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Altered Kinetics of Contraction in Skeletal Muscle Fibers Containing a Mutant Myosin Regulatory Light Chain with Reduced Divalent Cation Binding

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ABSTRACT We examined the kinetic properties of rabbit skinned skeletal muscle fibers in which the endogenous myosin regulatory light chain (RLC) was partially replaced with a mutant RLC (D47A) containing a point mutation within the Ca^{2+}/Mg^{2+} binding site that severely reduced its affinity for divalent cations. We found that when approximately 50% of the endogenous RLC was replaced by the mutant, maximum tension declined to ~60% of control and the rate constant of active tension redevelopment (k_{tr}) after mechanical disruption of cross-bridges was reduced to ~70% of control. This reduction in k_{tr} was not an indirect effect on kinetics due to a reduced number of strongly bound myosin heads, because when the strongly binding cross-bridge analog *N*-ethylmaleimide-modified myosin subfragment1 (NEM-S1) was added to the fibers, there was no effect upon maximum k_{tr}. Fiber stiffness declined after D47A exchange in a manner indicative of a decrease in the number of strongly bound cross-bridges, suggesting that the force per cross-bridge was not significantly affected by the presence of D47A RLC. In contrast to the effects on k_{tr}, the rate of tension relaxation in steadily activated fibers after flash photolysis of the Ca^{2+} chelator diazo-2 increased by nearly twofold after D47A exchange. We conclude that the incorporation of the nondivalent cation-binding mutant of myosin RLC decreases the proportion of cycling cross-bridges in a force-generating state by decreasing the rate of formation of force-generating bridges and increasing the rate of detachment. These results suggest that divalent cation binding to myosin RLC plays an important role in modulating the kinetics of cross-bridge attachment and detachment.

INTRODUCTION

In striated muscles contraction consists of Ca^{2+}-sensitive interactions between myosin cross-bridges and actin. There are predominantly two models that are invoked to account for Ca^{2+} regulation of the number of force-generating myosin cross-bridges. In the first of these, Ca^{2+} acts as a "switch" in that Ca^{2+} binding to the troponin C (TnC) subunit of troponin associated with the actin filament is thought to increase the number of available myosin-binding sites on actin, thereby recruiting more cross-bridges. In this model, the increase in isometric force observed with increased $[Ca^{2+}]$ directly reflects an increase in the number of cycling cross-bridges. In the second model Ca^{2+} regulates the distribution of cross-bridges between force-generating and non-force-generating states by regulating the kinetics of transitions within the cross-bridge cycle, i.e., the total number of cycling bridges remains constant. The rate of tension redevelopment (k_{tr}) after mechanical disruption of cross-bridges in steadily activated fibers (Brenner, 1988; Brenner and Eisenberg, 1986) has been shown to be highly dependent on the level of Ca^{2+} activation in both skeletal (Brenner, 1988; Metzger et al., 1989; Swartz and Moss, 1992) and cardiac (Wolff et al., 1995) muscle, suggesting that Ca^{2+} regulates a rate-limiting step or steps in the cross-bridge cycle during isometric force development. These mechanisms are not mutually exclusive, and normal regulation of contraction by Ca^{2+} may involve some combination of the two. Although in the recruitment model of Ca^{2+} regulation of force Ca^{2+} is thought to act primarily by binding to TnC, evidence indicates that regulation of the kinetics of force development may occur through Ca^{2+} binding to sites other than TnC, such as the regulatory light chain (RLC or LC_2) of myosin. Vertebrate RLC shares considerable sequence (Collins, 1976) and structural (Rayment et al., 1993) homology with other Ca^{2+}-binding proteins such as troponin C and calmodulin and contains a single high-affinity Ca^{2+}/Mg^{2+} binding site. Previous studies have shown that partial extraction of RLC from skeletal muscle fibers significantly increased k_{tr} during submaximum activations (Metzger and Moss, 1992), but partial extraction of TnC had no effect on k_{tr} (Metzger and Moss, 1991). Increased free $[Mg^{2+}]$ has been shown to reduce k_{tr} at submaximum Ca^{2+} concentrations, and this effect was abolished by partial extraction of RLC but not TnC, leading to the hypothesis that Ca^{2+} binding to a Ca^{2+}/Mg^{2+} site on the RLC was involved in the modulation of k_{tr} (Metzger and Moss, 1992).

To investigate the role of the RLC divalent cation-binding site in the regulation of contraction we previously (Diffee et al., 1995a) replaced endogenous RLC in rabbit skinned psoas fibers with an avian mutant RLC containing a single point mutation within the divalent cation-binding site that renders it incompetent to bind Ca^{2+} (Reinach et al.,

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1986). In fibers containing this mutant RLC (D47A), both maximum tension and stiffness were significantly reduced compared to control values, suggesting that the number of strongly bound, force-generating cross-bridges was reduced when the RLC lacked the ability to bind Ca^{2+} or Mg^{2+}. In the present study we have extended these investigations to determine the effect of this nondivalent cation-binding mutant RLC on the rates of kinetic transitions between non-force-generating and force-generating states. Experiments were also done to determine whether the observed reduction in the number of force-generating cross-bridges after D47A exchange was the result of a decrease in the overall number of cycling bridges or whether the presence of the mutant altered the kinetic properties of fibers. We found that the mutant RLC-induced decline in tension can be explained by a reduction in the rate of formation of force-generating cross-bridges as well as an apparent increase in the rate of cross-bridge detachment. A preliminary account of this work has appeared previously (Diffee et al., 1995b).

MATERIALS AND METHODS

Skinned fiber preparation and experimental setup

Complete details of the mounting procedures, experimental setup, and tension measurements have been described previously (Metzger et al., 1989; Diffee et al., 1995a). Briefly, bundles of ~50 fibers dissected from the psoas muscle of adult New Zealand rabbits were tied to glass capillary tubes and stored at −22°C for up to 21 days in relaxing solution containing 50% (vol/vol) glycerol. Before each experiment, an individual fiber was pulled from the end of the bundle, a control segment was saved for later protein analysis, and an experimental segment 1.5–2.5 mm in length was mounted to the mechanical apparatus. One end of the fiber was mounted to a force transducer (model 407; Cambridge Technology, Inc., Cambridge, MA; sensitivity, 0.2 mV/μN; 1–99% response time, 100 μs; resonant frequency ~5 kHz) and the other end to a DC torque motor (model 6350; Cambridge Technology, Inc.). The resting sarcomere length was set to 2.5–2.6 μm by adjusting the overall length of the fiber segment.

Relaxing and activating solutions contained 7 mM EGTA, 1 mM free Mg^{2+}, 20 mM imidazole (pH 7.00), 4.42 mM total ATP, 14.5 mM creatine phosphate, various free Ca^{2+} concentrations between 10^{-9} M (relaxing solution; pCa 9.0) and $10^{-4.5}$ M (maximally activating solution; pCa 4.5), and sufficient KCl to adjust the ionic strength to 180 mM. The computer program of Fabiato (1988) was used to calculate the final concentrations of each metal, ligand, and metal-ligand complex based on the stability constants listed by Godt and Lindley (1982). The apparent stability constant for Ca^{2+}-EGTA (2.39×10^6 M^{-1}) was corrected for ionic strength, pH, and an experimental temperature of 10°C.

Measurement of the rate constant of tension redevelopment

The experimental protocol for measuring the rate constant of tension redevelopment (k_{tr}) was a modification of the technique described by Brenner and Eisenberg (1986). A fiber was first transferred into activating solution at 10°C and allowed to develop steady isometric force. As illustrated in Fig. 1, the fiber was then rapidly slackened by ~20% of fiber length to reduce tension to zero, and the fiber was allowed to shorten without load for ~20 ms. The fiber was then rapidly reextended to its initial length. As a result of the restretch, force transiently increased and then rapidly declined to zero. The subsequent redevelopment of force reflects the rate of cross-bridge transition to strongly bound force-producing states. To avoid underestimating k_{tr} because of end compliance within the preparation (Brenner and Eisenberg, 1986), sarcomere length was held constant during the redevelopment of force. Sarcomere length was clamped to within 0.5 nm/half-sarcomere of the isometric length measured just before release. The control system involved servocontrol of the position of the first-order line of a laser (He-Ne, 10 mW; Melles Griot, Irvine, CA) diffraction pattern projected onto a lateral effects photodiode (LSC-5D; United Detector Technologies, Culver City, CA), as described previously (Metzger et al., 1989). Fiber length, sarcomere length, and tension data were all stored on a computer at 5000 Hz. Force redevelopment traces were well fit by the single-order exponential equation, $F_t = F_o(1 - e^{-kt})$, where F_t is the force at time t, F_o is the maximum force, and k is k_{tr}.

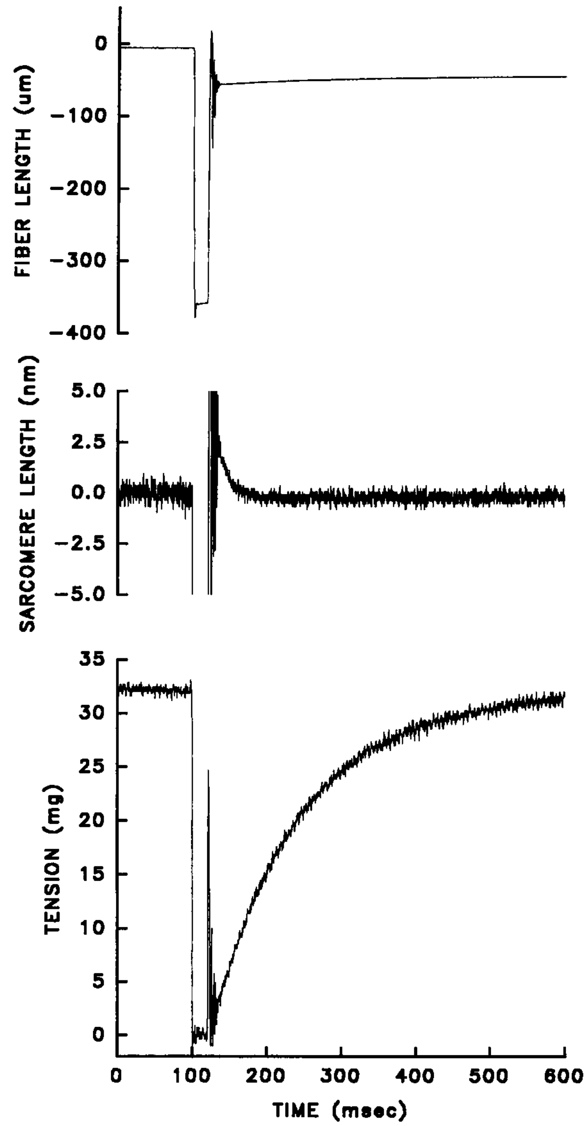

FIGURE 1 Protocol for measuring the rate constant of tension redevelopment (k_{tr}). After achieving steady isometric tension in activating solution, the position of the motor was changed, thereby decreasing fiber length by 350 μm and causing the tension to fall to zero. After 20 ms of unloaded shortening, the fiber length was reextended to the original length. After reextension, the sarcomere length as monitored by the position of the first-order line of the laser diffraction pattern was rapidly clamped to within 0.5 nm/half-sarcomere of the sarcomere length before the release and was maintained at this length for the duration of tension redevelopment.

In a number of experiments, effects on the kinetics of tension redevelopment due to increasing the number of strongly bound cross-bridges was determined by incorporating into fibers the strongly binding cross-bridge analog *N*-ethylmaleimide-modified myosin subfragment 1 (NEM-S1). NEM-S1 was prepared from rabbit skeletal muscle according to the method of Swartz and Moss (1992). Incorporation of NEM-S1 into the fibers was accomplished by incubating fibers in relaxing solution containing 6 μM NEM-S1 for 20 min at room temperature, and the NEM-S1 content of the fiber was maintained throughout the rest of the experiment by soaking the fiber between activations in relaxing solution containing 6 μM NEM-S1. NEM-S1 was incorporated into either control fibers or into fibers into which D47A had already been exchanged.

Stiffness measurements

Sarcomere stiffness was determined from force and sarcomere length responses to small, rapid step changes in fiber length (Huxley and Simmons, 1971; Ford et al., 1977). Step changes in fiber length (amounting to +0.5% to −1.0% of fiber length) were complete in 0.6 ms (Fig. 4). Sarcomere length was monitored using the position of the first-order line of the laser diffraction pattern. The relationship between the maximum change in tension (T_1) after the length step and the corresponding change in sarcomere length was plotted (Fig. 5), and the slope of this relationship ($\Delta F/\Delta$SL) was calculated as the sarcomere stiffness. These experiments were conducted at 7°C to improve the linearity of the T_1/ΔSL relationship by slowing the phase of tension recovery (phase 2) after the step. To eliminate effects on stiffness caused by potential changes in lattice spacing, 4% Dextran was added to all solutions used for stiffness measurements (Goldman and Simmons, 1978).

Rate of tension relaxation

The rate of tension relaxation was determined at 10°C by flash photolysis of the photolabile Ca^{2+} chelator diazo-2 (Adams et al., 1989). Upon photolysis with UV light, diazo-2 is rapidly converted (>3000 s^{-1}) from a Ca^{2+} chelator of relatively low affinity (K_d = 2.2 μM) to one of relatively high affinity (K_d = 73 nM) (Simnett et al., 1993). After an initial assessment of maximum tension in a solution of pCa 4.5, the fibers were placed in a low-EGTA (70 μM) relaxing solution. The fibers were then loaded with diazo-2 in a relaxing solution containing no EGTA but containing 600 μM $CaCl_2$ and 2 mM diazo-2. Fibers generated ~65% of maximum tension in this loading solution. After 4 min in loading solution the fibers were transferred to a quartz-walled photolysis chamber (80 μl) filled with silicone oil, and the fibers were immediately exposed to a flash of UV light (~360 nm) from a xenon lamp (Optoelektronic, Hamburg, Germany) directed at the quartz-walled chamber. Once the tension response was recorded, the fibers were transferred back to normal relaxing solution. Tension data were captured and stored on a digital oscilloscope (model 310; Nicolet Instruments, Madison, WI), and the tension relaxation data were analyzed with commercially available curve-fitting software (SigmaPlot; Jandel Scientific, San Rafael, CA). The tension data was fit with a double-exponential equation of the form

$$F = A(e^{-k_1{*}t}) + B(e^{-k_2{*}t}) + C,$$

where F is force, A and B are amplitudes, k_1 and k_2 are apparent rate constants, and C is the residual.

Preparation of proteins

The mutant (D47A) and wild-type (WT) RLC were prepared by expression of the full-length chicken skeletal myosin RLC gene in *Escherichia coli* as described previously (Reinach et al., 1986; Diffee et al., 1995a). The D47A mutant was constructed by replacing aspartate-47 with alanine by oligonucleotide-directed mutagenesis. This mutation has been shown to result in minimal Ca^{2+} binding to the light chain (Reinach et al., 1986).

Rabbit native skeletal RLC was purified from rabbit fast-twitch muscle according to the method of Wagner (1982). Purified TnC was prepared from rabbit fast muscle by the method of Greaser and Gergely (1971).

Exchange of exogenous RLCs into fibers

Exogenous RLCs (D47A RLC, WT RLC, or purified native rabbit skeletal RLC) were exchanged into skinned fibers as described previously (Diffee et al., 1995a). Fibers were warmed to 37°C and bathed for 30 min in a solution containing 25 mM KCl, 10 mM EDTA, 10 mM imidazole (pH 7.0), 2 mM dithiothreitol, and 2–3 mg/ml of exogenous RLC. A small number of fibers had a relatively high resting tension at pCa 9.0 after RLC exchange. If this increased resting tension (>1% of active tension) persisted, the fiber was discarded. Because a small amount of TnC was extracted during the exchange procedure, the fibers were bathed in relaxing solution containing 0.5 mg/ml purified TnC, and tension was then measured at pCa 4.5 to assess the extent of TnC recombination before postexchange mechanical measurements. This cycle of TnC soak and maximum activation was repeated until there was no significant increase in active tension. In a number of fibers, mechanical measurements in RLC-exchanged fibers were followed by reexchange of purified rabbit skeletal RLC back into the fibers to assess the reversibility of the observed effects of RLC exchange. The exchange procedure for this additional step was identical to that described above.

After mechanical experiments, the control and experimental fiber segments were placed in sodium dodecyl sulfate (SDS) sample buffer and stored at −80°C for later analysis. The extent of exchange of exogenous RLCs into the fibers was monitored by using SDS-polyacrylamide gel electrophoresis (PAGE) and an ultrasensitive silver stain technique described previously (Giulian et al., 1983). The stained gels were densitometrically scanned (model GS-670; Bio-Rad), and the peak areas were integrated using BioRad image analysis software. The extent of exchange of exogenous RLC was calculated from the ratio of exogenous RLC to total RLC determined from the gel. The stoichiometry of the exchange was assessed by calculating the total RLC present as a fraction of the total alkali light chain content (LC_1 and LC_3), because the alkali light chains are not affected by the exchange procedure (Moss et al., 1983). The TnC content of the fibers was determined by calculating the TnC/TnI ratio.

RESULTS

Rate of tension redevelopment

As we have reported previously (Diffee et al., 1995a), and as illustrated in Fig. 2, the replacement of endogenous RLC with the D47A mutant RLC reduced the tension-generating capability of the fibers. In 15 experiments, the mean (±SEM) ratio of D47A RLC/total RLC was 0.53 (± 0.11) after the exchange protocol, and the average maximum tension (at pCa 4.5) in these fibers was 61% (± 9%) of control values. We have previously shown the decline in maximum isometric tension to be highly correlated to the degree of exchange of the D47A mutant into the fiber (Diffee et al., 1995a). In addition to the decline in maximum isometric tension, there was a reversible reduction in the maximum rate of isometric tension redevelopment (k_{tr}). In the fiber shown in Fig. 2, maximum k_{tr} under control conditions was 11.7 s^{-1}, whereas in the same fiber after D47A exchange, maximum k_{tr} was reduced to 5.8 s^{-1}. After partial exchange of native RLC back into the fiber, k_{tr} was 8.7 s^{-1}. In 11 fibers, the mean (±SEM) k_{tr} at maximum activation (pCa 4.5) was 10.9 (±1.1) s^{-1}, whereas in the same fibers after D47A exchange, maximum k_{tr} declined to

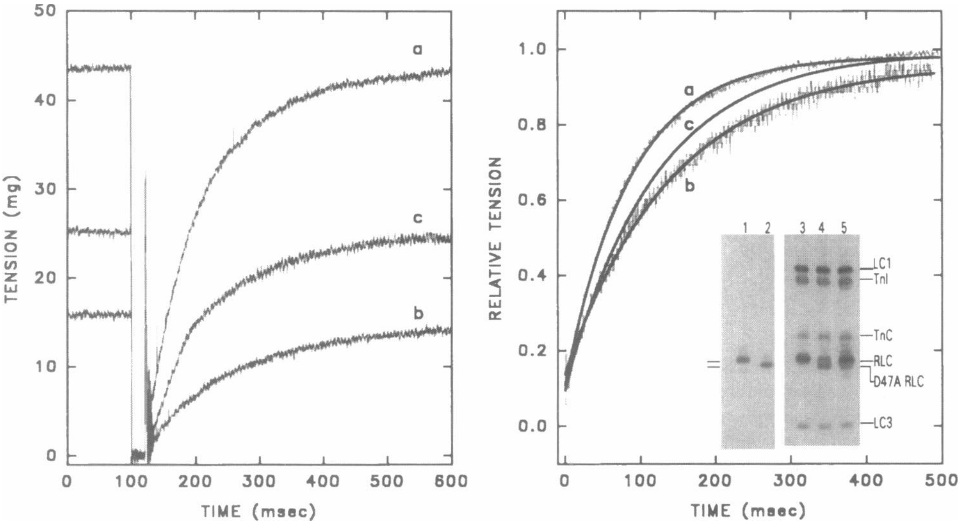

FIGURE 2 Effect of D47A RLC exchange on the rate constant of tension redevelopment (k_{tr}) at pCa 4.5. On the left are the tension responses during the k_{tr} protocol in a representative fiber before (*a*) and after (*b*) exchange of D47A into the fiber, and then after reexchange of native RLC back into the fiber (*c*). On the right is an expanded view of the tension redevelopment phase, showing tension normalized to the steady-state tension for each condition (*dotted lines*) and the computer-derived best fit (*solid lines*) to the exponential equation in Materials and Methods. For clarity, only the best fit line is presented for the native RLC exchange trace (*c*). In this fiber k_{tr} was 11.6 s^{-1} in the control condition (*a*), 5.9 s^{-1} in the same fiber after D47A RLC incorporation (*b*), and 8.5 s^{-1} in the same fiber after partial reexchange of native RLC back into the fiber (*c*). (*Inset*) Lower portion of gel from SDS-PAGE analysis of this fiber illustrating the extent of reversible RLC exchange. Lanes 1 and 2 are purified rabbit skeletal RLC and D47A RLC, respectively. Lane 3 is a control segment of this fiber. Lane 4 is a segment of the same fiber after exchange of D47A RLC into the fiber. Lane 5 shows the reexchange of rabbit skeletal RLC back into the same fiber. The D47A RLC/total RLC ratio in lane 4 is 0.65; in lane 5 this ratio is reduced to 0.15.

7.5 (±1.4) s^{-1}. To determine whether the effects of D47A RLC exchange on k_{tr} were specific to the presence of the mutation, we exchanged WT RLC into a number of fibers ($n = 4$) and found that there was no significant effect on k_{tr} (data not shown). We previously demonstrated that exchange of WT RLC into fibers did not have a specific effect on either maximum or submaximum tension (Diffee et al., 1995a).

In addition to inducing a reduction in maximum k_{tr}, exchange of D47A RLC into fibers reduced k_{tr} at submaximum activation. Fig. 3 *A* shows absolute rates of tension redevelopment as a function of pCa. At pCa levels that, in control fibers, yielded tensions greater than 50% of maximum, there was a significant reduction of k_{tr} in the D47A-exchanged fibers compared to control, whereas at lower levels of activation there was no significant difference in k_{tr}. As shown in Fig. 3 *B*, the relationship between k_{tr} and steady isometric tension is quite steep in control fibers at tensions near the maximum. After D47A exchange, both maximum k_{tr} and submaximum k_{tr} were reduced, and the slope of the relationship between k_{tr} and tension was reduced.

Effect of NEM-S1

Swartz and Moss (1992) have demonstrated that k_{tr} can be increased to maximum values irrespective of [Ca^{2+}] by incorporating into fibers an analog of strongly binding cross-bridges (NEM-S1), indicating that k_{tr} is extremely sensitive to cooperative activation of the thin filament by strongly bound myosin heads. To determine whether the observed reductions in k_{tr} were a direct effect of D47A RLC or an indirect effect due to a reduced number of strongly bound cross-bridges, we examined the effects of added NEM-S1 on fibers containing endogenous or D47A RLC. Fig. 3 *B* shows that incubation of control fibers in 6 μM NEM-S1 greatly increased k_{tr} at low levels of activation (~50% of P_o) but had no effect on k_{tr} at maximum activation, similar to the effect observed by Swartz and Moss (1992). In D47A-exchanged fibers, the presence of NEM-S1 increased both steady-state tension and k_{tr} by small amounts at submaximum [Ca^{2+}] but had no effect on either tension or k_{tr} at pCa 4.5. Thus, the decline in maximum k_{tr} associated with D47A exchange cannot be accounted for on the basis of reduced activation of the thin filament by strongly bound cross-bridges, because increasing the number of strongly binding myosin heads did not increase k_{tr} to control levels.

Stiffness measurements

In a previous study (Diffee et al., 1995a) we demonstrated that decreases in maximum and submaximum isometric tension induced by D47A exchange were accompanied by a proportionate decrease in stiffness, suggesting that the tension decline could be explained by a reduced number of force-generating cross-bridges. In the present study we measured stiffness over a wider range of length changes (+0.5% to −1.0% of fiber length) using rapid step changes in fiber length to determine whether D47A exchange altered me-

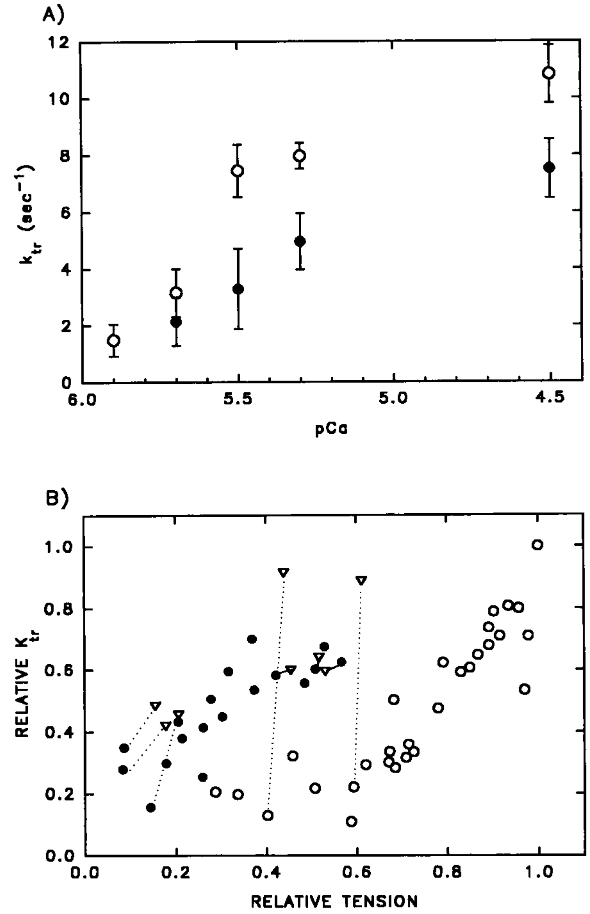

FIGURE 3 Mean data from five fibers showing the relationship between k_{tr} and the level of activating Ca^{2+} before and after the exchange of D47A RLC into the fibers. (*A*) Relationship between k_{tr} and pCa, showing that k_{tr} is reduced both at maximum and submaximum [Ca^{2+}]. (*B*) Plot of individual data points showing the relationship between the tension generated by the fiber (relative to control maximum tension) and k_{tr} (relative to control maximum). Open circles are measurements made in control fibers; closed circles are measurements from the same fibers after D47A exchange. Triangles are data points obtained after treatment with NEM-S1 (see Materials and Methods). Lines connect data points collected from the same fiber at the same activation level before and after NEM-S1 treatment.

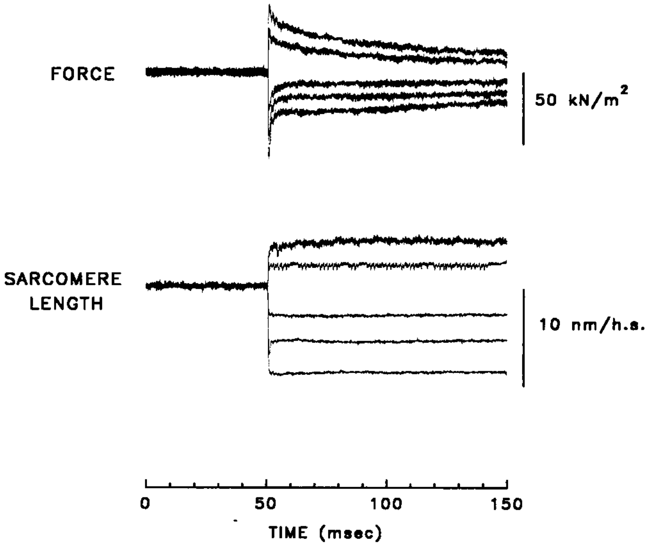

FIGURE 4 Force (*top*) and sarcomere length (*bottom*) traces obtained during a series of length steps to determine fiber stiffness. The extreme tension reached after the length step was T_1. Length steps were made in the range of +0.5% to −1.0% of fiber length. Sarcomere length was monitored from the position of the first-order line of a laser diffraction pattern.

chanical properties of cross-bridges that were not apparent during the smaller amplitude (<0.1% of fiber length) sinusoidal stiffness measurements. Force and sarcomere length responses to step changes in length were measured before and after exchange of D47A. Fig. 4 shows records of force (*top trace*) and sarcomere length (*bottom trace*) in a representative fiber subjected to step changes in length. The tension transients exhibited several phases, as observed previously (Huxley and Simmons, 1971; Ford et al., 1977). The extreme tension reached in response to a given length step (T_1) is indicative of fiber stiffness. Fig. 5 shows the relationship between T_1 and sarcomere length for a control fiber under both maximum (pCa 4.5) and submaximum (pCa 5.9) activating conditions, as well as for the same fiber (maximally activated) after D47A exchange. For both control and D47A-exchanged fibers, the relationship between T_1 and sarcomere length change was linear for stretches and small releases. The relationship became nonlinear when larger releases were imposed, as observed previously for both intact frog fibers (Huxley and Simmons, 1971; Ford et al., 1977) and in rabbit skinned psoas fibers (Chase et al., 1993; Martyn and Gordon, 1992; Martyn and Chase, 1995).

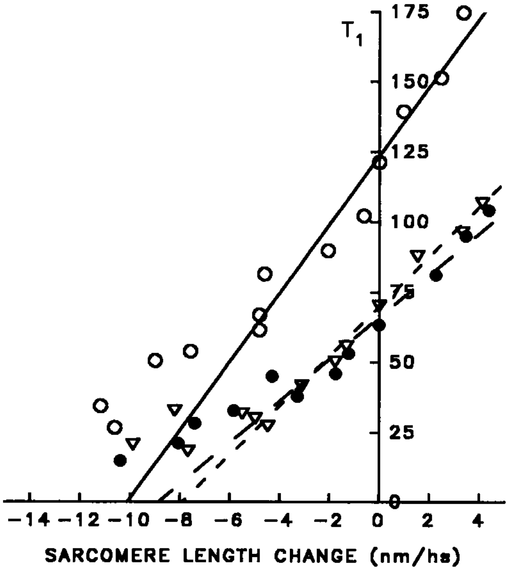

FIGURE 5 Relationship between the maximum change in force after a length step (T_1) and the corresponding change in sarcomere length for a representative fiber. The units on the T_1 axis are of force (kN · m^{-2}). The slope of the linear portion of this relationship gives fiber stiffness, which for this fiber was 33.7 N/m for control pCa 4.5 (○), 21.3 N/m for control pCa 5.9 (▽), and 20.5 N/m at pCa 4.5 after D47A exchange (●).

The linear portion of each curve was fit by least-squares linear regression analysis, and the slope of the resulting line ($\Delta F/\Delta SL$) was taken as fiber stiffness. In six fibers the mean (±SEM) stiffness at pCa 4.5 was 39 ± 7 N/m. In these same fibers, activation at pCa 5.9 reduced tension to 57 ± 3% of P_o and stiffness to 62 ± 8% of maximum, whereas D47A exchange reduced maximum tension to 54 ± 6% and stiffness to 60 ± 9% of control values. Thus, the stiffness of D47A-exchanged fibers is similar to the stiffness of control fibers activated to the same relative tension by reducing Ca^{2+}, indicating that the reduced stiffness in fibers with mutant light chain is most likely due to fewer numbers of attached cross-bridges.

Tension relaxation

To assess the effect of D47A RLC exchange on the kinetics of cross-bridge detachment, we measured the rate of tension relaxation after a rapid decrease in free [Ca^{2+}] induced by flash photolysis of the caged Ca^{2+}-chelator diazo-2. Fig. 6 shows representative tension traces after flash photolysis of diazo-2 in a control fiber and then in the same fiber after D47A exchange. Tension traces after photolysis of diazo-2 were well fit by a double exponential which has previously been observed (Palmer et al., 1991; Walker et al., 1992; Simnett et al., 1993). In the control fiber shown in Fig. 6, the fast phase of tension decline had an apparent rate constant of 1.4 s^{-1} and accounted for 65% of the relaxation amplitude. The slow phase had an apparent rate constant of 0.21 s^{-1}. In this same fiber after D47A exchange the fast phase rate constant was 2.9 s^{-1} (amplitude 61%), and the slow phase rate constant was 0.41 s^{-1}. Table 1 summarizes the rate constants and amplitudes for each of the two exponential phases of tension decline for five control fibers and in the same fibers after exchange of D47A RLC. Exchange of D47A into the fibers increased the rate of decline in both phases by approximately twofold, with no significant change in the amplitude of either phase.

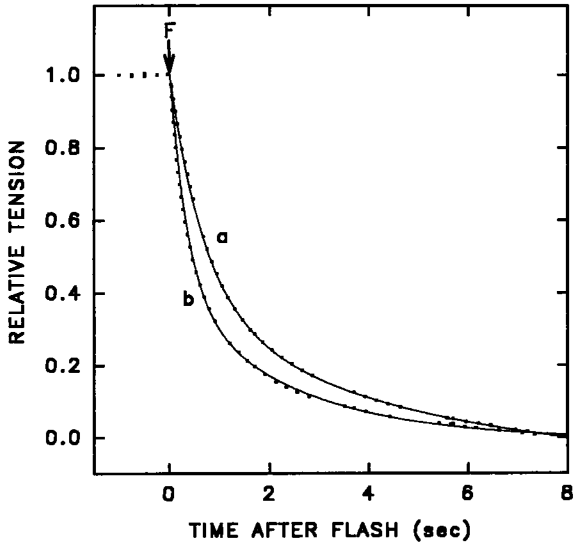

FIGURE 6 Rate of tension relaxation in a representative fiber before and after D47A exchange. Tension is normalized to steady-state tension for each condition, i.e., control (*a*) and in the same fiber after D47A exchange (*b*). Relaxation of tension was induced by flash photolysis of diazo-2 (F). Dotted lines represent tension data, and solid lines are from the best fit of the data to the equation in Materials and Methods.

The rates of tension relaxation after photolysis of diazo-2 observed in the present study are significantly slower than those reported previously for skinned skeletal muscle fibers from frog (Palmer et al., 1991; Simnett et al., 1993) and rabbit (Walker et al., 1992). This discrepancy may be attributed in part to species differences, because the rates reported by Walker et al. (1992) for rabbit muscle are also slower than rates obtained for frog muscle, although there was significant variability in the rates reported for frog muscle (Palmer et al., 1991; Simnett et al., 1993). Furthermore, we measured tension relaxation rates at a lower temperature (10°C) than in the previous studies to allow comparison of these rate constants with our k_{tr} measurements, and relaxation rates exhibit a high degree of temperature dependence (Walker et al., 1992). Also in the present study, diazo-2 was photolyzed and tension relaxation was measured while the fibers were in silicone oil, but in previous work photolysis was done in air (Palmer et al., 1991; Walker et al., 1992; Simnett et al., 1993). When we photolyzed diazo-2 in air at 15°C, the tension relaxation rates we obtained (data not shown) were comparable to those reported in other studies on rabbit psoas muscle (Walker et al., 1992).

To summarize, the results of D47A exchange presented here are that replacement of ~50% of the endogenous myosin RLC with D47A RLC reduces both maximum and submaximum tension and decreases fiber stiffness in proportion to the tension decrease. In addition, k_{tr} is reduced at all activation levels above half-maximum in a manner that cannot be attributed to the decreased number of strongly bound cross-bridges, and the rate of relaxation after flash photolysis of diazo-2 is increased by approximately twofold after D47A exchange.

DISCUSSION

The results of our previous study (Diffee et al., 1995a) indicated that when endogenous regulatory light chains in skeletal muscle fibers were replaced with mutant RLC incapable of binding divalent cations (D47A RLC), the tension-generating capability of the fibers was significantly reduced. Based on the proportionate decline in stiffness and force, we concluded in the earlier study that the decrease in tension could be attributed to a decrease in the number of force-generating cross-bridges rather than a change in the force-generating capabilities of individual cross-bridges. However, it remained unclear whether cross-bridges containing the D47A mutant RLC simply lose the ability to form force-generating cross-bridges and thus “drop out” of

TABLE 1 Comparison of rates of tension relaxation after flash photolysis of diazo-2 in control and D47A-exchanged fibers

	k_1 (s^{-1})	A	k_2 (s^{-1})	B	C
Control	1.49 ± 0.09	0.671 ± 0.059	0.261 ± 0.026	0.393 ± 0.071	0.058 ± 0.016
D47A-exchanged	2.79 ± 0.19	0.656 ± 0.092	0.397 ± 0.015	0.339 ± 0.084	0.011 ± 0.004

Data are means ± SEM from four fibers. Parameters A, B, and C are amplitudes given as fractions of total tension and were calculated from the best fits of data to the equation in Materials and Methods.

the pool of cycling bridges, or whether the decrease in tension can be explained by a D47A-induced change in the rate of kinetic transitions between non-force-generating and force-generating states. This distinction is important in determining whether the divalent cation-binding site on myosin RLC is involved in maintaining the structural integrity of the cross-bridge or if it has a role in modulating cross-bridge interaction kinetics. Our aim in the present study was to answer this question by examining the kinetic properties of fibers containing D47A RLC.

In a simple two-state cross-bridge model similar to that described by Huxley (1957), cross-bridges cycle between a force-generating state and a non-force-generating state. The turnover of cross-bridges between these two states can be described by the rate constants f_{app} (for the transition to the force-generating state) and g_{app} (for the return to the non-force-generating state), similar to the rate constants f and g in Huxley's model. The fraction of cycling cross-bridges that are in the force-generating state is given by the ratio $f_{app}/(f_{app} + g_{app})$, and steady-state isometric force (P) is then given by

$$P = nF \cdot f_{app}/(f_{app} + g_{app}), \tag{1}$$

where n is the number of cycling cross-bridges and F is the average force per cross-bridge (Brenner, 1988). Thus, the decline in force observed after incorporation of D47A RLC must result from a decrease in n, F, or the ratio $f_{app}/(f_{app} + g_{app})$. The results of the present study, in which D47A RLC reduced k_{tr}, reduced stiffness in proportion to force, and increased the rate of tension relaxation, suggest that the decline in tension can be explained on the basis of a reduced rate of formation of force-generating cross-bridges coupled with an increased rate of detachment. These changes in kinetics would both lead to a decrease in the proportion of cycling cross-bridges that are in force-producing states.

Effects of D47A on the rate of tension redevelopment

According to the simple two-state model outlined above, the rate of tension redevelopment after the mechanical disruption of cross-bridges (k_{tr}) is governed by the rate of the force-generating transition. Thus, k_{tr} is equal to the sum of the forward and reverse rate constants ($k_{tr} = f_{app} + g_{app}$). Previous studies have shown that k_{tr} is highly dependent on [Ca^{2+}] in both skeletal muscle fibers (Brenner, 1988; Metzger et al., 1989) and cardiac trabeculae (Wolff et al., 1995). In the present study we found that D47A decreased k_{tr} at maximum activation to 70% of control as well as decreasing k_{tr} at all levels of activation above half-maximum. There was no change in k_{tr} at lower levels of activation. Thus, the effect of D47A incorporation was to reduce the absolute rate of tension redevelopment and to reduce its variation with Ca^{2+} concentration.

Analysis of the effects of D47A exchange on k_{tr} was made difficult by the steep dependence of k_{tr} on the level of activation. Swartz and Moss (1992) showed that k_{tr} was highly dependent on the cooperative effects of strongly bound myosin heads to activate the thin filament. Thus, in the present study, the effect of D47A incorporation to reduce k_{tr} might be explained as an indirect effect of D47A exchange to reduce the number of strongly bound force-generating cross-bridges, because our previous results indicated that the number of force-generating cross-bridges decreased after D47A exchange (Diffee et al., 1995a). The results of the experiments with NEM-S1 (Fig. 3 *B*) indicate that this was not the case, because NEM-S1 did not increase maximum k_{tr} in D47A-exchanged fibers. Instead, our finding that the mutant RLC reduced the rate of cross-bridge turnover implies that the divalent cation-binding ability of RLC is important in modulating the absolute rate of force development as well as its Ca^{2+} dependence.

D47A effects on stiffness

The effects of D47A RLC to reduce force and k_{tr} might be explained by a D47A-induced increase in cross-bridge compliance but no change in cross-bridge number. We previously measured stiffness in D47A-exchanged fibers using small-amplitude, high-frequency sinusoidal length changes and found that stiffness declined in proportion to force. Because high-frequency fiber stiffness is thought to reflect the number of attached cross-bridges, we concluded that D47A exchange reduced the number of attached bridges after D47A exchange but had no effect on the average force per cross-bridge. In the present study we sought to complement our earlier sinusoidal stiffness measurements by assessing the effect of D47A exchange on fiber stiffness, measured with small step changes in fiber length (Huxley and Simmons, 1971; Ford et al., 1977). Similar to our earlier results (Diffee et al., 1995a), we found that D47A incorporation again reduced stiffness in a way that was consistent with a decrease in the number of attached cross-bridges rather than a decrease in the force developed by each of the attached bridges.

It must be noted that both sinusoidal and length-step stiffness measurements provide imperfect measurements of the number of attached cross-bridges, particularly in light of recent observations of substantial thin-filament compliance in skinned skeletal muscle fibers (Higuchi et al., 1995). Furthermore, neither method can accurately distinguish between a change in the number of attached cross-bridges versus a change in the stiffness (or compliance) of individual attached cross-bridges. Thus, the results of the present study are also consistent with an increase in the compliance of individual cross-bridges as a result of D47A incorporation. Clearly, an independent measure of the number of attached cross-bridges that is not confounded by the stiffness of attached cross-bridges is necessary, and the technique of Duong and Reisler (1989), based on the decreased proteolytic susceptibility of attached cross-bridges, may eventually prove helpful in addressing this question. However, the fact that in the present study tension and stiffness were both reduced to ~50% of control values by either exchange of D47A or by reducing the [Ca^{2+}] (Fig. 5) argues that the decline in stiffness in both cases is most likely due to a reduction in the number of attached cross-bridges. And the fact that the decrease in force due to D47A exchange can be explained entirely by changes in the rates of cross-bridge attachment and detachment (see below) argues against a change in the force (or stiffness) per cross-bridge.

Tension relaxation kinetics

The decrease in k_{tr} after D47A exchange indicates that the overall rate of cross-bridge cycling is reduced, because k_{tr} gives a measure of the sum of the forward and reverse rate constants ($f_{app} + g_{app}$) of the two-state cross-bridge cycle. To accurately predict the effect of altered cycling rate on force, it is necessary to separate the effects of D47A incorporation on f_{app}, the rate constant for cross-bridge attachment, and g_{app}, the rate constant for cross-bridge detachment. As an approximation of the rate of cross-bridge detachment, we examined the kinetics of tension relaxation in fibers before and after exchange of D47A. Tension was relaxed in steadily activated fibers by rapidly lowering [Ca^{2+}] via flash photolysis of diazo-2. Because tension relaxation after flash photolysis of diazo-2 involves a number of complex processes, including Ca^{2+} dissociation from TnC, detachment of cross-bridges, and possible recycling of detached bridges, the relaxation rate may not provide a direct measure of g_{app}. Clearly, though, the rate of tension relaxation is related to the rate of cross-bridge detachment, and the rates of relaxation obtained in the present study yield rate constants that are similar to estimates of g_{app} in previous studies (Brenner, 1988; Brenner and Eisenberg, 1986). Thus, our finding that the rate of relaxation is increased twofold after D47A exchange suggests that D47A incorporation into fibers increases the rate of cross-bridge detachment. This change in the rate of relaxation is not thought to be an indirect effect of the lower force after D47A exchange, because previous studies have indicated that the rate of tension relaxation induced by photolysis of diazo-2 is insensitive to the starting level of activation and to the extent of relaxation (Zhang et al., 1995; Walker et al., 1992).

D47A effects on cross-bridge cycling rates

As illustrated by Eq. 1 above, steady-state force can be modulated by a change in the number of cycling cross-bridges (n), a change in the average force per cross-bridge (F), or a change in the proportion of cycling cross-bridges that are in the force-generating state ($f_{app}/(f_{app} + g_{app})$). Our finding that the incorporation of D47A RLC into fibers reduces k_{tr} demonstrates that the overall rate of cross-bridge cycling is reduced by D47A. However, our measurements regarding tension relaxation rates suggest that D47A increases the rate of cross-bridge detachment by twofold. Thus, the results of the present study indicate that D47A incorporation decreases the rate of cross-bridge attachment and increases the rate of detachment. Assuming that k_{tr} is a measure of the sum of $f_{app} + g_{app}$ and that the fast rate of tension relaxation induced by photolysis of diazo-2 approximates g_{app}, our results indicate that D47A incorporation causes a decrease in $f_{app} + g_{app}$ to 70% of control while at the same time increasing g_{app} by as much as twofold compared to control. This results in a decrease in the ratio $f_{app}/(f_{app} + g_{app})$ to ~60% of the control ratio. Thus, the D47A-induced decline in tension (to ~60% of control tension) can be explained entirely on the basis of alterations in the rate constants of cross-bridge attachment and detachment. The fact that the effects of D47A RLC on tension are apparently manifestations of changes in cross-bridge kinetics argues against gross structural abnormalities in the cross-bridge as a result of D47A incorporation, because it appears that cross-bridges containing D47A remain part of the pool of cycling cross-bridges and that the force per attached cross-bridge is unchanged.

Implications for the role of RLC in contraction

The results of the present study demonstrate that a functional divalent cation-binding site on RLC is important in the development of strongly bound cross-bridges and that impaired divalent cation binding to the RLC alters the kinetics of cross-bridge cycling. At present the mechanism for these effects is unknown. Studies of the RLC from scallop myosin suggest that in the absence of divalent cation binding, the RLC is likely to dissociate from the heavy chain (Xie et al., 1994; Houdusse and Cohen, 1996). However, it is important to note that the effects of replacement of ~50% of endogenous RLC with D47A RLC are very different from the effects due to extraction of up to ~50% of endogenous RLC. Partial extraction of myosin RLC from skeletal muscle fibers has been shown to increase both force and the rate of tension development under submaximum conditions (Hofmann et al., 1990; Metzger and Moss, 1992), effects that are opposite those seen in the present study. Thus it appears unlikely that the effects of D47A incorporation are due to incomplete binding to the heavy chain. The earlier findings of the effects of RLC extraction (Hofmann et al., 1990; Metzger and Moss, 1992) led to the hypothesis that Ca^{2+} binding to the Ca^{2+}/Mg^{2+} site on RLC was an important modulator of the rate and extent of tension development. We have previously shown that partial extraction of RLC eliminated the Ca^{2+} dependence of the rate of tension development (Patel et al., 1996). The fact that D47A RLC exchange also reduced the variation of k_{tr} with Ca^{2+} (Fig. 3) implies that divalent cation binding to the RLC plays an important role in conferring Ca^{2+} sensitivity on the activation dependence of the kinetics of tension development. The observations that the mutant RLC slowed the rate of cross-bridge attachment and sped the rate of detachment imply that in the absence of divalent cation binding to myosin RLC the preferred configuration for the cross-bridge is to be detached.

With this mutant RLC we are unable to distinguish between effects due to alterations in Ca^{2+} versus Mg^{2+} binding at the divalent cation-binding site, because this mutation substantially reduces both Ca^{2+} and Mg^{2+} binding to the isolated light chain (da Silva et al., 1995). Given the relative Mg^{2+}- and Ca^{2+}-binding affinities of skeletal muscle native RLC, it is likely that Ca^{2+} replaces Mg^{2+} at the divalent cation site only during periods of prolonged elevation of $[Ca^{2+}]$ (Bagshaw and Reed, 1977; Holroyde et al., 1979). We have recently observed that partial extraction of RLC alters the effect of variations in $[Mg^{2+}]$ on steady-state tension in skeletal muscle fibers (Patel et al., 1996), suggesting that occupancy of the RLC divalent cation-binding site by Mg^{2+} may in itself play a role in modulation of tension or that the RLC may play a different role in modulating tension, depending on whether Mg^{2+} or Ca^{2+} is bound. Resolution of this question would be aided by incorporation of a RLC with substantially different affinities for Ca^{2+} versus Mg^{2+}, but this requires a greater understanding of the factors affecting ion specificity in "EF hand"-type divalent cation-binding sites. In any case, the results of the present study indicate that the divalent cation-binding site on RLC is an important component in the mechanism of modulation of cross-bridge cycling kinetics in skeletal muscle fibers.

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