_{10}^+ = 0–50\ s^{-1}$	$k_{10}^- = 15.0\ s^{-1(6)}$
$k_{11}^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_{11}^- = 100.0\ s^{-1(7)}$
$k_{12}^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_{12}^- = 100.0\ s^{-1(7)}$
$k_{13}^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_{13}^- = 100.0\ s^{-1(7)}$
$k_{14}^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_{14}^- = 100.0\ s^{-1(7)}$
$k_{15}^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_{15}^- = 100.0\ s^{-1(7)}$
$k_{16}^+ = 10.0^6\ M^{-1}\ s^{-1}$	$k_{16}^- = 0.1\ s^{-1(7,8)}$
$k_{17}^+ = 0–50\ s^{-1}$	$k_{17}^- = 15.0\ s^{-1(1)}$
$k_{18}^+ = 0–50\ s^{-1}$	$k_{18}^- = 15.0\ s^{-1(1)}$
$k_{19}^+ = 0–50\ s^{-1}$	$k_{19}^- = 0.15\ s^{-1(1,8)}$

$[actin]_{total} = 200\ \mu M^{(10)}$
$[myosin\ heads]_{total} = 100\ \mu M$
$[MgATP] = 1\ mM$
$[MgADP] = 0$ or $0.1\ mM$
$[P_i] = 0$ or $1\ mM^{(9)}$

(1)Rate constants of hydrolysis step from biochemical studies (e.g., Rosenfeld and Taylor, 1984; Stein, 1996; White et al., 1997).
(2)Rosenfeld and Taylor (1984); Millar and Homsher (1990); Stein (1996).
(3)To match rate constant of isometric ATPase in skinned fibers under same experimental conditions (Brenner, 1986).
(4)From biochemical studies (Siemankowski et al., 1985).
(5)Solution studies (White and Taylor, 1976); fiber studies (Goldman et al., 1982).
(6)Basic assumption of mechanism of regulation. The forward rate constant varied from 0 to 50 s^{-1} to simulate the increase in k_{fluor} with Ca^{++} concentration. The reverse rate constant was assumed to be independent of Ca^{2+}.
(7)Rate constants were assumed 1) to match fast association/dissociation kinetics seen in fibers and 2) to match in conjunction with assumed actin concentration the fraction of weakly attached cross-bridges seen in fibers (not critical for fractions >1–2%). To study the effects of a lower fraction of weakly attached cross-bridges, either lower values for the forward rate constants or faster rate constants for dissociation were used.
(8)By detailed balancing with the assumption that $A_{on}MDP_i^{II}$ is strongly attached (cf. Millar and Homsher, 1990; Stein, 1996).
(9)For testing k_{redev} versus rate constants of tension transients in response to release of P_i from caged P_i.
(10)Concentrations of actin = twice the concentration of myosin heads; absolute values of actin and myosin concentration (100 and 200 mM, respectively) were used as an approximation of actin and myosin concentrations in the contractile system. If an effective actin concentration in the mM range had been used (e.g., Brenner et al., 1986b), the second-order rate constants for actin binding (k_{11}^+, k_{12}^+, k_{13}^+, k_{14}^+, k_{15}^+, and k_{16}^+) had to be reduced accordingly, to match the fraction of weakly attached cross-bridges in fibers. Because of this coupling between actin as well as myosin concentration and the second-order rate constant for actin binding, the selection of absolute values is not critical.

IANBD fluorescence change) when we switch to unloaded shortening conditions. The apparent rate constant for the change in occupancy of A_{off} states was taken as the reciprocal of the time required to reach $1 - 1/e$ of the observed difference between isometric and isotonic steady-state lev-

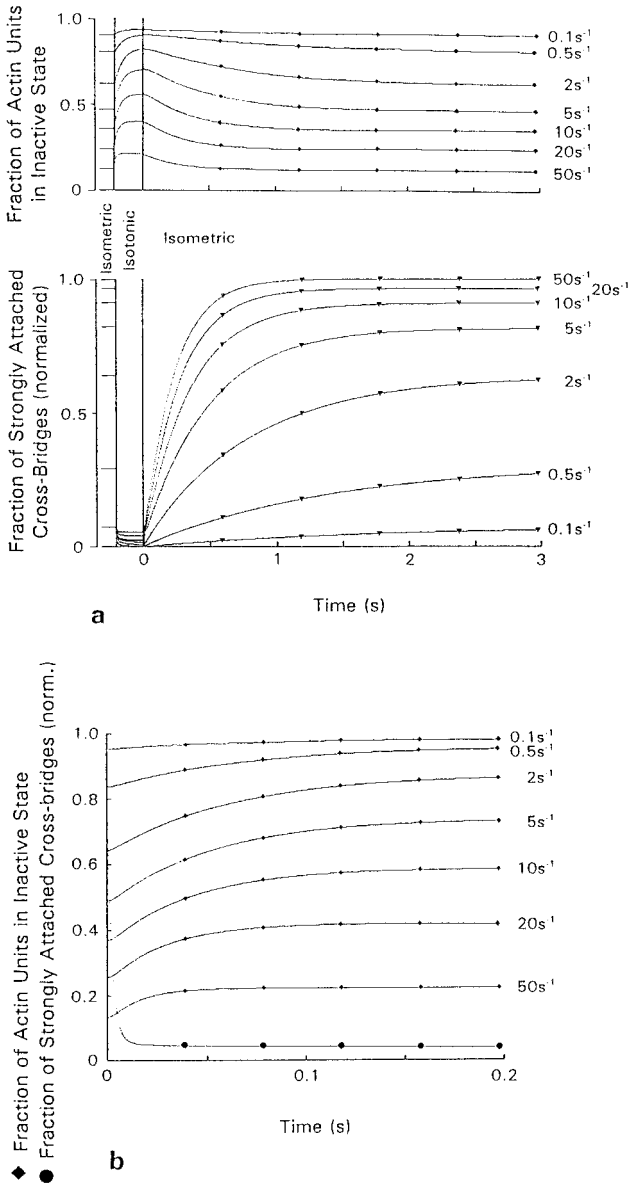


FIGURE 7 Model responses. (*a*) Modeled time course of the fraction of actin units in the inactive state ($A_{off} + A_{off}MT + A_{off}MDP_i^I + A_{off}MDP_i^{II}$; *top panel*) and of the fraction of cross-bridges in actin-attached strong binding states, $F_{st} = A_{on}MDP_i^{II} + A_{on}MD + A_{on}M$ (*bottom panel*). Numbers next to each trace are rate constants for k_{10}^+ and related forward rate constants (k_{17}^+, k_{18}^+, k_{19}^+) that describe equilibration between the assumed two states of actin (A_{off} and A_{on}). The fraction of activating (strong binding) cross-bridges, F_{st}, normalized to the fraction at a high "activation level," i.e., for $k_{10}^+ = k_{17}^+ = k_{18}^+ = k_{19}^+ = 50\ s^{-1}$. Note the close agreement with original data traces shown in Fig. 1. (*b*) Fraction of actin units in inactive states (A_{off} states, as defined in *a*), with an expanded time scale showing part from switchover to isotonic conditions up to 200 ms into isotonic period. The trace labeled with filled circles is the fraction of activating (strong binding) cross-bridges as defined in *a*. Note the fraction of activating (strong binding) cross-bridges reaches a new steady state within some 10 ms, as experimentally observed (Fig. 4). With increasing values for k_{10}^+ the fraction of actin units in inactive states reaches a new steady state faster (cf. fluorescence traces in Fig. 5). Also note the small lag that is due to the finite time required for the fraction of activating (strong binding) cross-bridges to fall toward the new low steady-state value.

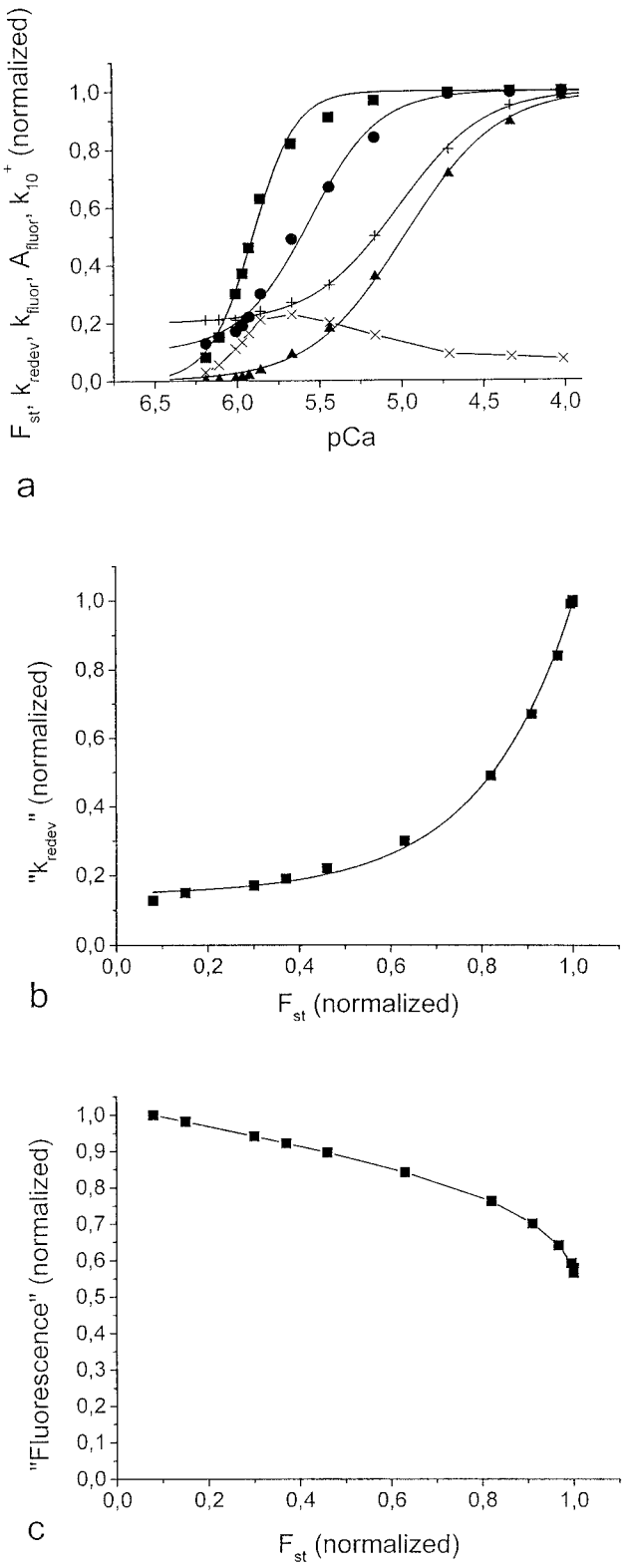


FIGURE 8 Summary of model response. (*a*) Relative positions of F_{st}-pCa (■), k_{redev}-pCa (●), k_{fluor}-pCa (+), A_{fluor}-pCa (x), and k_{10}^{+}-pCa relations (▲). F_{st} = fraction of cross-bridges in activating (strong binding states). All data, except A_{fluor}, are normalized to the value at maximum activation. Note that our modeling quite closely predicts the relative positions of force-pCa, k_{redev}-pCa, k_{fluor}-pCa, and A_{fluor}-pCa relations, as well as the magnitudes of k_{redev} and k_{fluor} at maximum activation level, with 4.11 s^{-1} and 70 s^{-1} for k_{redev} and k_{fluor}, respectively. The largest change in A_{fluor} is slightly larger than the fluorescence change seen experimentally (Fig. 6 *a*). (*b*) k_{redev} versus F_{st} for different degrees of activation (different k_{10}^{+} values), and F_{st} and k_{redev} normalized to their values at "saturating" conditions (k_{10}^{+} = 50 s^{-1}). Note the close match with the experimentally recorded plot of k_{redev} versus isometric force (Fig. 6 *b*). (*c*) Occupancy of A_{off} states versus isometric force for different degrees of activation (different k_{10}^{+}). Note the agreement with the experimentally observed plot of steady-state fluorescence versus isometric force (Fig. 6 *c*).

els. All data, except the amplitude of the fluorescence change (change in occupancy of A_{off} states, A_{fluor}), were normalized to the maximum observed at the highest [Ca^{2+}]. The amplitude of fluorescence changes (change in A_{off} states, A_{fluor}) is defined as in Fig. 7 *a*. Note the very close agreement between model and data (cf. Fig. 6), specifically the match of the relative positions of the force-pCa, k_{redev}-pCa, and k_{fluor}-pCa relations. Also note the rather close agreement for the amplitude of the fluorescence change. It should be considered, however, that because of the small amplitude of the fluorescence change at low and high activation levels, substantial scatter is present in the experimental data, especially for the kinetics of the fluorescence change at high activation levels.

Fig. 8 *b* shows, for different levels of Ca^{2+} activation, the plot of the observed rate constant for the reapproach to the isometric steady-state occupancy of strongly attached cross-bridge states (i.e., the equivalent of the rate constant of force redevelopment, k_{redev}) versus isometric steady-state occupancy of strongly attached states, F_{st} (i.e., versus the equivalent of isometric force). Different activation levels again result from changing the values for $k_{10}^{+} = k_{17}^{+} = k_{18}^{+} = k_{19}^{+}$. Note the pronounced nonlinearity as observed experimentally. Also note that the only variable was the value of $k_{10}^{+} = k_{17}^{+} = k_{18}^{+} = k_{19}^{+}$. All other parameters were kept unchanged; i.e., we did not assume any different or additional mechanisms for low and high levels of activation.

In Fig. 8 *c* the fraction of actin units in A_{off} states (in our modeling the equivalent of IANBD fluorescence) for isometric steady-state conditions is plotted versus the steady-state fraction of strongly attached cross-bridges, F_{st} (i.e., the equivalent of isometric force). It should be noted that judging only from the isometric force level, maximum activation, i.e., Ca^{2+} independence of k_{redev} and k_{fluor}, may not have been reached (cf. Fig. 8 *a*). Instead, further increases in Ca^{2+} concentration may result in further changes in k_{redev}, k_{fluor}, and steady-state fluorescence, while the steady-state isometric force is little affected. For this reason one might miss the nonlinearity in the experimental data (Fig. 6 *c*), i.e., in the high-force range of the plot of steady-state fluorescence versus isometric force.

The close match between our modeling and our data required only the three assumptions described earlier. For example, as long as equilibration between different states of actin is fast compared to force redevelopment and Ca^{2+} dependent, we can fully account for the Ca^{2+} dependence of force redevelopment, independently of the actual magnitude

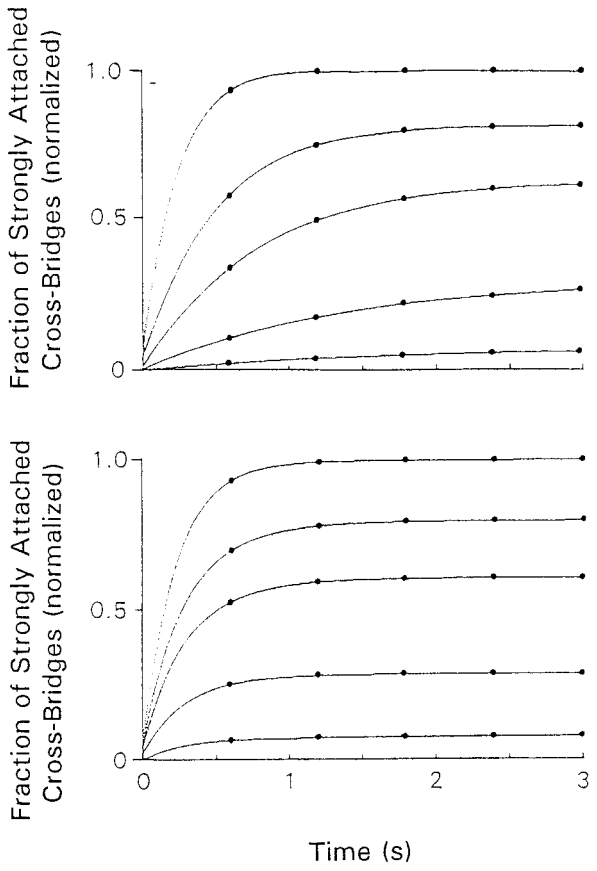


FIGURE 9 Time course of force redevelopment predicted by our model, assuming the values for k_{10}^{+} and k_{10}^{-} 1) as shown in Fig. 7 *a* (*top panel*) or 2) 100-fold smaller values, i.e., values that are an order of magnitude slower than the rate constant of force redevelopment. Note that with the second assumption (*bottom panel*), as previously pointed out (Brenner, 1988), force redevelopment is independent of the degree of Ca^{2+}-activation (i.e., independent of the value of k_{10}^{+} and related rate constants).

of the rate constants for this equilibration. Thus it is not the small value for k_{10}^{+} (and the related rate constants k_{17}^{+}, k_{18}^{+}, and k_{19}^{+}) that is rate limiting for force redevelopment at low Ca^{2+}, but rather the low occupancy of the $A_{on}MDP_i^{II}$ state that results from the small equilibrium for step 19 at low Ca^{2+}. Only the need to also account for the observed kinetics of the fluorescence changes sets the rate constants for the equilibration between the different states of the actin filament (steps 10, 17, 18, 19) to the values reported here. If, however, the equilibration between the active and inactive forms of actin is made slow, k_{redev} becomes independent of activation (Fig. 9, *bottom panel*; cf. Brenner, 1988). Other data also support the idea that the two states of activation of actin are rapidly interconvertible. Trybus and Taylor (1980) estimated the kinetics of actin interconversion using the same fluorescent probe but in solution studies with reconstituted thin filaments. Their derived rate constants are even somewhat faster than presented here. Part of the difference with our results may be that we have utilized native thin filaments in structurally intact skinned fibers in which the fluorescent probe was incorporated by an exchange procedure. Another possible difference is that strain was imposed on attached cross-bridges by the myofilament lattice in our fibers. In any case, it is reasonable that the interconversion of the actin states is rapid.

Interestingly, the simulations were rather insensitive to several other changes to the model. The following were not required to simulate the experimentally observed pCa dependence of k_{redev} and k_{fluor} and their positions relative to the force-pCa relation:

1. Assumptions about whether the MDP_i^{II} state has high or low affinity for actin (note the very low occupancy of the $A_{on}MDP_i^{II}$ state) or whether binding of this state to the inactive form of actin (A_{off}) is incorporated into the model were not required (again note the very low occupancy of the $A_{off}MDP_i^{II}$ state).

2. Assumptions about whether the hydrolysis step or one of the P_i release steps is the slowest step in the cycle were also not required. However, if one assumes that P_i release is not rate limiting (cf. White et al., 1997), it is possible to simulate the approximately twofold difference between k_{redev} and the rate constant of the transients in response to the release of phosphate from caged phosphate (k_{Pi}) (data not shown; Millar and Homsher, 1990; Dantzig et al., 1992).

3. The assumption of a large fraction of weakly attached cross-bridges was also not required. The simulations are reasonable, even if one assumes that as little as ~2% of the cross-bridges in nonactivating (weak binding) states are attached to actin. This extent of binding is quite realistic, even for physiological ionic strengths and high temperature (Kraft et al., 1995a).

4. The assumption of no difference in rate constants for association/dissociation (affinity essentially unchanged) for MT and MDP_i binding to the inactive versus active forms of actin (A_{off} and A_{on}, respectively) is also not an essential feature in accounting for the Ca^{2+} dependence of force, k_{redev}, and k_{fluor}. Such differences have been observed experimentally with ATPγS in the absence (A_{off}) and presence (A_{on}) of saturating Ca^{2+} (Kraft et al., 1992) but are not required for the successful modeling of the experimental data shown here.

5. Including a third state of the regulated thin filament (e.g., a blocked state; McKillop and Geeves, 1993) again allowed simulation of the data shown here, as long as equilibration between the different states of the actin filament is rapid and Ca^{2+}-controlled, as discussed in this section.

Three simplifying assumptions have been made in the present modeling. First, the strain dependence of kinetics has been ignored. The close agreement of the model response with the data in the absence of the additional adjustable parameters required to describe strain dependence is surprising, because the rate constants certainly exhibit strain dependence. However, as discussed previously (Brenner, 1990), close spacing of binding sites on actin results in only narrow distributions of different strains. Nevertheless, in future studies in which strain dependence becomes a relevant factor, the modeling will need expansion to include

such strain dependence. Second, we have so far not accounted for the fact that one troponin-tropomyosin complex controls seven actin monomers. This effect will have to be included when trying to account for all of the detailed relations from Ca^{2+} binding to troponin C up to Ca^{2+} dependence of force and the rate constant of force redevelopment. Finally, cooperative activation of actin filaments by strong cross-bridge attachment has also not been included, although such cooperativity was proposed to exist in solution (e.g., Bremel and Weber, 1972; Greene and Eisenberg, 1980; Nagashima and Asakura, 1982; Williams et al., 1988) and in fibers (Swartz et al., 1990). However, it was shown later that in fibers cooperative activation by strong cross-bridge attachment appears to be rather limited (Cantino et al., 1993; Kraft et al., 1995b). This is supported by the rather small changes in fluorescence observed when we switched to isotonic conditions (low occupancy of strongly attached cross-bridge states). Thus it appears that the effect of strong cross-bridge attachment on the activation of native thin filaments in Ca^{2+}-activated fibers under our experimental conditions is small. This lack of a large effect by the strong attachment of native cross-bridges within the myofilament lattice may not be very surprising because the potential to activate thin filaments was proposed to depend on the ability to bind with much higher affinity to the active form of actin compared to binding to the inactive form. However, the strain of strongly attached cross-bridges in the myofilament lattice in fibers very much reduces binding strength and thus might result in much reduced potential to activate the thin filament.

Relation to previous work

In our previous solution studies we showed that Ca^{2+} has little effect on the binding of nonactivating (weak binding; e.g., myosin-ATP) states to actin-troponin-tropomyosin (Chalovich et al., 1981; Chalovich and Eisenberg, 1982), although the steady-state ATPase rate at saturating actin was increased by Ca^{2+}. From these observations we suggested that some process other than cross-bridge attachment (e.g., a horizontal step in the common kinetic schemes) is regulated in striated muscle. On the basis of detailed balance arguments we suggested that steps associated with phosphate release were likely to be regulated by Ca^{2+}-dependent blocking or unblocking. There are two ways that P_i release could be regulated. In one case, the rate constant for P_i release is directly controlled (increased or decreased). In the alternative case, which we presented in a formal model (Hill et al., 1981), P_i release is indirectly regulated by Ca^{2+}-controlled switching between active and inactive states of actin, with P_i release being fast when myosin is attached to the active form of actin. That is, an increase in free Ca^{2+} causes the actin filament to shift toward the active form, and a greater fraction of ATP hydrolysis occurs along the pathway that has a rapid rate constant for P_i release. Rosenfeld and Taylor (1985) subsequently showed that the steady-state rate of P_i release becomes faster with Ca^{2+}, but their results did not distinguish between the two possible mechanisms described above. In fact, because of the lack of pre-steady-state kinetic data, no previous biochemical study could distinguish between regulation via the number of actively cycling myosin heads (recruitment principle; Podolsky and Teichholz, 1970) or regulation via reaction kinetics, e.g., via control of the (apparent) rate constant for the transition of cross-bridges into their (strong binding) activating states (Julian, 1969). It was by using the rate constant of force redevelopment as a measure of cross-bridge cycling kinetics in skinned fibers (Brenner and Eisenberg, 1986) that the first experimental evidence was provided that in skeletal muscle fibers Ca^{2+} controls the probability that cross-bridges enter the force-generating states (rate modulation concept; Brenner, 1988). Concerning the mechanism by which calcium can exert such control, we proposed that it is by control of equilibration between inactive and active states of actin, as defined in the model of Hill et al. (1981), and that equilibration between the different states of the actin filament is Ca^{2+} controlled and fast compared to force redevelopment (Brenner, 1988).

Several other alternatives for Ca^{2+}-based regulation have been eliminated from our consideration on the basis of other experimental evidence. Ca^{2+} control over the affinity of nonactivating (weak binding) states of myosin, such as M·ATP or M·ADP·P_i, for actin appeared unlikely for several reasons. As already mentioned, this disagrees with our earlier solution studies in which this binding was directly measured. It is also inconsistent with our fiber studies showing that the actin affinity of nonactivating (weak binding) states of myosin is not altered much by Ca^{2+}. For example, in the presence of the slowly hydrolyzable ATP-analog ATPγS (Goody and Eckstein, 1971; Bagshaw et al., 1973), essentially all cross-bridges occupy weak binding (nonactivating) states of the myosin head, independent of the Ca^{2+} concentration. Although another laboratory initially reported a strong Ca^{2+} effect on actin affinity in the presence of ATPγS (Dantzig et al., 1988), it was later shown that this effect resulted from incomplete nucleotide saturation in the presence of Ca^{2+}; with saturating ATPγS concentrations, Ca^{2+} has little if any effect on the actin affinity of myosin heads with this nucleotide analog (Kraft et al., 1992). Thus it appeared unlikely that a higher actin affinity of nonactivating (weak binding) states in the presence of Ca^{2+} is a main factor in regulation.

Direct modulation of the rate constant of P_i release also appeared unlikely because several laboratories have measured the effect of Ca^{2+} on the force transients that occur in response to release of P_i from caged P_i (Millar and Homsher, 1990; Dantzig et al., 1992; Walker et al., 1992). These results cannot be explained by a variable rate constant of P_i release along a single pathway, but they are consistent with the model of Hill et al. (1981) (Scheme 4).

As a further possibility to account for Ca^{2+} effects on the rate constant of force redevelopment, a cooperative model was proposed by Campbell (1997). The model predicts that

the rate constant of force redevelopment should become faster when higher loads are applied during the isotonic period before force redevelopment. However, contrary to that prediction, the rate constant of force redevelopment has been shown to be independent of the load during the preceding isotonic shortening (Brenner, 1988).

Other proposals have recently been made to explain the Ca^{2+} dependence of force redevelopment. These other proposals are somewhat similar to the concept we had developed based on the model of Hill et al. (1981). One of these proposals is that Ca^{2+} controls the rate constant of the hydrolysis step or of a hypothetical step leading to the AM·ADP·P_i state from which P_i is released (Regnier et al., 1995). In this model, however, it was not specified how the movement of tropomyosin might alter this rate constant. One might assume that this hypothetical reaction step occurs more quickly when actin is in an active form and that Ca^{2+} controls a rapidly established distribution between inactive and active forms of actin. With these assumptions, such a concept would be identical with ours except that the controlled step is not one of the P_i release reactions but an earlier step in the cycle. The hydrolysis step cannot serve such a purpose because it occurs almost as fast when the myosin head is detached from actin and thus cannot be effectively regulated by a change in the state of the thin filament. Nevertheless, an extra step between hydrolysis and P_i release reactions could serve such a purpose if it only occurs when the myosin head is attached to the active form of actin but is essentially blocked when the head is detached from or attached to the inactive form of actin.

Another group of models (e.g., Landesberg and Sideman, 1994; Hancock et al. 1997; Regnier et al., 1998) are also based on the assumption that actin exists in two forms, only one of which allows cross-bridges to effectively enter the force-generating states. In that sense these models are not different from our proposal (Brenner, 1988) based on the model of Hill et al. (1981). The only difference is that in these models the various intermediates defined in the model of Hill et al. (1981) are not specified, but rather several reaction steps are consolidated into apparent rate constants (e.g., f, f', g, and g'). It should be noted that the definition of the rate constants (f, f', g, and g') used by Hancock et al. (1997), Landesberg and Sideman (1994), and Regnier et al. (1998) differs from our notation used in our earlier work (Brenner, 1988). To demonstrate that Ca^{2+} affects the probability that cross-bridges enter force-generating states we had defined f_{app} as the average probability that a cross-bridge in weak binding (nonactivating) states, whether free or bound to either the active or inactive forms of actin, enters the activating (strong binding) states. This definition was based on the assumption that the interchange between different forms of actin as well as the binding/dissociation of myosin in nonactivating (weak binding) states to and from actin are fast processes compared to active cycling. Hancock et al. (1997), Landesberg and Sideman (1994), and Regnier et al. (1998), however, defined their rate constants f and g separately for the inactive and active forms of actin. Thus, for instance, f_{app} (by our definition) equals $f/(1 + k^-/k^+)$ of their models, where k^+ and k^- are the rate constants of the interchange between the two states of actin in their models. Aside from this difference, these other models are simplified versions of the model of Hill et al. (1981), and thus their ability to predict the Ca^{2+} effect on the rate constant of force redevelopment is not surprising but supports our original proposal that rapid equilibration between different states of the actin filament is a possible mechanism that can account for the Ca^{2+} dependence of force redevelopment and the proposed rate-modulation principle of regulation. As already pointed out, the Ca^{2+} dependence of force redevelopment is not a sensitive parameter for the actual magnitude of the rate constants of equilibration, as long as equilibration is fast and Ca^{2+} controlled. Thus it is not surprising that there is some variability in the magnitude of these rate constants assumed for these different models.

Toward the question of how tropomyosin could exert its regulatory effect on a molecular level, we have discussed in our previous work (Brenner et al., 1999) the possibility that attachment of a myosin head in its nonactivating (weak binding) states to activated thin filaments results in distortion of the catalytic domain of the myosin head, thereby destabilizing the binding of the γ-phosphate to the nucleotide binding pocket and thus promoting the release of inorganic phosphate after the hydrolysis step. Such distortion within the catalytic domain induced upon binding of myosin in nonactivating (weak binding) states to the activated thin filament could reflect a molecular basis for the acceleration of P_i release by actin (actin activation) once the γ-phosphate has been cleaved from the ATP molecule. In this view, one essential role of attachment of cross-bridges in nonactivating (weak binding) states to the activated thin filament is the promotion of the phosphate release and stabilization of a subsequent force-generating conformation of the actomyosin complex, which is consistent with our previous observation that weak cross-bridge attachment is an essential intermediate for force generation (Brenner et al., 1991; Kraft et al., 1995a) and consistent with the "two-step" phosphate release mechanism derived from phosphate transients (Millar and Homsher, 1990; Kawai and Halvorson, 1991; Dantzig et al., 1992).

In conclusion, in our previous work we had pointed out that regulation of muscle contraction by modulation of the probability that cross-bridges enter the force-generating states (rate modulation principle) can be accounted for on the basis of the model of Hill et al. (1981) if the interconversion between the two states of actin is fast and is Ca^{2+} controlled. With our newly developed approach to the characterization of kinetics of changes in IANBD-TnI fluorescence at different Ca^{2+} concentrations we now provided experimental evidence that equilibration among different states of the thin filament might indeed be fast compared to active cross-bridge cycling and Ca^{2+} controlled. We have shown that the model of Hill et al. (1981) can fully account for the Ca^{2+} dependence of various parameters measured in

a muscle fiber. Other modeling, not shown here, indicates that this concept can also account for changes in the Ca^{2+} dependence of force redevelopment in the presence of different concentrations of inorganic phosphate, including the Ca^{2+} independence of force redevelopment at intermediate P_i concentrations (Tasche et al., 1998). The model described here is simple, with only two states of the actin filament. The major tenants of this model are that the rate constants controlling the distribution between these two states are rapid and Ca^{2+} controlled. There is no need to postulate control over the attachment of nonactivating (weak binding) cross-bridges or the presence of additional intermediates in the pathway of ATP hydrolysis. Furthermore, the present results support the contention that the rate constant of force redevelopment is a measure of cross-bridge cycling kinetics, i.e., of the probabilities that cross-bridges pass from nonactivating (weak binding) to activating (strong binding) states of the myosin head (e.g., f_{app} in our previous notation; Brenner, 1988), and back from the activating to the nonactivating states (e.g., g_{app}).

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