Role of Ca^{2+} and Cross-Bridges in Skeletal Muscle Thin Filament Activation Probed with Ca^{2+} Sensitizers

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ABSTRACT Thin filament regulation of contraction is thought to involve the binding of two activating ligands: Ca^{2+} and strongly bound cross-bridges. The specific cross-bridge states required to promote thin filament activation have not been identified. This study examines the relationship between cross-bridge cycling and thin filament activation by comparing the results of kinetic experiments using the Ca^{2+} sensitizers caffeine and bepridil. In single skinned rat soleus fibers, 30 mM caffeine produced a leftward shift in the tension-pCa relation from 6.03 ± 0.03 to 6.51 ± 0.03 pCa units and lowered the maximum tension to 0.60 ± 0.01 of the control tension. In addition, the rate of tension redevelopment (k_{tr}) was decreased from 3.51 ± 0.12 s^{-1} to 2.70 ± 0.19 s^{-1}, and V_{max} decreased from 1.24 ± 0.07 to 0.64 ± 0.02 M.L./s. Bepridil produced a similar shift in the tension-pCa curves but had no effect on the kinetics. Thus bepridil increases the Ca^{2+} sensitivity through direct effects on TnC, whereas caffeine has significant effects on the cross-bridge interaction. Interestingly, caffeine also produced a significant increase in stiffness under relaxing conditions (pCa 9.0), indicating that caffeine induces some strongly bound cross-bridges, even in the absence of Ca^{2+}. The results are interpreted in terms of a model integrating cross-bridge cycling with a three-state thin-filament activation model. Significantly, strongly bound, non-tension-producing cross-bridges were essential to modeling of complete activation of the thin filament.

INTRODUCTION

Contraction in striated muscle is initiated by the binding of Ca^{2+} to troponin C. This triggers a number of conformational changes in the thin filament that ultimately allow the formation of tension producing actomyosin cross-bridges. Furthermore, evidence has accumulated that at least some cross-bridge states can affect the Ca^{2+} sensitivity of the thin filament. Apparent calcium binding to the thin filament is decreased during shortening contractions (Gordon and Ridgway, 1987), and cross-bridge detachment during relaxation is accompanied by an increase in the free Ca^{2+} (Caputo et al., 1994). These results indicate that the presence of attached or cycling cross-bridges alters the Ca^{2+} affinity of the thin filament. Moreover, Ca^{2+} binding to fluorescently labeled TnC has been shown to increase in the presence of bound myosin (Guth and Potter, 1987; Morimoto, 1991). Hence it appears that myosin binding plays an important role in thin filament activation and may be required for full activation of the thin filament.

Based on tropomyosin movements observed by fluorescence polarization (Borovikov et al., 1993) and EM reconstructions (Vibert et al. 1997), three distinct structural states of the thin filament have been identified, corresponding to the presence or absence of Ca^{2+} and bound cross-bridges. The presence of at least three tropomyosin positions is also suggested by x-ray diffraction studies (Popp and Maeda, 1993). This has led to a model in which the thin filament exists in three distinct states (McKillop and Geeves, 1993). These three states can be interpreted in terms of the movement of tropomyosin across the thin filament (Holmes, 1995). In the absence of Ca^{2+}, myosin binding to actin is "blocked" by tropomyosin. Upon binding Ca^{2+}, the tropomyosin moves to an intermediate "closed" position in which the myosin binding site on actin is only partially exposed, allowing only weakly bound cross-bridges. Strongly bound, tension-producing cross-bridges occur only after cross-bridge binding causes an additional movement of tropomyosin, thereby producing the thin-filament "open" state. Thus, in this three-state model, full activation of the thin filament requires the presence of both Ca^{2+} and bound cross-bridges. There is an extensive literature devoted to the cross-bridge cycle, and a number of models with multiple cross-bridge states have been proposed. It has yet to be determined which cross-bridge states are responsible for the transition of the thin filament from the closed to the open state. It is our goal to understand the interaction between the thin-filament activation mechanism and cross-bridge cycling.

Conceptually, two distinct mechanisms for increasing the Ca^{2+} sensitivity of contraction can be inferred from the three-state model of thin-filament activation outlined above. The Ca^{2+} affinity of TnC can be increased, leading to a greater percentage of the thin filament in the closed state and available for cross-bridge binding. Alternatively, the number of cross-bridges binding to the thin filament in the closed state can be increased independently of Ca^{2+} binding, promoting the transition to the open state and increasing tension production. By comparing kinetics of contraction in the presence of Ca^{2+} sensitizers that differentially act on the independent activation mechanisms of Ca^{2+} binding and cross-bridge binding, it should be possible to identify cross-bridge states that are involved in thin-filament activation. We have examined the kinetics of contraction in skinned

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fibers in the presence of two known Ca^{2+} sensitizers, caffeine and bepridil, in an attempt to better define the mechanism of thin-filament activation.

Caffeine has been used extensively in muscle research, primarily because of its ability to mobilize intracellular Ca^{2+}. In intact fibers caffeine also alters the mechanics of contraction in the form of increased tetanic tension and a prolonged relaxation (e.g., Allen and Westerblad, 1995; Westerblad and Allen, 1996). These effects are typically attributed to caffeine-mediated changes in intracellular Ca^{2+} handling. However, caffeine has been shown to exert effects on the myofilaments as well. In skinned fibers caffeine produces an increase in the Ca^{2+} sensitivity of tension production (Wendt and Stephenson, 1983; Powers and Solaro, 1995; Palmer and Kentish, 1996, 1997), while decreasing the maximum tension (Wendt and Stephenson, 1983) and MgATPase activity (Powers and Solaro, 1995), apparently in the absence of changes in the binding of Ca^{2+} to troponin C (Powers and Solaro, 1995). More recently, caffeine has been shown to increase the rate of relaxation in skinned cardiac trabeculae (Palmer and Kentish, 1997). These skinned fiber studies strongly suggest that some of the effects of caffeine may be mediated through changes in cross-bridge kinetics. To address this possibility, we examined the effects of caffeine on cross-bridge kinetics.

In contrast to the apparent cross-bridge-mediated effects of caffeine, bepridil produces an increase in the Ca^{2+} sensitivity through direct effects on troponin C (Solaro et al., 1986; Kleerekoper et al., 1998). Therefore, bepridil is predicted to produce effects on kinetics solely through thin-filament-mediated mechanisms. In this study, we use caffeine and bepridil to differentially alter the distribution of thin filament activation states (i.e., blocked, closed, and open). By comparing the effects of caffeine and bepridil on the kinetics and Ca^{2+} sensitivity of contraction, the relationship between the cross-bridge cycle and thin-filament activation is revealed.

MATERIALS AND METHODS

Skinned fiber preparation

Multiple activations and relaxations were required to demonstrate the reversibility of the experiments performed in this study. For this reason soleus fibers, which are particularly robust, were used. Single skinned fibers were obtained from the soleus muscle of female Sprague-Dawley rats as previously described (Wahr et al., 1997). Briefly, small bundles of fibers were soaked in relaxing solution containing 50% glycerol at 4°C overnight and stored at −20°C until use within 1 month. On the day of the experiment, individual fibers were carefully pulled from a bundle and mounted between a force transducer and galvanometer. The sarcomere length was set at 2.50–2.55 μm by adjusting the overall fiber length. A HeNe laser was directed through the fiber to monitor the sarcomere diffraction position. Feedback control of either the tension or sarcomere length was provided by a computer software system that simultaneously recorded tension, galvanometer deviation, and the position of the sarcomere diffraction pattern (Wahr et al., 1997).

Instantaneous stiffness

Instantaneous stiffness was measured by applying a small (~0.1% of the fiber length) 1-kHz sinusoidal length change to the preparation. A frequency of 1 kHz was chosen because the stiffness is not dependent on the frequency between 1 kHz and 3 kHz in this preparation (unpublished observation). The amplitudes of 20 peaks in the tension and length traces were averaged, and the stiffness was defined as the average tension change divided by the average change in fiber length. Changes in active instantaneous stiffness were assumed to be proportional to changes in the number of strongly bound cross-bridges (Huxley and Simmons, 1971).

Solutions

All experiments were performed at 15°C. Solutions were prepared using the program of Fabiato (1988) to calculate the concentration of metals, ligands, and metal-ligand complexes. Solutions for activating (pCa 4.5) and relaxing (pCa 9.0) the fibers contained 7 mM EGTA, 20 mM imidazole, 1 mM Mg^{2+}, 4 mM MgATP, and 14.5 mM creatine phosphate (pH 7.0). Ionic strength was adjusted to 180 mM by added KCl. Intermediate pCa levels were obtained by mixing pCa 4.5 and pCa 9.0 solutions in appropriate amounts. It was important that the pCa levels were identical in both the presence and absence of drug to ensure that changes in tension were due to the presence of the drug and not to small changes in the Ca^{2+} level. Therefore, intermediate pCa solutions were mixed without added caffeine or bepridil. These solutions were then divided into smaller volumes, and appropriate amounts of caffeine or bepridil were added to these small volumes. Caffeine was added as a powder, and bepridil was added from a freshly made 10 mM ethanol stock. At the levels of bepridil used in this study, concentrations of ethanol in the bepridil solutions were ≤1%. Control experiments performed with 1% ethanol indicated that there is no discernible effect of the ethanol on contractile function (data not shown).

Rate of tension redevelopment (k_{tr})

The rate constant of tension redevelopment (k_{tr}) was obtained by rapidly releasing the activated fiber by 15% of the initial fiber length for 20 ms and then rapidly reextending the fiber to its initial length. The resulting tension redevelopment after the reextension was then fit by a single exponential equation in the form $P = P_{init} + A \exp(-k_{tr} * t)$, where P is tension, P_{init} is an initial tension, t is time, and A is the amplitude of the redeveloped tension with rate constant k_{tr} (Brenner, 1988). The sarcomere length during k_{tr} measurements was maintained by feedback control of the laser diffraction pattern at 2000 Hz (Wahr et al., 1997).

Unloaded shortening velocity

The maximum unloaded shortening velocity was obtained either by extrapolation from the force-velocity curves (V_{max}) or by slack test (V_0). Force-velocity curves were constructed by maximally activating the fiber at pCa 4.5 and then allowing the fiber to shorten at a submaximum tension for 1 s (Fig. 7, *inset*). The preselected level of submaximum tension was feedback controlled at 2000 Hz. The fiber was then relaxed in pCa 9.0 solution, and the process was repeated at a different tension level. Shortening velocities were then calculated as the slopes of fiber length versus time. V_{max} was then extrapolated by fitting Hill's hyperbolic equation to the force-velocity plots in the form $V = (b(P_0 + a)/(P + a)) - b$, where P and P_0 are tension and maximum tension and a and b are parameters with units of tension and velocity, respectively. Curvature was quantified as a/P_0. Slack tests were performed by rapidly shortening maximally activated fibers beyond the slack length and then noting the time required for tension to develop just above the baseline. This was repeated for 10 different length steps. The size of the length step was then plotted versus the time required for tension to appear. The slope of this plot then gave V_0.

Data collection, curve fitting, modeling, and statistics

Multiple data sets were obtained from each fiber. In general, experiments under control conditions preceded and followed experiments with either caffeine or bepridil. In this way fibers served as their own control. Likewise, comparison of the control experiments before introduction of a drug and after its removal indicated that the effects of caffeine and bepridil were completely reversible. The various concentrations of the drugs were introduced in no particular order, and fibers were washed twice in relaxing solution containing a new drug concentration before data were collected.

Curves were fit using the program Igor (WaveMetrics, Lake Oswego, OR). Tension-pCa curves were fit to the Hill equation in the form $P = P_0/(1 + 10^{(-nH(pK - pCa))})$, where P_0 is the maximum developed tension, n_H is the Hill coefficient, and pK is the pCa at 0.5 P_0. The model was evaluated numerically using the computer programs KFIT/KSIM (Dr. Neil Millar).

The data are presented as mean ± SEM, unless otherwise indicated. Significance was determined by a paired Student's *t*-test or a pairwise ANOVA, depending on the number of data sets. The confidence level was set at $p < 0.05$.

TABLE 1 Summary of tension-pCa fits in the presence of 0–30 mM caffeine or 0–100 μM bepridil

Concentration	P/P_0	n_H	pCa_{50}	N
[caffeine] (mM)				
0	1.0	1.54 ± 0.07	6.03 ± 0.03	22
10	0.86 ± 0.01*	1.42 ± 0.05	6.37 ± 0.03*	19
20	0.71 ± 0.01*	1.38 ± 0.06	6.47 ± 0.03*	19
30	0.60 ± 0.01*	1.37 ± 0.05	6.51 ± 0.03*	19
[bepridil] (μM)				
0	1.0	1.57 ± 0.07	6.03 ± 0.02	10
10	0.98 ± 0.02	1.73 ± 0.13	6.08 ± 0.03	3
50	0.96 ± 0.02	1.51 ± 0.11	6.17 ± 0.03*	5
100	0.92 ± 0.02*	1.34 ± 0.20*	6.30 ± 0.02*	5

Values given as mean ± SEM.
*Significantly different from control (ANOVA, $p < 0.05$).

RESULTS

Tension-pCa

In agreement with the results of other laboratories, caffeine produced a significant increase in the Ca^{2+} sensitivity of tension production (Fig. 1 and Table 1). The application of 30 mM caffeine produced a leftward shift of the tension-pCa relation of 0.47 ± 0.03 pCa units ($n = 19$), with no significant change in the Hill coefficient. Bepridil (100 μM) produced a similar shift of 0.27 ± 0.02 pCa units ($n = 5$; Fig. 2). Thus both caffeine and bepridil are effective Ca^{2+} sensitizers. However, the application of caffeine also resulted in a significant decrease (~40% at 30 mM) in tension at all concentrations studied (Table 1). Furthermore, from Fig. 1 it appears that a greater concentration of caffeine is required to suppress tension than is required to shift the tension pCa curve, indicating that caffeine affects at least two processes in contraction. In contrast, bepridil had no statistically significant effect on maximum tension production up to 50 μM and produced only a small (~5%) decrease at 100 μM (Table 1). The effects of both caffeine and bepridil were easily reversed by washing the fiber in caffeine- and bepridil-free relaxing solution.

Instantaneous stiffness

Instantaneous stiffness is thought to be related to the number of strongly interacting cross-bridges (Huxley and Simmons, 1971). Because the decreased tension observed with caffeine could be the result of a decrease in the number of strongly bound cross-bridges, it was of interest to examine

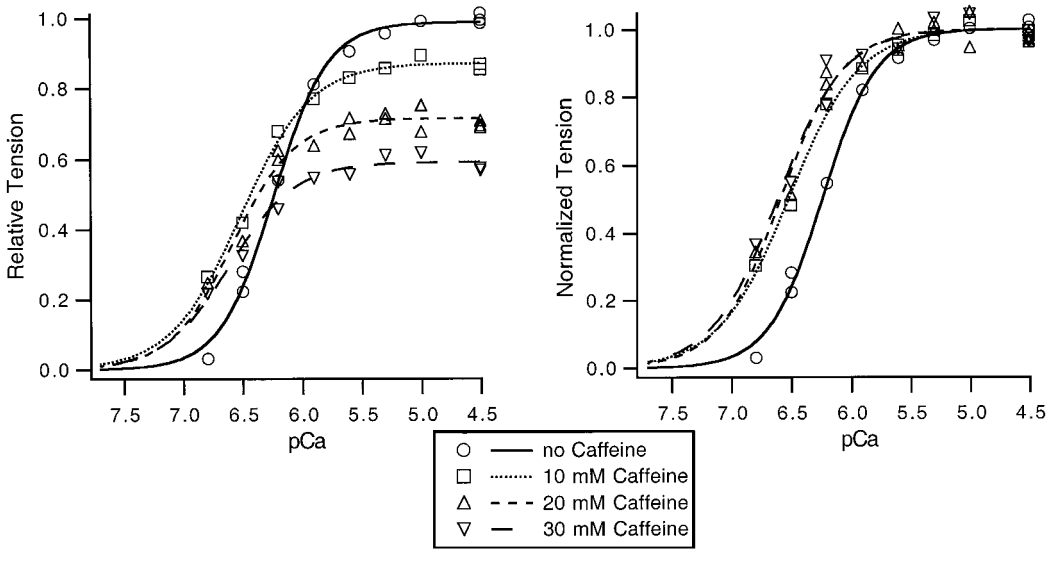

FIGURE 1 Example of tension-pCa relations from a single rat soleus fiber at 15°C in the presence of 0–30 mM caffeine. Data are fit by the Hill equation. (*Left*) Tension shown relative to the maximum control tension (pCa 4.5, no caffeine). (*Right*) Curves normalized to maximum tension to emphasize shifts in Ca^{2+} sensitivity.

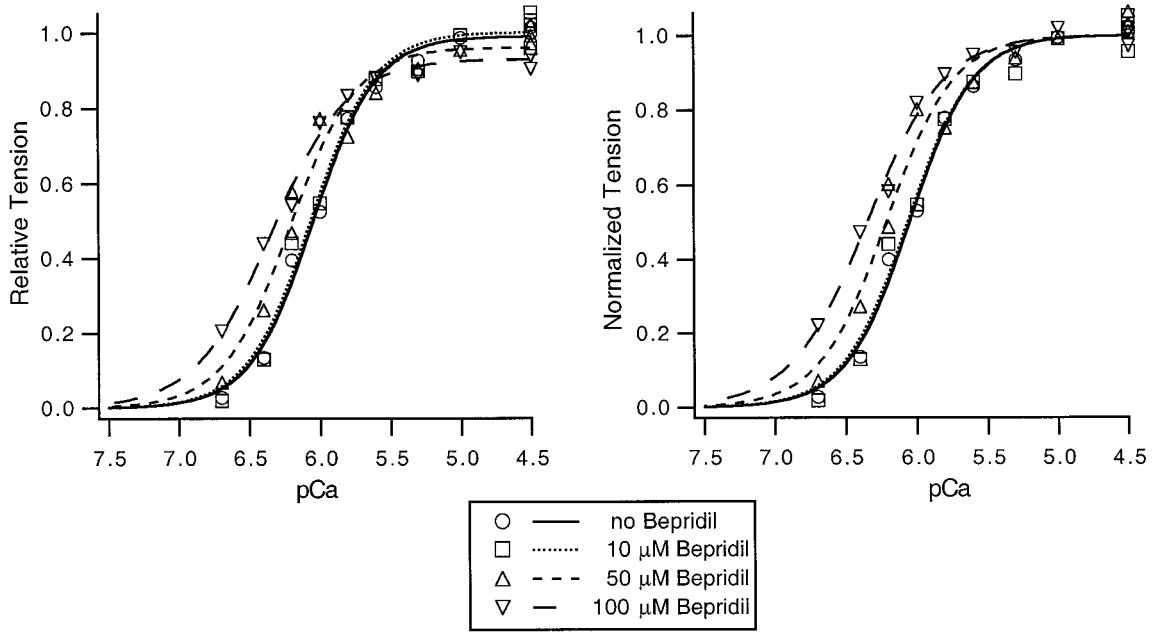

FIGURE 2 Example of tension-pCa relations from a single rat soleus fiber at 15°C in the presence of 0–100 μM bepridil. Data are fit by the Hill equation. (*Left*) Tension shown relative to the maximum control tension (pCa 4.5, no bepridil). (*Right*) Curves normalized to maximum tension to emphasize shifts in Ca^{2+} sensitivity.

the effects of caffeine on the instantaneous stiffness of the fibers. Bepridil did not produce the decrease in tension observed with caffeine; therefore stiffness studies with bepridil were not performed. If the caffeine-mediated decrease in tension is due solely to a decrease in the number of strongly bound cross-bridges, the slope of the tension-stiffness plots will be identical in the presence or absence of caffeine. As shown in Fig. 3, the decrease in tension observed with the application of caffeine was not accompanied by a similar change in stiffness. There is a significant decrease in the slope of the tension-stiffness plots, to 75% of the control slope when 30 mM caffeine was used, indicating that the decrease in tension is not due solely to a decrease in the number of cross-bridges. Although there was a significant decrease in tension at all caffeine concentrations studied, there was no decrease in stiffness until 20 mM caffeine was applied, and at 30 mM caffeine the decrease in maximum stiffness was only 16% (Table 2), which is considerably less than the 40% (Table 1) decrease in maximum tension seen in the same fibers under identical conditions.

Interestingly, 30 mM caffeine significantly increased the stiffness of relaxed fibers (pCa 9.0) by 75 ± 7% (n = 9) (Fig. 4), indicating that caffeine leads to the formation of strong cross-bridges, even in the absence of Ca^{2+} activation. This view is further supported by a similar increase in Ca^{3+} independent tension of 74 ± 11% in these fibers at 30 mM caffeine. The increase in Ca^{3+} independent stiffness and tension with caffeine was observed regardless of whether the fiber had been previously activated and was abolished upon return to caffeine-free relaxing solution. In conjunction with the increased stiffness at low pCa, the stiffness-pCa curves showed a lower Hill coefficient in the presence of caffeine (Table 2). This decreased n_H was not observed in the tension-pCa curves, indicating that there is a population of strongly bound cross-bridges producing low tension at low Ca^{2+} in the presence of caffeine.

Rate of tension redevelopment

To characterize the effects of caffeine on cross-bridge kinetics, the rate of tension redevelopment (k_{tr}) (Brenner, 1988) was examined. As illustrated in Fig. 5 and Table 3, caffeine produced a marked decrease in k_{tr}. At maximum Ca^{2+} activation, k_{tr} was reduced from 3.51 ± 0.12 s^{-1} (n = 12) to 2.70 ± 0.19 s^{-1} (n = 9) with the addition of 30 mM caffeine. Because k_{tr} has been shown to be decreased by a reduction in the thin-filament activation level (Brenner, 1988; Wahr et al., 1997), which results in lower tension generation, it might be argued that the decreased k_{tr} with caffeine is a reflection of a thin-filament effect that decreases both the tension and k_{tr}. A plot of k_{tr} versus tension (Fig. 6) indicates that the slowing of k_{tr} with caffeine is not mediated by the effect of caffeine on tension, and at a given submaximum tension k_{tr} is increased. This uncoupling of tension and k_{tr} by caffeine indicates an action at the level of cross-bridges and not at the thin filament. In contrast, bepridil had no significant effect on k_{tr}, at either maximum or submaximum tension levels (Fig. 6), indicating no effect of bepridil at the level of the cross-bridges.

Unloaded shortening velocity

Force-velocity curves were constructed at maximum Ca^{2+} activation in the presence of 0–30 mM caffeine and in the

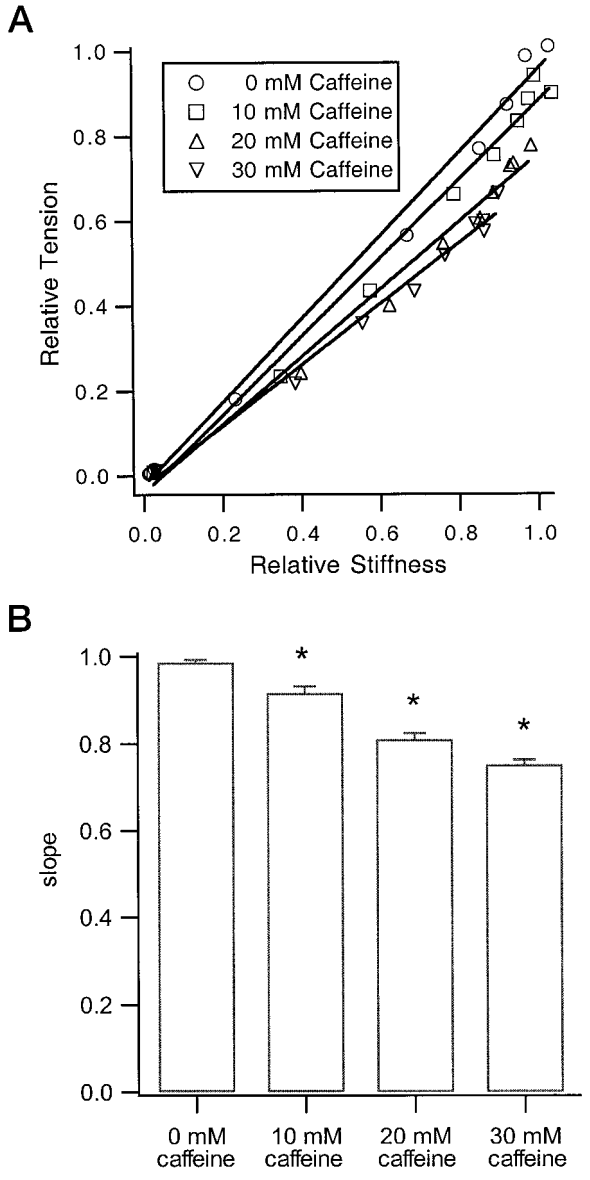

FIGURE 3 Relation between tension and stiffness in the presence of 0–30 mM caffeine. (*A*) Relative tension versus relative stiffness from a single rat soleus fiber. Tension and stiffness are shown relative to the maximum control tension and stiffness (pCa 4.5, no caffeine). (*B*) Summary of slopes of relative tension versus relative stiffness from six fibers. * Significantly different from control.

presence of 0–100 μM bepridil (Fig. 7 and Table 4). Bepridil had no significant effect on the force-velocity curves. However, although caffeine had no effect on the curvature (a/P_0), the unloaded shortening velocity (V_{max}) extrapolated from these curves was significantly reduced by caffeine. At maximum Ca^{2+} activation, 30 mM caffeine reduced V_{max} from 1.24 ± 0.07 (n = 7) to 0.64 ± 0.02 (n = 4) M.L./s. This dramatic decrease in maximum shortening velocity was confirmed by slack tests (Fig. 8), in which 30 mM caffeine produced a decrease in V_0 from 1.53 ± 0.03 to 0.85 ± 0.01 M.L./s (n = 5).

It has been reported that at submaximum Ca^{2+} activation levels, the slack test plots become biphasic, with the shortening velocity for longer steps being slowed compared to the velocity of shorter step lengths. This effect is dependent on the level of thin-filament activation (Moss, 1986; Martyn et al., 1994). This biphasic effect was not observed with caffeine (data not shown). This is consistent with the interpretation that caffeine reduces tension and velocity through a pathway that is distinct from the thin-filament Ca^{2+} binding mechanism.

TABLE 2 Summary of stiffness-pCa fits in the presence of 0–30 mM caffeine

[caffeine] (mM)	S/S_0	n_H	pCa_{50}	N
0	1.0	1.62 ± 0.05	6.13 ± 0.01	10
10	1.01 ± 0.01	1.41 ± 0.05*	6.50 ± 0.02*	10
20	0.93 ± 0.01*	1.30 ± 0.03*	6.61 ± 0.03*	10
30	0.84 ± 0.01*	1.30 ± 0.07*	6.66 ± 0.03*	10

Values given as mean ± SEM.
*Significantly different from control (ANOVA, $p < 0.05$).

Caffeine at varied ATP

Caffeine has been shown to act as an inhibitor of adenosine receptors (Bruns et al., 1983; Fredholm, 1995). Therefore, because many of the preceding results could be explained if caffeine acts as a competitive inhibitor with ATP in cross-bridge cycling, the effect of caffeine was tested while the ATP concentration was varied. As shown in Table 5, there was no significant decrease in the effects of 20 mM caffeine on tension or V_0 as ATP varied from 0.5 to 10 mM. Indeed, the only significant difference over this range was an increased reduction in V_0 at the higher ATP concentration. These results are not consistent with the hypothesis that

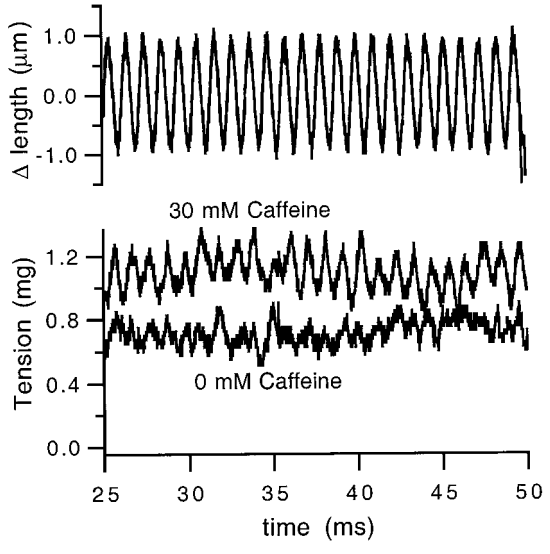

FIGURE 4 Example of increased stiffness in a relaxed fiber (pCa 9.0) upon application of caffeine. (*Top*) Sinusoidal length change applied to fiber. (*Bottom*) Tension trace in response to length change in the presence of 0 and 30 mM caffeine.

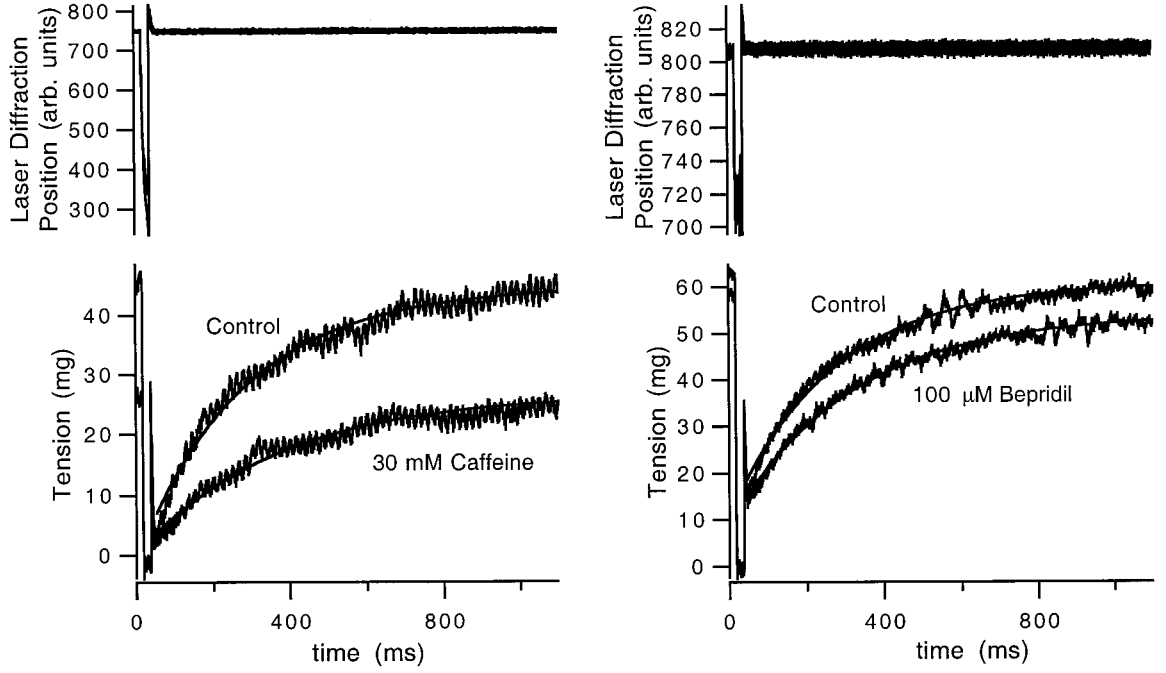

FIGURE 5 Examples of k_{tr} from a single rat soleus fiber with 0 and 30 mM caffeine (*left*) and 0 and 100 μM bepridil (*right*) at 15°C and pCa 4.5. Sarcomere length was held constant during tension redevelopment through feedback control of the first-order laser diffraction pattern (*top*). Tension redevelopment traces after a 20-ms shortening step of 15% of the initial fiber length (*bottom*) are fit by a single exponential.

caffeine acts as a competitive inhibitor of ATP at the active site of myosin. Therefore, it appears that caffeine and ATP have distinct myosin-binding sites.

DISCUSSION

In the three-state model of thin filament activation proposed by McKillop and Geeves (1993), activation of the thin filament occurs in two steps, a Ca^{2+}-induced blocked-to-closed transition and a subsequent transition from closed to open coupled to strong cross-bridge binding. Although the primary effect of Ca^{2+} is on the first transition, both transitions are described as Ca^{2+} sensitive. Because the transition from blocked to closed does not involve cross-bridge binding, any change in the relative population of blocked to closed thin-filament states will alter the Ca^{2+} sensitivity of contraction without altering the apparent kinetics of contraction. Because strong cross-bridge states are allowed only in the open state of the thin filament whereas weak cross-bridges are allowed in the closed state, the transition to the open state of the thin filament must require the binding of specific cross-bridge states to the closed thin filament. In this model, altering the number of cross-bridges in these specific states will alter the Ca^{2+} sensitivity of contraction by altering the transition from the closed to the open state. In addition, because cross-bridge binding is involved in the closed-to-open transition, the apparent kinetics of contraction may be affected as well. The primary goal of this paper is to identify cross-bridge states that may be responsible for the transition between closed and open states by examining the effects of compounds known to increase the Ca^{2+} sensitivity of contraction.

TABLE 3 Summary of k_{tr} fits in the presence of 0–30 mM caffeine or 0–100 μM bepridil, pCa 4.5

[caffeine] (mM)	k_{tr}	N	[bepridil] (μM)	k_{tr}	N
0	3.51 ± 0.12	12	0	3.36 ± 0.18	6
10	2.97 ± 0.12*	9	10	3.05 ± 0.78	2
20	2.46 ± 0.10*	9	50	3.18 ± 0.16	6
30	2.70 ± 0.19*	9	100	3.10 ± 0.18	6

Values given as mean ± SEM.
*Significantly different from control (ANOVA, $p < 0.05$).

Bepridil had no effect on the kinetics of contraction (i.e., k_{tr} and V_{max}). Because bepridil increases the Ca^{2+} sensitivity of contraction by increasing the binding of Ca^{2+} to TnC (Solaro et al., 1986), it is apparent that the TnC-Ca^{2+} affinity does not influence the kinetics of contraction as measured in the present study. Thus bepridil appears to produce changes in the activation state of the thin filament by increasing the transition from blocked to closed. In contrast, caffeine, which appears not to affect Ca^{2+} binding to TnC (Powers and Solaro, 1995), produces changes in both the Ca^{2+} sensitivity and the kinetics of contraction, as measured by k_{tr} and V_{max}, making it a candidate for altering the closed-to-open transition. The k_{tr} and V_{max} results indicate that caffeine directly influences the cross-bridge interaction and support the hypothesis that at least some cross-bridge states influence the Ca^{2+} sensitivity of contraction.

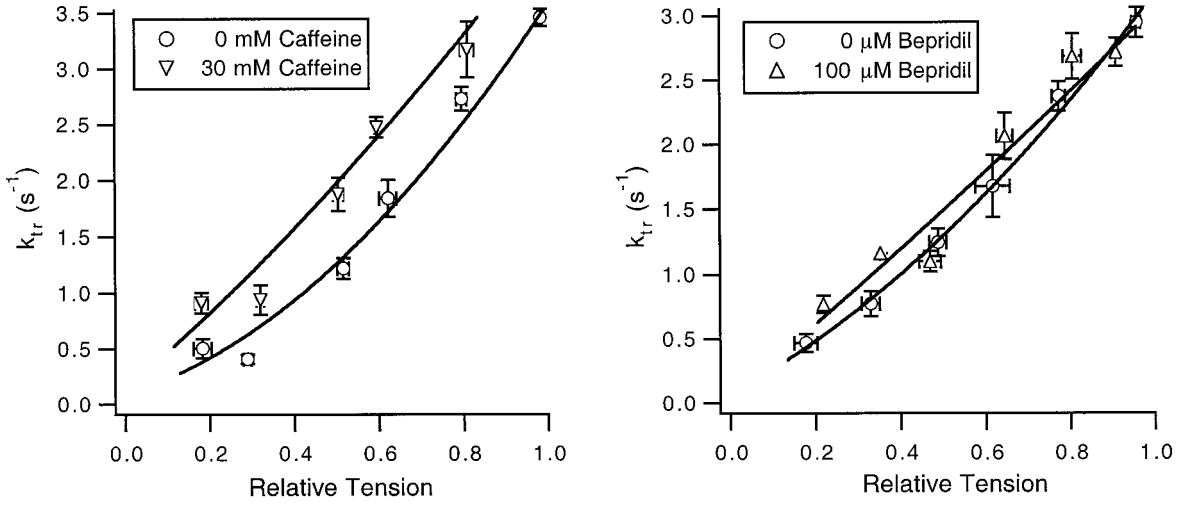

FIGURE 6 k_{tr} versus relative tension from rat soleus fibers with 0 and 30 mM caffeine (*left*) and with 0 and 100 μM bepridil (*right*). Data from 12 (caffeine) or three (bepridil) fibers were normalized to maximum control tension (pCa 4.5, no caffeine) and pooled into six bins with a width of $0.15P_0$ and averaged in tension and k_{tr}. Error bars indicate SEM.

Integrated model of cross-bridge cycling with thin-filament activation

To describe the interaction between cross-bridge states and thin filament regulation, it is necessary to first define a specific cross-bridge model. The cross-bridge model used here, shown in Fig. 9, is based on that of Regnier et al. (1995), as adapted by Wahr et al. (1997), and includes a strongly bound (i.e., stiffness bearing), non-tension-producing AM′·ADP·P_i state. Simpler models that did not include such a strongly bound, non-tension-bearing state were unable to reproduce the independent changes in tension and stiffness induced by caffeine. Earlier kinetic studies require step 3 of the cross-bridge cycle to be Ca^{2+} sensitive (Regnier et al., 1995; Wahr et al., 1997). Because the transition from the blocked to the closed state of the thin filament is the primary effect of Ca^{2+} binding to the thin filament (McKillop and Geeves, 1993), we have assumed that step 3 in Fig. 9 is the one that is blocked in the absence of Ca^{2+}. Thus, in the working model shown in Fig. 9, Ca^{2+} binding to TnC has no direct effects on the kinetics of cross-bridge cycling, which is supported by our results in the presence of bepridil. Instead, Ca^{2+} binding to TnC influences contractile kinetics through the interaction between the thin-filament Ca^{2+} binding kinetics and cross-bridge cycling. To

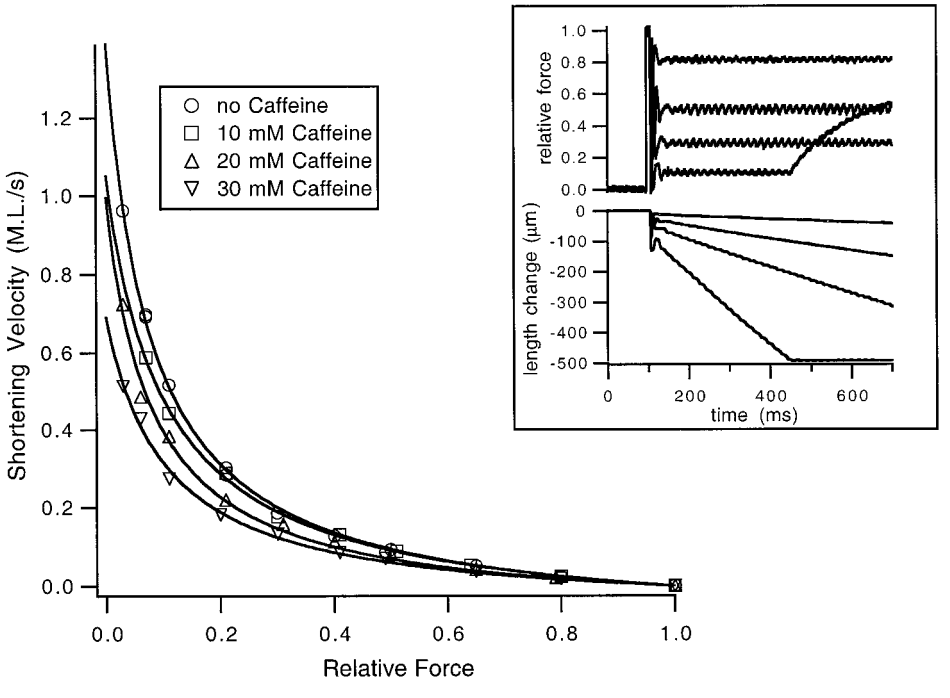

FIGURE 7 Force-velocity curves from a single fiber at 15°C with 0–30 mM caffeine, pCa 4.5. (*Inset*) Protocol for determining shortening velocity curves from a fiber under control conditions (see Materials and Methods). (*Top*) Tension versus time for isotonic shortening at 10%, 30%, 50%, and 80% P_0. (*Bottom*) Length versus time corresponding to the isotonic traces at top.

TABLE 4 Effect of caffeine and bepridil on unloaded shortening velocity at pCa 4.5

Concentration	V_{max} (M.L./s)	a/P_0	N	V_0 (M.L./s)	N
[caffeine] (mM)					
0	1.24 ± 0.07	0.068 ± 0.005	7	1.53 ± 0.03	5
10	1.22 ± 0.16	0.089 ± 0.015	4	1.34 ± 0.04*	5
20	0.98 ± 0.15	0.072 ± 0.004	4	1.06 ± 0.03*	5
30	0.64 ± 0.02*	0.098 ± 0.006*	4	0.85 ± 0.01*	5
[bepridil] (μM)					
0	0.88 ± 0.07	0.092 ± 0.008	10		
50	0.96 ± 0.20	0.090 ± 0.018	5		
100	0.75 ± 0.05	0.106 ± 0.009	5		

Values are given as mean ± SEM.
*Significantly different from control (ANOVA, $p < 0.05$).

TABLE 5 Effect of 20 mM caffeine at varied ATP concentrations

[ATP] (mM)	P (Caffeine)/P_0	ΔV_0 (caffeine-control)	$\%V_0$ (caffeine/control)	n
0.5	0.731 ± 0.014	−0.002 ± 0.058*	104 ± 11*	5
4	0.721 ± 0.006*	−0.464 ± 0.058	70 ± 3	5
10	0.758 ± 0.007	−0.498 ± 0.051	71 ± 2	5

*Significantly different from 10 mM ATP (ANOVA, $p < 0.05$).

put it another way, specific thin-filament states are required for the cross-bridge cycle to proceed. Myosin is therefore limited in its ability to bind actin by the Ca^{2+}-dependent kinetics of the thin-filament state transitions. Furthermore, we characterize the non-tension-producing, strongly bound state as that which binds to the thin filament in the closed state of McKillop and Geeves (1993) and promotes the transition to the open state (see below). Although the model proposed by McKillop and Geeves (1993) does not allow cross-bridge binding in the blocked state, there is evidence for weak cross-bridge binding in the absence of Ca^{2+} (Schoenberg, 1988). Therefore, as illustrated in Fig. 9, we have modified the McKillop and Geeves model to allow the weakly binding AM·ATP and AM·ADP·P_i cross-bridges in the blocked thin-filament state. An alternative formulation of the model would be to allow weak cross-bridge binding in the absence of Ca^{2+} only in the portion of the thin filaments that are in the closed state. These alternatives are functionally indistinguishable because the short-lived, non-stereospecific AM·ATP and AM·ADP·P_i states, which do not appear in the McKillop and Geeves model, do not contribute to the mechanical responses of the fiber under physiological conditions. In the model shown in Fig. 9, weak ionic cross-bridge interactions are allowed in the absence of Ca^{2+}, but step 3 is blocked. With Ca^{2+} binding the thin filament moves to the closed state and step 3 is allowed, leading to the binding of non-tension-producing, strongly bound AM′·ADP·P_i cross-bridges. This produces the transition to the open state, in which all cross-bridge states are allowed.

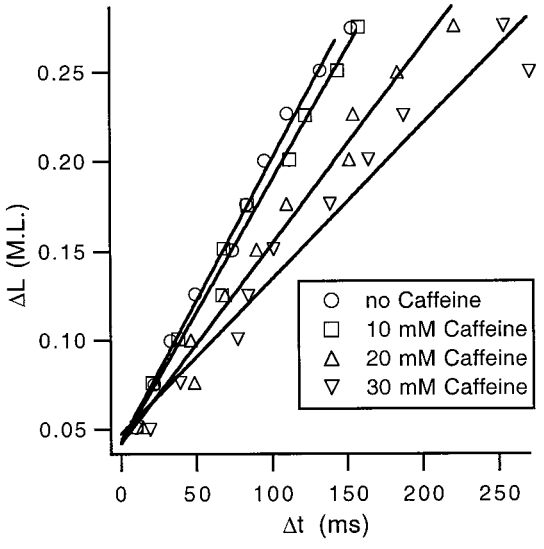

FIGURE 8 Example of slack-test data from a single fiber exposed to 0–30 mM caffeine at maximum Ca^{2+} (pCa 4.5). The time for tension to develop after a step length change is plotted against the step size. The slope of a line fit to these data is taken as V_0.

In summary, in Fig. 9 we propose a working model incorporating three thin filament states corresponding to the blocked, closed, and open states of McKillop and Geeves (1993). These states are 1) in the absence of both Ca^{2+} and strongly bound cross-bridges; 2) with Ca^{2+} bound, but no tension production; and 3) in the presence of both Ca^{2+} and tension-producing cross-bridges. The transition between these thin filament states is caused by the binding of Ca^{2+} (blocked to closed) and the binding of cross-bridges in a specific state (AM′·ATP·P_i, closed to open). Furthermore, we have identified the cross-bridge states that are allowed under these three thin-filament states, permitting us to assign roles to specific cross-bridge states in thin-filament activation. This represents an improvement over previous attempts at linking thin filament activation and cross-bridge kinetics that have used greatly simplified cross-bridge models, typically with only two cross-bridge states (Brenner, 1988; Hancock et al., 1997; Landesberg and Sideman, 1994; McKillop and Geeves, 1993).

The proposed model was quantitatively evaluated by numerically solving the differential rate equations for the cross-bridge and thin-filament transitions shown in Fig. 9. For simplicity, the ATP cleavage steps were combined into a single kinetic step (*step 2* in Fig. 9). The rates of the cross-bridge transitions were taken from Wahr et al. (1997), with the modification that the forward rates of steps 3 and 4 (k_{+3} and k_{+4}) were written as bimolecular rate constants dependent on the activation state of the thin filament. The closed or open state of the thin filament was necessary for k_{+3}, and k_{+4} required the open state. The rates of the thin-filament transitions were chosen to give a ratio of blocked:closed:open of 5:75:20 under conditions of rapid shortening and high Ca^{2+}, corresponding to the ratios reported in the solution studies of McKillop and Geeves (1993). This resulted in values for K_{BC} and K_{CO} of 14.9 and

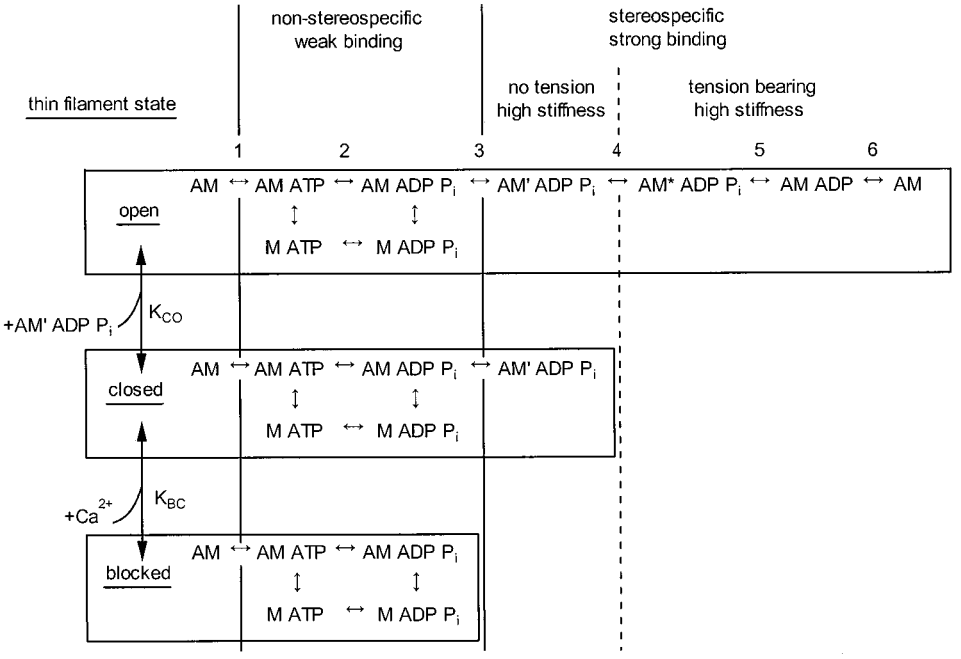

FIGURE 9 Working model integrating cross-bridge cycling and thin filament activation. Cross-bridge states that are allowed under blocked, closed, and open states of the thin filament are as shown in boxes. Weak (low stiffness, low tension) and strong (high stiffness) binding and tension-producing states are as marked. Numbers above the scheme in the open box refer to transitions between cross-bridge states discussed in the text. K_{BC} and K_{CO} refer to the equilibration constants of the blocked to closed and closed to open transitions of the thin filament, respectively. Cross-bridge rate constants used to simulate control (isometric, high Ca^{2+}) conditions (derived from Wahr et al., 1997): $k_{+1} = 1000$, $k_{-1} = 0.001$, $k_{+2} = 50$, $k_{-2} = 10$, $k_{+3} = 0.16$, $k_{-3} = 0.6$, $k_{+4} = 3$, $k_{-4} = 20$, $k_{+5} = 40$, $k_{-5} = 9.1$, $k_{+6} = 0.9$, $k_{-6} = 0$, $k_{+BC} = 1$, $k_{-BC} = 5.5$, $k_{+CO} = 5$, $k_{-CO} = 330$.

0.27, respectively, in good agreement with the McKillop and Geeves (1993) values. The ratios of thin filament states shifted to 3.5:53:43.5 under isometric conditions at high Ca^{2+}. The forward rate of K_{BC} was assumed to be Ca^{2+} dependent and was varied to simulate submaximum Ca^{2+} conditions. Cross-bridge rate constants are assumed to be Ca^{2+} independent. It is assumed that all strong, stereospecifically bound cross-bridges are capable of maintaining the open state of the thin filament. Therefore, the forward rate of K_{CO} is a bimolecular rate dependent on the concentration of cross-bridges in strongly bound conformations (AM′·ATP·P_i through AM in Fig. 9). Simulations using this model adequately predict the Ca^{2+}-sensitive behavior of tension, stiffness, and k_{tr}. Allowing K_{CO} to vary with Ca^{2+}, as indicated by McKillop and Geeves, produced no qualitative changes in these simulations and was therefore not included. No near-neighbor cooperativity along the thin filament is included in this model. Consequently, the Hill coefficients are expected to be underestimated. Specific modifications necessary to mimic the results with caffeine and bepridil are discussed below.

Caffeine and stiffness

The increase in stiffness produced by caffeine in the absence of Ca^{2+} and the small decrease in the Hill coefficient of the stiffness-pCa curves (Table 2) in the presence of caffeine are the result of strong, non-tension-generating cross-bridges binding in the small number (~22%; McKillop and Geeves, 1993) of closed thin filament units that exist even in the absence of Ca^{2+}. A slight increase in step 3 of the proposed model produces such an increase in stiffness at low Ca^{2+}. At high Ca^{2+}, there was a significant decrease in both the tension and the stiffness with the addition of caffeine. However, the decrease in stiffness is only observed at the higher concentrations of caffeine, whereas the tension was significantly decreased at all caffeine levels. Furthermore, the relative decrease in stiffness was much less than the relative decrease in tension. It can therefore be concluded that the decrease in tension observed with caffeine is not primarily a result of caffeine inhibiting the formation of strongly bound cross-bridges, but results from a decrease in the tension per cross-bridge interaction. A decrease in tension per interaction could be produced by a shift in the distribution of cross-bridges to favor an increase in strongly bound, low-tension-generating states, e.g., a decrease in the equilibrium constant of step 4. Alternatively, the decrease in tension per interaction could be the result of a decrease in the tension produced by a single cross-bridge state without a change in the distribution of states. The kinetic changes seen with the application of caffeine (see below) indicate that caffeine produces a combination of these two effects.

An alternative mechanism for the increased stiffness at low Ca^{2+} and the apparent decrease in the tension per cross-bridge interaction in the presence of caffeine would be if caffeine competes with ATP for the nucleotide binding site. Such a competition would be expected to lead to a rigor-like state with caffeine bound to the cross-bridges. This type of competition is suggested by the observation that caffeine inhibits adenosine receptors (Bruns et al., 1983; Fredholm, 1995). This appears not to be the case, however, because altering the ATP concentration from 0.5 to 10 mM failed to produce results consistent with competitive inhibition (Table 5). Therefore, caffeine appears to alter the distribution of attached states after ATP binding.

Ca^{2+} sensitizers and kinetics

Bepridil had no effect on either the maximum k_{tr} or the relation between k_{tr} and tension (Table 3 and Fig. 6). This is consistent with bepridil increasing the Ca^{2+} sensitivity through effects on TnC. In the proposed model, Ca^{2+} acts to alter k_{tr} and tension only by regulating access to thin filament binding sites and leaves the inherent cross-bridge kinetics unaffected (this assumes no direct interaction of Ca^{2+} with the cross-bridges as described by Metzger and Moss, 1992). Therefore, in the absence of changes to cross-bridge kinetics, k_{tr} in this model will be determined by the proportion of thin-filament sites available for cross-bridge binding. The effects of bepridil can be accounted for by simply increasing K_{BC}. Bepridil, by increasing the TnC Ca^{2+} affinity, acts only to shift the k_{tr}-pCa relation to lower Ca^{2+} levels without changing the tension-k_{tr} relation (Fig. 6). This is in contrast to results reported for the Ca^{2+} sensitizer calmidazolium, which significantly decreased the maximum k_{tr} and increased k_{tr} at submaximum tension levels (Regnier et al., 1996). Initially, the calmidazolium results are puzzling, because the main impact on the myofibrillar proteins of both bepridil and calmidazolium is to increase the Ca^{2+} affinity of TnC. However, upon close examination, calmidazolium produced a decrease in tension that is lacking in the bepridil results presented here. Furthermore, small changes in myosin ATPase were reported (Regnier et al., 1996). These observations suggest that calmidazolium exerts some additional effects on the cross-bridges that are not present with bepridil. These additional effects, and not the change in Ca^{2+} sensitivity, are likely to be responsible for the change in k_{tr} with calmidazolium.

The reduction in k_{tr} produced by caffeine is most easily explained as a decrease in the rate of transition from weak, non-tension-bearing to strong, tension-producing cross-bridge states, i.e., step 4 in Fig. 9. A decreased maximum shortening velocity (Figs. 7 and 8), as observed with caffeine, is typically described as either a decreased cross-bridge detachment as limited by the ADP release (step 6) or a decrease in the rate of ATP cleavage. Caffeine has been shown to have no effect on myosin Mg^{2+}-ATPase (Powers and Solaro, 1995), implying that ATP cleavage is unaffected by caffeine. Therefore, a decreased rate of cross-bridge detachment is most likely responsible for the decreased shortening velocity produced by caffeine. The rate of relaxation would also be expected to be dependent on the rate of cross-bridge detachment and therefore to be slowed in the presence of caffeine. This has been observed in intact muscle fibers (e.g., Allen and Westerblad, 1995), although the origin of this effect is obscured by the prolongation of the Ca^{2+} transient. However, caffeine has been reported to increase the rate of relaxation after photolysis of a caged Ca^{2+} chelator (Palmer and Kentish, 1997). The origin of this discrepancy is unclear.

Mimicking the effects of caffeine on kinetics in our numerical model requires alterations in at least two steps. The decrease in V_{max} requires a decrease in k_{+6}, and the decrease in k_{tr} can be accomplished through a decrease in either k_{+3} or k_{+4}. In addition to the decrease in kinetics, caffeine produced a leftward shift in the tension-pCa plots (Fig. 1). Because caffeine is not known to affect the thin filament, caffeine is expected to alter the tension-pCa relation through changes in the cross-bridge kinetics. Leftward shifts in the tension-pCa relation are accomplished in two ways: 1) the proportion of open thin filament states available for strong cross-bridge binding is increased or 2) the population of cross-bridges is shifted to increase the number of tension-producing states (AM*·ADP·P_i, AM·ADP, and AM) bound to the open state. Thus decreasing k_{+6} from 0.9 to 0.5 s^{-1} to decrease V_{max} by 45% leads to an increase in Ca^{2+} sensitivity of 0.08 pCa units by increasing the proportion of tension-producing states. However, this also leads to an increase in maximum tension, which was not observed. Decreasing k_{tr} by a decrease in k_{+4} leads to a decrease in tension as well, thereby partially offsetting the increased tension produced by the decreased k_{+6} required to decrease V_{max}. However, decreasing k_{+4} produces a rightward shift in the tension-pCa plots by altering the distribution of strongly attached cross-bridges. Decreasing k_{tr} by a decrease in k_{+3} instead of k_{+4} leads to a decreased number of thin-filament units in the open state and a similar rightward shift in the tension-pCa plots. Besides, as indicated above, the increase in stiffness at low Ca^{2+} suggests that k_{+3} does not decrease, but rather increases slightly. Thus, to simultaneously account for the changes in both cross-bridge kinetics and Ca^{2+} sensitivity, caffeine must alter additional rate constants. The minimum change that is able to account for the leftward shift in the tension-pCa plots and the slowed kinetics is a decrease in k_{-4} from 20 s^{-1} to 1 s^{-1} in addition to a decrease in k_{+4} and k_{+6} to 1.1 s^{-1} and 0.4 s^{-1}, respectively, effectively shifting the equilibrium of step 4 to favor the tension-producing AM*·ADP·P_i state. This produces a decrease in the simulated k_{tr} from 3.49 s^{-1} to 2.70 s^{-1} and a leftward shift in the tension-pCa plot of 0.3 pCa units, similar to the changes seen with 30 mM caffeine (Table 1). However, these changes in rates lead to a predicted increase in tension of 27%, whereas tension is seen to decrease by 40% with caffeine. Thus, in addition to altering the distribution of cross-bridge states, the model requires a reduction in the tension-generating capability of one or more of the tension-bearing states in the presence of caffeine.

SUMMARY

By utilizing Ca^{2+} sensitizers we were able to produce a model combining thin-filament activation and cross-bridge cycling. In this way we identified a strongly bound, non-tension-producing cross-bridge state that is necessary for full activation of the thin filament.

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REFERENCES

Allen, D. G., and H. Westerblad. 1995. The effects of caffeine on intracellular calcium, force and the rate of relaxation of mouse skeletal muscle. *J. Physiol. (Lond.).* 487:331–342.

Borovikov, Y. S., E. Nowak, M. I. Khoroshev, and R. Dabrowska. 1993. The effect of Ca^{2+} on the conformation of tropomyosin and actin in regulated actin filaments with or without bound myosin subfragment 1. *Biochim. Biophys. Acta.* 1163:280–286.

Brenner, B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. *Proc. Natl. Acad. Sci. USA.* 85:3265–3546.

Bruns, R. F., J. J. Katims, Z. Annau, S. H. Snyder, and J. W. Daly. 1983. Adenosine receptor interactions and anxiolytics. *Neurophamacology.* 22:1523–1529.

Caputo, C., K. A. P. Edman, F. Lou, and Y.-B. Sun. 1994. Variation in myoplasmic Ca^{2+} concentration during contraction and relaxation studied by the indicator fluo-3 in frog muscle fibres. *J. Physiol. (Lond.).* 478:137–148.

Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.* 157: 378–417.

Fredholm, B. B. 1995. Adenosine, adenosine receptors and the actions of caffeine. *Pharmacol. Toxicol.* 76:93–101.

Gordon, A. M., and E. B. Ridgway. 1987. Extra calcium on shortening in barnacle muscle. *J. Gen. Physiol.* 90:321–340.

Guth, K., and J. D. Potter. 1987. Effect of rigor and cycling cross-bridges on the structure of troponin C and on the Ca^{2+} affinity of the Ca^{2+}-specific regulatory sites in skinned rabbit psoas fibers. *J. Biol. Chem.* 262:13627–13635.

Hancock, W. O., L. L. Huntsman, and A. M. Gordon. 1997. Models of calcium activation account for differences between skeletal and cardiac force redevelopment kinetics. *J. Muscle Res. Cell Motil.* 18:671–681.

Holmes, K. C. 1995. The actomyosin interaction and its control by tropomyosin. *Biophys. J.* 68:2s–7s.

Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism of force generation in striated muscle. *Nature.* 233:533–538.

Kleerekoper, Q., W. Liu, D. Choi, and J. A. Putkey. 1998. Identification of binding sites for bepridil and trifluoroperazine on cardiac troponin C. *J. Biol. Chem.* 273:8153–8160.

Landesberg, A., and S. Sideman. 1994. Mechanical regulation of cardiac muscle by coupling calcium kinetics with cross-bridge cycling: a dynamic model. *Am. J. Physiol.* 267:H779–H795.

Martyn, D. A., P. B. Chase, J. D. Hannon, L. L. Huntsman, M. J. Kushmerick, and A. M. Gordon. 1994. Unloaded shortening of skinned muscle fibers from rabbit activated with and without Ca^{2+}. *Biophys. J.* 67:1984–1993.

McKillop, D. F. A., and M. A. Geeves. 1993. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys. J.* 65:693–701.

Metzger, J. M., and R. L. Moss. 1992. Myosin light chain 2 modulates calcium-sensitive cross-bridge transitions in vertebrate skeletal muscle. *Biophys. J.* 63:460–468.

Morimoto, S. 1991. Effect of myosin cross-bridge interaction with actin on the Ca^{2+}-binding properties of troponin C in fast skeletal myofibrils. *J. Biochem.* 109:120–126.

Moss, R. L. 1986. Effects on shortening velocity of rabbit skeletal muscle due to variations in the level of thin-filament activation. *J. Physiol. (Lond.).* 377:487–505.

Palmer, S., and J. C. Kentish. 1996. Developmental differences and regional similarities in the response of rat cardiac skinned muscles to acidosis, inorganic phosphate and caffeine. *J. Mol. Cell. Cardiol.* 28: 797–805.

Palmer, S., and J. C. Kentish. 1997. Differential effects of the Ca^{2+} sensitizers caffeine and CGP 48506 on the relaxation rate of rat skinned cardiac trabeculae. *Circ. Res.* 80:682–687.

Popp, D., and Y. Maeda. 1993. Calcium ions and the structure of muscle actin filament: an x-ray diffraction study. *J. Mol. Biol.* 229:279–285.

Powers, F. M., and R. J. Solaro. 1995. Caffeine alters cardiac myofilament activity and regulation independently of Ca^{2+} binding to troponin C. *Am. J. Physiol.* 268 (*Cell Physiol.* 37):C1348–C1353.

Regnier, M., D. A. Martyn, and P. B. Chase. 1996. Calmidazolium alters Ca^{2+} regulation of tension redevelopment rate in skinned skeletal muscle. *Biophys. J.* 71:2786–2794.

Regnier, M., C. Morris, and E. Homsher. 1995. Regulation of the cross-bridge transition from a weakly to strongly bound state in skinned rabbit muscle fibers. *Am. J. Physiol.* 269:C1532–C1539.

Schoenberg, M. 1988. Characterization of the myosin adenosine triphosphate (M·ATP) crossbridge in rabbit and frog skeletal muscle fibers. *Biophys. J.* 54:135–148.

Solaro, R. J., P. Bousquet, and J. D. Johnson. 1986. Stimulation of cardiac myofilament force, ATPase activity and troponin C Ca^{++} binding by bepridil. *J. Pharmacol. Exp. Ther.* 238:502–507.

Vibert, P., R. Craig, and W. Lehman. 1997. Steric-model for activation of muscle thin filaments. *J. Mol. Biol.* 266:8–14.

Wahr, P. A., H. C. Cantor, and J. M. Metzger. 1997. Nucleotide-dependent contractile properties of Ca^{2+}-activated fast and slow skeletal muscle fibers. *Biophys. J.* 72:822–834.

Wendt, I. R., and D. G. Stephenson. 1983. Effects of caffeine on ca-activated force production in skinned cardiac and skeletal muscle fibres of the rat. *Pflugers Arch.* 398:210–216.

Westerblad, H., and D. G. Allen. 1996. Mechanisms underlying changes of tetanic $[Ca^{2+}]_i$ and force in skeletal muscle. *Acta Physiol. Scand.* 156: 407–416.