Effects of a Non-Divalent Cation Binding Mutant of Myosin Regulatory Light Chain on Tension Generation in Skinned Skeletal Muscle Fibers

Gary M. Diffee,* Marion L. Greaser,‡ Fernando C. Reinach,§ and Richard L. Moss*
*Department of Physiology and ‡Muscle Biology Laboratory, University of Wisconsin, Madison, WI 53706 USA; and §Instituto de Química, Universidade de São Paulo, Brazil.

ABSTRACT Each myosin molecule contains two heavy chains and a total of four low-molecular weight light chain subunits, two "essential" and two "regulatory" light chains (RLCs). Although the roles of myosin light chains in vertebrate striated muscle are poorly understood at present, recent studies on the RLC have suggested that it has a modulatory role with respect to Ca^{2+} sensitivity of tension and the rate of tension development, effects that may be mediated by Ca^{2+} binding to the RLC. To examine possible roles of the RLC Ca^{2+}/Mg^{2+} binding site in tension development by skeletal muscle, we replaced endogenous RLC in rabbit skinned psoas fibers with an avian mutant RLC (D47A) having much reduced affinity for divalent cations. After replacement of up to 80% of the endogenous RLC with D47A RLC, maximum tension (at pCa 4.5) was significantly reduced compared with preexchange tension, and the amount of decrease was directly related to the extent of D47A exchange. Fiber stiffness changed in proportion to tension, indicating that the decrease in tension was due to a decrease in the number of tension-generating cross-bridges. Decreases in both tension and stiffness were substantially, although incompletely, reversed after reexchange of native RLC for D47A. RLC exchange was also performed using a wild-type RLC. Although a small decrease in tension was observed after wild-type RLC exchange, the decrease was not proportional to the extent of RLC exchange and was not reversed by reexchange of the native RLC. D47A exchange also decreased the Ca^{2+} sensitivity of tension and reduced the apparent cooperativity of tension development. The results suggest that divalent cation binding to myosin RLC plays an important role in tension generation in skeletal muscle fibers.

INTRODUCTION

In vertebrate striated muscle, myosin is a hexamer comprised of two high-molecular weight (~200 kDa) heavy chains and four low-molecular weight (~20 kDa) light chain subunits. The light chains of myosin have been classified based on the conditions required for their differential dissociation from myosin (Weeds and Lowey, 1971). One class of light chains can be chemically dissociated from the myosin molecule using the reagent 5,5′-dithio-2-nitrobenzoic acid (DTNB) and are thus called the DTNB light chains, also known as the "regulatory light chain" (RLC) or light chain 2 (LC_2). Only one isoform of LC_2 is expressed in mammalian fast-twitch skeletal muscle, and is present in a ratio of 2 mol LC_2/mol myosin. A second class of light chains can be dissociated using alkaline solutions and are thus termed the alkali, or "essential" light chains. In mammalian fast-twitch muscles there are two distinct alkali light chains, LC_1 and LC_3, that are present in a molar ratio of 2 mol of alkali light chains/mol of myosin and 2 mol LC_1/mol LC_3 (Lowey and Risby, 1971). At the present time, the roles of the myosin light chains in contraction of vertebrate striated muscles are generally unknown. However, in vertebrate smooth muscles, in nonmuscle cells, and in most invertebrate muscles, the myosin light chains appear to be a primary molecular component of the Ca^{2+} regulatory system. In many smooth muscles and nonmuscle systems, contraction is initiated by Ca^{2+}/calmodulin-dependent phosphorylation of the RLC (thus this light chain has also been termed the "P-light chain"). In molluscan muscles, contraction is initiated by direct binding of Ca^{2+} to myosin, a mechanism that requires the RLC either directly or as an accessory protein (Goodwin et al., 1990; Kwon et al., 1990; Simmons and Szent-Gyorgi, 1978).

In vertebrate striated muscles, Ca^{2+} regulation of contraction occurs primarily via Ca^{2+} binding to the thin filament regulatory protein troponin (Tn). Ca^{2+} binds to low-affinity sites on the TnC subunit of Tn, which through a series of events involving the other Tn subunits and the regulatory protein tropomyosin, activates the binding of cross-bridges to actin in the thin filament. While it has long been believed that Ca^{2+} has a switch-like regulatory role in muscle, recent findings suggest that regulation of contraction is more complicated in that tension is modulated by a number of factors such as cooperative binding of Ca^{2+} to TnC due to interactions among proteins of the thin filament (Grabarek et al., 1983), and cooperative effects of strong-binding myosin cross-bridges to enhance Ca^{2+} binding and to facilitate additional cross-bridge attachments (Bremel and Weber, 1972; Greene and Eisenberg, 1980; Grabarek et al., 1983). In addition, results of a number of studies suggest that some thick filament-associated proteins may have a role in conferring Ca^{2+} sensitivity to contractile properties. Lehman (1978) observed that the ATPase activity of myosin and presumably unregulated actin in solution was substantially increased by the addition of Ca^{2+} and from this concluded that there was a thick filament-linked Ca^{2+} regulatory mechanism in vertebrate striated muscle. Studies using both biochemical

Received for publication 23 June 1994 and in final form 20 January 1995.
Address reprint requests to Dr. Gary M. Diffee, Department of Physiology, University of Wisconsin School of Medicine, 1300 University Ave., Madison, WI 53706. Tel.: 608-262-7586; Fax: 608-265-5512; E-mail: gmdiffee@facstaff.wisc.edu.

0006-3495/95/04/1443/10 $2.00

(Wagner, 1984; Margossian et al., 1983) and physiological (Moss et al., 1982, 1983; Hofmann et al., 1990; Metzger and Moss, 1992) approaches have concluded that the RLC of myosin may play a role in modulating contraction and in regulating contractile properties.

Extraction of up to 50% of the endogenous RLC has been shown to decrease maximum shortening velocity (V_{max}) in skinned skeletal muscle fibers (Moss et al., 1982, 1983; Hofmann et al., 1990). Consistent with these findings, the sliding velocity of actin in an in vitro motility assay was much reduced in the presence of RLC-deficient myosin as compared with light chain-replete myosin (Lowey et al., 1993). In skinned fibers, extraction of RLC had no effect on either maximum tension or stiffness, but increased both tension and stiffness during submaximal activations (Hofmann et al., 1990), indicating that the Ca^{2+} sensitivity of tension was increased. Finally, Metzger and Moss (1992) found that the Ca^{2+} sensitivity of the rate constant of tension redevelopment (k_{tr}), initially described by Brenner (1986), is modulated by partial extraction of RLC from skinned skeletal muscle fibers. Extraction had no effect on k_{tr} during maximal activation but significantly increased k_{tr} during submaximal activations. They concluded that Ca^{2+} binding to a Ca^{2+}/Mg^{2+} site on the RLC was involved in this modulation of k_{tr} because increased free [Mg^{2+}] reduced k_{tr} at submaximal Ca^{2+} concentrations, an effect that was abolished by partial extraction of RLC but not TnC.

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. The present study was designed to directly assess the importance of the RLC divalent cation binding site in modulating tension development in vertebrate striated muscle. This was done by replacing endogenous RLC in skinned skeletal muscle fibers with a mutant RLC (D47A, containing a single point mutation in the Ca^{2+}/Mg^{2+} site), that has previously been shown to have a substantially decreased ability to bind Ca^{2+} (Reinach et al., 1986) and presumably Mg^{2+} (Strynadka and James, 1989). A preliminary account of this work appeared previously (Diffee et al., 1994).

MATERIALS AND METHODS

Mechanical measurements on skinned fibers

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% (v/v) glycerol. Before each experiment, a bundle was placed in relaxing solution containing 0.5% (w/v) Brij-58 for 30 min to disrupt the sarcoplasmic reticulum. 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 attached 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 a DC torque motor (model 6350; Cambridge Technology, Inc.). The resting sarcomere length was set to 2.3–2.6 μm by adjusting the overall length of the fiber segment. Complete details of the mounting procedure and experimental setup have been described previously. (Moss et al., 1983).

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 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 and pH, and for an experimental temperature of 15°C.

Before tension measurements, the fiber was bathed for 15 s in relaxing solution containing 0.5 mg purified TnC/ml to insure the fiber was fully TnC replete. Tension was first measured in pCa 4.5, and then in randomly selected submaximal pCa solutions, with every fourth activation made in pCa 4.5 to assess any decline in fiber performance. For each activation steady tension was allowed to develop, after which the fiber was slackened and subsequently transferred to relaxing solution. The difference between steady developed tension and the tension baseline immediately after the slack step was measured as total tension. Active tension was calculated by subtracting resting tension at pCa 9.0 from total tension. Tensions at each pCa are expressed as fractions of the maximum tension (pCa 4.5) obtained for that fiber under the same conditions, i.e., control or following exchange of RLC.

In-phase cross-bridge stiffness was measured during maximal activation at 10°C by applying a small amplitude (<0.1% of fiber length) 3.3 kHz sinusoidal length change at one end of the fiber and measuring the resultant changes in force. Sarcomere length was measured using 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). This system has a resolution of 0.5–1.0 nm/half-sarcomere with sufficient intensity of the first-order line to allow sarcomere length measurements at all levels of activation. The peak-to-peak amplitudes of sarcomere length (ΔSL) and tension (ΔT) were measured as the averages of 10 consecutive oscillations, and stiffness was calculated as $\Delta T/\Delta SL$.

Preparation of proteins

The mutant RLC (D47A) and wild-type (WT) RLC were prepared as described previously (Reinach et al., 1986) by expression of the full-length chicken skeletal myosin RLC gene in *Escherichia coli*. A plasmid, pLcI-IFXMLC, was constructed, which directed the synthesis of a fusion protein consisting of the N-terminal 31 amino acid residues of the λ *c*II protein, the blood coagulation factor X_a recognition site, and the complete chicken myosin RLC polypeptide. The fusion protein was digested with thrombin, and purified RLC was obtained by DEAE cellulose chromatography. The D47A mutant was constructed by replacing aspartate-47 with alanine by oligonucleotide-directed mutagenesis. The amino acid D47 is believed to be at the "−Z" coordination position in the Ca^{2+}/Mg^{2+} binding loop of the RLC. This mutation has been shown to result in dramatically reduced Ca^{2+} binding to the light chain (Reinach et al., 1986). A mutation to alanine at this position would eliminate the −Z coordination position and thus would also be likely to decrease Mg^{2+} binding to the RLC (Strynadka and James, 1989), although the Mg^{2+} binding of this mutant has not been measured directly. Reinach et al. (1986) measured the Ca^{2+} binding capability of the D47A mutant in solution but not when the mutant RLC was bound to myosin in skinned fibers. Our preliminary experiments using the Ca^{2+} indicator fluo-3 and flash photolysis of caged Ca^{2+} indicate that when 50% of endogenous RLC in rabbit psoas fibers is replaced with the mutant, Ca^{2+} binding to RLC decreases by ~50% (Patel et al., 1994).

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

Maximum tension, maximum stiffness, and tension-pCa relationships were measured under control conditions for each fiber, and these measurements

were then repeated in the same fiber after the exchange of exogenous RLC into the fiber. Exogenous RLCs (D47A RLC, WT RLC, or purified native rabbit skeletal RLC) were exchanged using a modification of the method used to extract RLC from skinned fibers (Hofmann et al., 1990). Fibers were warmed to 37°C and bathed in a solution containing 25 mM KCl, 10 mM EDTA, 10 mM Imidazole (pH 7.0), 2 mM DTT, and 2–3 mg/ml of exogenous RLC, for 30 min. The fibers quickly developed rigor tension in the exchange solution. To minimize the deleterious effects of maintained rigor tension at elevated temperature, the fibers were slackened during the period of exchange. After the 30-min exchange procedure, the chamber was again cooled to 15°C and the fiber was transferred to relaxing solution. Fiber length was adjusted if necessary to attain the original sarcomere length for subsequent mechanical measurements. A small number of fibers had a high level of resting tension at pCa 9.0 after RLC exchange. If this increased resting tension (>1% of active tension) persisted the fiber was discarded. Since 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. This cycle of TnC soak and maximal activation was repeated until there was no significant increase in active tension. Maximum tension, stiffness, and the tension-pCa relationship were then measured in the RLC-exchanged fibers. In a number of cases, 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 assessed using SDS-polyacrylamide gel electrophoresis (PAGE) and an ultrasensitive silver stain technique described previously (Giulian et al., 1983). The stained gels were scanned with a densitometer, and the areas under the peaks were integrated using commercially available software (BioMed Instruments, Fullerton, CA). The extent of exchange of exogenous RLC was calculated from the ratio of exogenous RLC/total RLC on 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.

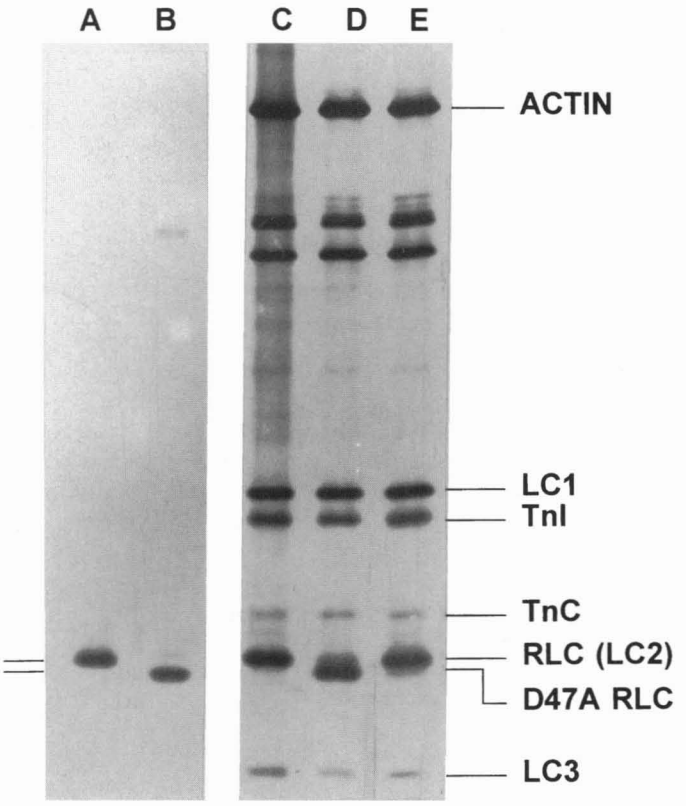

FIGURE 1 SDS-PAGE analysis of a fiber illustrating nearly stoichiometric exchange of RLC. Lanes A and B are purified rabbit native skeletal RLC and mutant D47A RLC, respectively. Lane C is a control segment of fiber #09091 taken before exchange experiments. Lane D is a segment of the same fiber after exchange of D47A RLC into the fiber. Lane E shows reexchange of rabbit skeletal RLC back into the same fiber. The D47A RLC/total RLC ratio is 0.833 in lane D and 0.174 in lane E, while the (total RLC)/(LC_1+LC_3) ratios are 1.03, 1.25, and 1.17 in lanes C, D, and E, respectively.

RESULTS

Results of SDS-PAGE analysis of RLC exchange

SDS-PAGE analysis of fiber segments showing the results of a representative exchange experiment is shown in Fig. 1. The D47A and WT RLC are avian gene products, and the resultant proteins had slightly greater mobility under these gel conditions than that shown by the rabbit fast skeletal RLC, because avian RLC has a slightly lower molecular weight than rabbit RLC. This ability to resolve rabbit RLC and exogenous RLC by SDS-PAGE allowed direct determination of the extent of RLC exchange in fiber segments used for mechanical measurements. In the fiber shown in Fig. 1, D47A RLC content after exchange was 83.3% of the total RLC (total RLC = endogenous RLC + exogenous RLC exchanged into the fiber). There was variability in the amount of exogenous RLC exchanged into the fibers; however, exchange of RLCs under conditions described in Materials and Methods consistently resulted in replacement of 60–90% of endogenous RLC with no significant effect on the (total RLC)/(LC_1+LC_3) ratio. The mean ± SEM of (total RLC)/(LC_1+LC_3) ratios was 0.902 ± 0.026 in control fibers and 1.012 ± 0.049 in exchanged fibers. Reexchange of rabbit skeletal RLC back into these fibers reduced D47A RLC content to a mean (±SEM) of 16.3% ± 1.1% of the total RLC. To extend the range of the percent RLC exchanged (see Fig. 3), some fibers were subjected to a lower temperature (~30°C) or a shorter incubation time (15 min) during the exchange procedure, resulting in lesser exchange. On the other hand, increasing the temperature or time of exchange did not significantly affect the amount of RLC exchanged into the fibers. It is apparent from SDS-PAGE analysis that the readdition of TnC was effective in keeping the fibers TnC replete even after multiple exchange procedures, because the TnC/TnI ratios were unchanged in both experimental segments.

Effects of RLC exchange on maximum isometric tension

The effects on isometric tension due to exchange of the mutant D47A RLC (Fig. 2 *A*) or WT RLC (Fig. 2 *B*) are shown for representative fibers. In the D47A experiment shown, tension was reduced to ~50% of control tension, and was not increased by subsequent TnC soaks. Tension recovered to

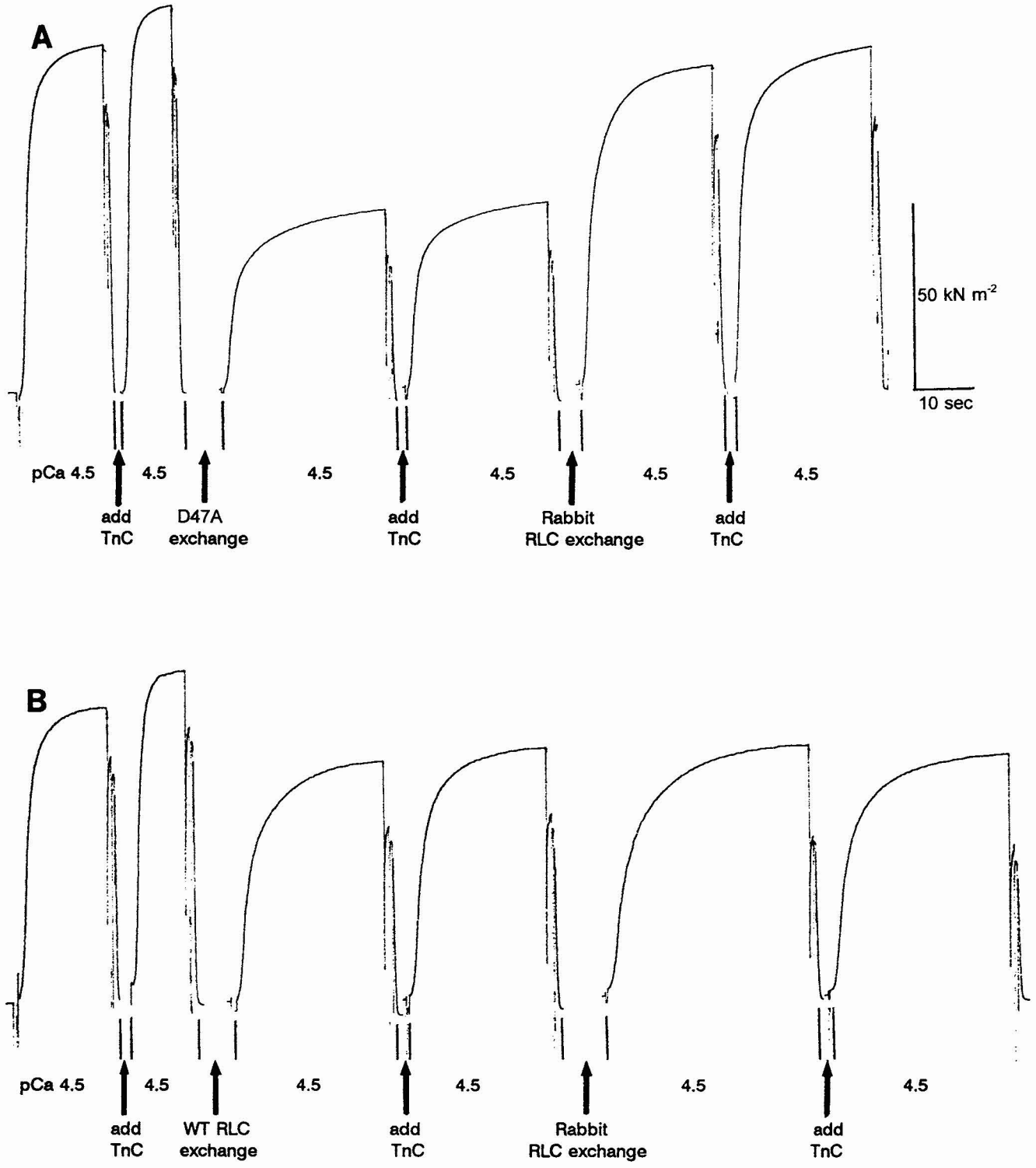

FIGURE 2 (*A*) Original slow-time base tension records showing reversible effect of D47A exchange into a rabbit psoas fiber. "add TnC" denotes multiple cycles of TnC addition and maximal activations as described in methods. Fiber #03301, $SL = 2.47$. (*B*) The same experiment on another fiber in which WT RLC was exchanged into the fiber. Fiber #03311, $SL = 2.45$. Scale bars are the same for both (A) and (B) traces. The extent of exogenous RLC exchange into these fibers was not assessed.

~80% of control tension after reexchange of rabbit RLC into the fiber. In the eight experiments in which reexchange was done, tension was increased after RLC exchange in all cases. The mean (±SEM) maximal tension was 50.4% ± 4.8% of control after D47A exchange and 75.4 ± 5.6% of control after reexchange of rabbit RLC into the fiber. Summary data from 19 fibers show that after exchange of the D47A into the fibers maximum tension (P_o) declined to an average of 47.3%

of control, and the magnitude of tension decline was proportional to the percent D47A exchanged (Fig. 3). The lines drawn in Fig. 3 are linear best fits to the data but should not be taken to indicate that the relationship between the extent of D47A RLC exchange and the decline in maximum tension is necessarily linear over the entire range, because we have no data for extents of exchange greater than ~80%.

To determine whether the observed effects of D47A exchange on tension were specifically due to the mutation, WT RLC was exchanged into some fibers. The results of one such WT RLC exchange experiment are shown in Fig. 2 *B*. In a total of nine fibers, exchange of WT RLC resulted in a small decline in maximum tension, to an average of 79.3% of control tension. However, this decrease was not reversed by reexchange of rabbit RLC back into the fiber (Fig. 2), and the decline in tension was not proportional to the extent of WT RLC exchange (Fig. 3). Thus, it is likely that in the case of WT RLC, the observed decrease in tension is due to a nonspecific decline in fiber performance associated with the exchange protocol, rather than a specific result of WT RLC exchange into the fiber. We also did a small number of experiments (n = 3) in which rabbit RLC was exchanged into the fibers. In these fibers, tension after exchange using rabbit RLC was an average of 89.9% of control tension.

Effects of RLC exchange on stiffness

To investigate whether the observed reductions in tension were due to changes in numbers of force-generating cross-bridges or in the force per attached cross-bridge, fiber stiffness was measured before and after exchange of D47A RLC. Fig. 4 shows original records of force and sarcomere length

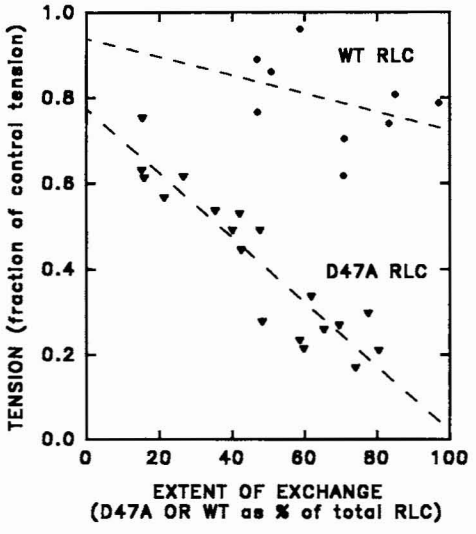

FIGURE 3 Correlation between the decline in tension after exchange and the incorporation of exogenous RLC in 28 fibers. Tension is expressed as a fraction of maximal tension in the same fiber before the exchange procedure (control tension). Lines are drawn by least-squares linear regression. There is a strong correlation (R = 0.93) between the extent of D47A RLC exchange and the decline in maximum tension. In the case of WT RLC exchange, R = 0.38.

obtained when a 3.3 kHz-length sinusoid (<0.1% of fiber length, i.e., ~2 nm/half sarcomere) was introduced at one end of a maximally activated fiber. In the fiber shown, stiffness at pCa 4.5 was 30.7 N/m before exchange and declined to 9.5 N/m in the same fiber after the exchange of D47A, which is 30.7% of control stiffness. In this same fiber maximum tension declined to 38.8% of control tension. Summary data in Fig. 5 shows that in 14 fibers tested the decline in stiffness observed following D47A RLC exchange was proportional to the decrease in maximum tension, suggesting that the decrease in tension can be accounted for on the basis of a decrease in the number of force-generating cross-bridges.

Tension-pCa relationship

The effects of D47A and WT RLC exchange on tension at submaximal concentrations of Ca^{2+} were also investigated and are shown for representative fibers in Fig. 6. It should be noted that tension is expressed relative to the maximum (pCa 4.5) tension obtained under the same conditions, i.e., control or exchanged. Thus, in the case of the D47A RLC exchange experiments, tension is expressed relative to the maximum tension after exchange, although the absolute maximum tension is less than control. D47A RLC exchange into the fibers resulted in significant depression in relative submaximal tensions so that the tension-pCa relationship was shifted to higher [Ca^{2+}] (Fig. 6 *A*). The right shift induced by D47A RLC exchange was partially reversed after subsequent reexchange of rabbit skeletal RLC back into the fiber, which in every case was less than stoichiometric. Fig. 6 *B* shows that exchange of WT RLC into the fiber did not have a significant effect on the tension-pCa relationship.

Summary tension-pCa data for 12 fibers showing the effects of D47A RLC exchange is presented in Fig. 7. Exchange of mutant RLC into these fibers resulted in a 0.2 pCa unit rightward shift in the pCa_{50}. This result is qualitatively different from that seen when the RLC is partially extracted from skeletal muscle fibers, which induces a leftward shift in the tension-pCa relationship (Hofmann et al., 1990).

In addition to the rightward shift of the pCa_{50}, D47A exchange reduced the steepness of the tension-pCa curve. Summary tension-pCa data from D47A-exchanged fibers were characterized by Hill plot analysis (Fig. 7 *B*), which is typically used to quantify the shape of the tension-pCa relationship (Shiner and Solaro, 1984). The Hill plot for data from control fibers is best fit by two straight lines that intersect at the pCa_{50}, as seen previously for skeletal muscle fibers (Moss et al., 1983). The Hill coefficients n_1 (for tensions >pCa_{50}) and n_2 (<pCa_{50}) are in good agreement with previously published values (Moss et al., 1983; Hofmann et al., 1990). After D47A exchange, both n_1 and n_2 were reduced, indicating a reduction in the apparent cooperativity of tension development in these fibers. The difference between n_1 and n_2 was also substantially reduced in D47A-exchanged fibers compared with control, so that n_1 and n_2 were more nearly equal.

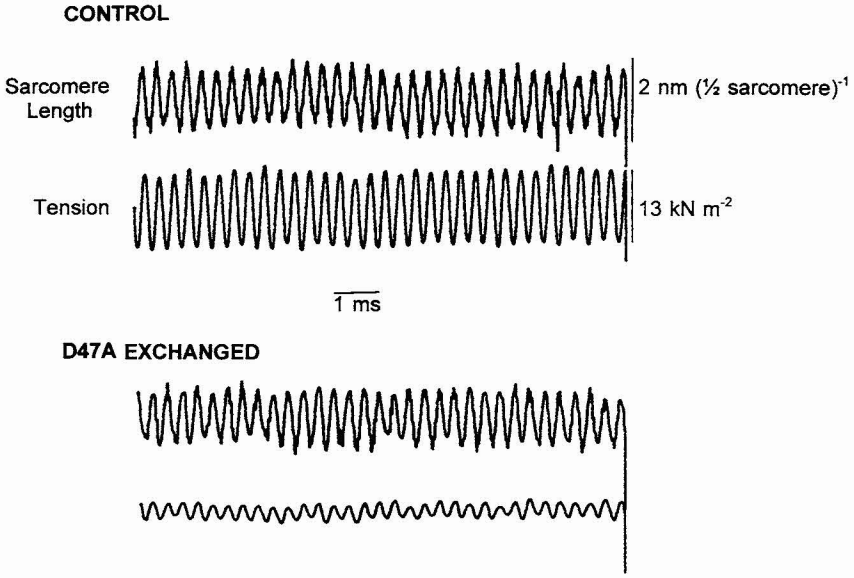

FIGURE 4 Original fast-time base recordings of tension and sarcomere length during stiffness measurements in pCa 4.5 at 10°C. Traces are shown for fiber #07021 under control conditions and for the same fiber following exchange of D47A RLC into the fiber. Data regarding extent of D47A exchange into this fiber are not available. Scale bars are the same for both control and D47A-exchanged traces.

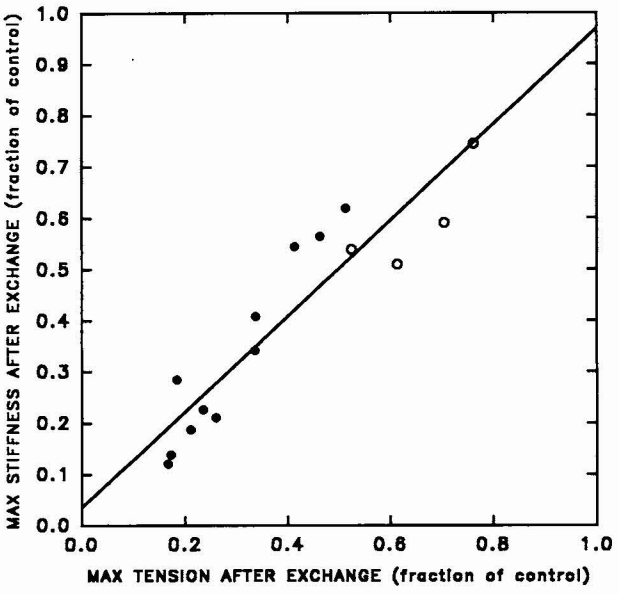

FIGURE 5 Plot comparing the change in maximum tension after D47A RLC exchange (●) with the change in maximum stiffness in a total of 11 fibers. (○) Data collected after reexchange of rabbit RLC back into the fiber after the initial D47A exchange. The solid line is drawn using least-squares linear regression and has a slope of 0.934 (R = 0.924).

DISCUSSION

RLC exchange

In this study, we have demonstrated the feasibility of near-stoichiometric exchange of exogenous RLCs into skinned skeletal muscle fibers under conditions that allow measurements of mechanical properties both before and after the exchange. When the D47A mutant RLC is incorporated into skinned fibers, tension-generating capability is reduced under both maximal and submaximal activating conditions, and the decline in maximal tension is proportional to the amount of D47A in the fiber. These effects appear to be due specifically to the presence of the mutant RLC in the fiber rather than a nonspecific effect of the exchange procedure. Although it might be expected that conditions of the exchange procedure, such as high temperature and low ionic strength, would result in significant deterioration of the fiber, control experiments clearly show that fiber damage cannot account for the observed effects of D47A exchange. For example, whereas we found the decline in tension to be proportional to the extent of incorporation of D47A RLC, control experiments in which wild-type RLC was exchanged into the fibers resulted in relatively small reductions in tension that were apparently unrelated to the amount of WT RLC exchanged. Because the fibers were subjected to identical exchange conditions, the greater reductions in tension with D47A incorporation are most likely due specifically to the mutant. Secondly, the reductions in tension with D47A were substantially reversed when most of the mutant RLC was replaced with rabbit native RLC by reexchange. While the finding that tension did not recover to 100% of the preexchange value indicates that some rundown of the fiber may have occurred, the large increases in tension after a second exchange protocol clearly indicate that fiber rundown cannot account for the observed effects of D47A on tension.

D47A-induced reductions in tension also cannot be attributed to net extraction of either total RLC or TnC as secondary effects of the exchange procedure. With respect to RLC, SDS-PAGE analysis of segments from the same fibers before and after exchange showed no change in the (total RLC)/(LC_1+LC_3) ratio, indicating that there was no change in total RLC content. Even if a small amount of RLC had been extracted, or if some of the D47A RLC was nonspecifically bound to myosin, effects such as those observed here would not be expected. Previous studies (Hofmann et al., 1990) showed that extraction of up to 50% of endogenous RLC had no effect on either maximum tension or

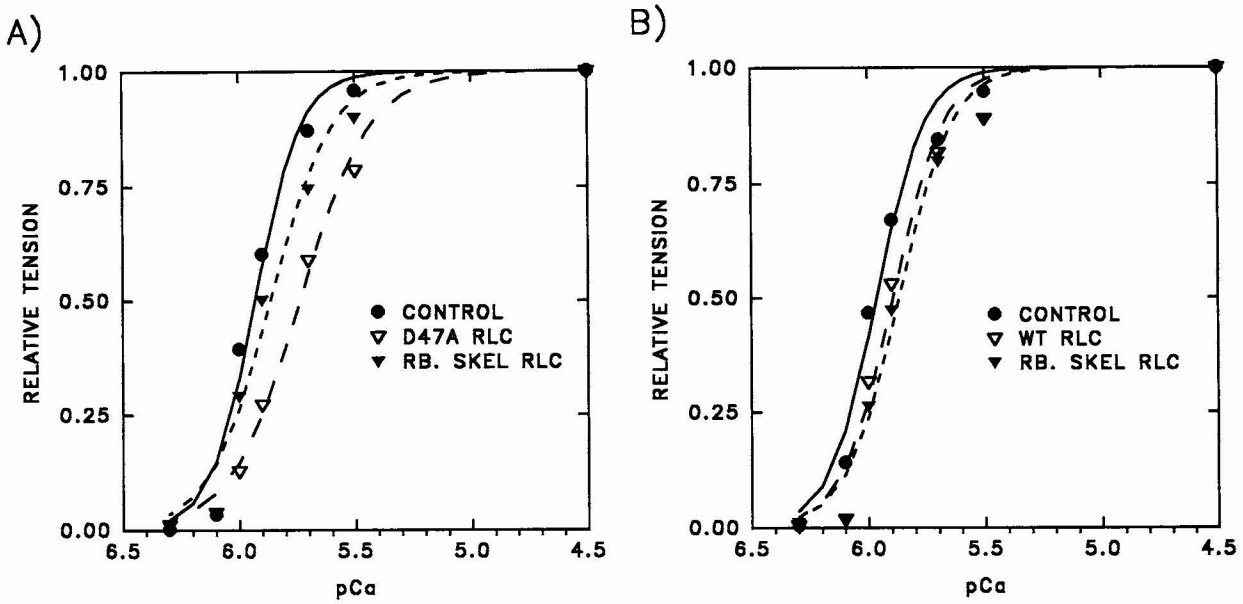

FIGURE 6 Representative tension-pCa relationships in fibers before and after the exchange of either (*A*) D47A RLC or (*B*) WT RLC into the fiber and again after reexchange of rabbit native skeletal RLC back into the fiber. Using constants derived from the Hill equation, curves were drawn by computer using the equation $P/P_o = [Ca^{2+}]^n/(k^n+[Ca^{2+}]^n)$ (Hofmann et al., 1991). (A) Fiber #05241. Absolute maximum tension in this fiber was 47.7% of control tension after D47A exchange (D47A content = 50.5% of total RLC) and recovered to 77.3% of control after reexchange of rabbit native RLC (D47A content reduced to 17.8% of total RLC). (B) Fiber #05251. Absolute maximum tensions were 84.1% and 81.3% of control after exchange of WT RLC and rabbit skeletal RLC, respectively. WT RLC content was 44.7% of total RLC after exchange and 13.4% following reexchange.

maximum stiffness but caused proportionate increases in tension and stiffness during submaximal activations. These effects are clearly opposite those seen in the present study as a result of D47A RLC exchange. On the other hand, the effects of D47A RLC to reduce maximum tension and induce a rightward shift of the tension-pCa relationship are qualitatively similar to the effects of partial extraction of TnC from skeletal muscle fibers (Moss et al., 1985). Although a small amount of TnC was extracted from the fibers during RLC exchange, this TnC was replaced by readdition, which we verified by SDS-PAGE analysis of fiber segments. Also, multiple soaks in relaxing solution containing TnC failed to

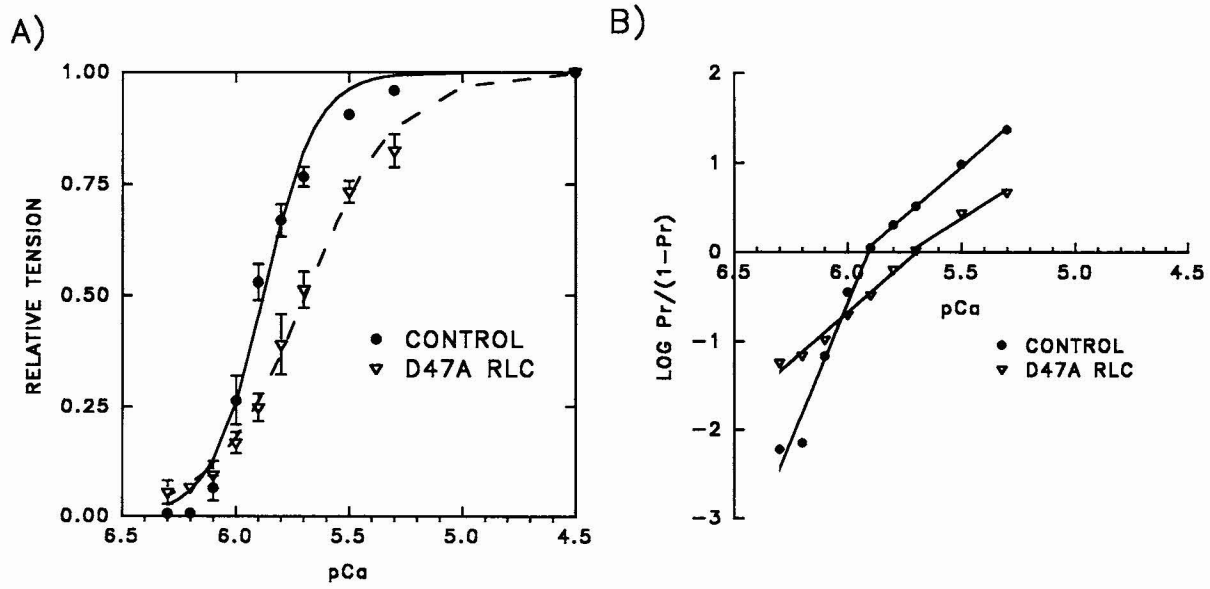

FIGURE 7 Summary data showing the effect of D47A RLC exchange on the tension-pCa relationship. Points are means ± SEM from 12 fibers. (*A*) Tension-pCa relationship showing the significant rightward shift following D47A exchange. The pCa_{50} values are 5.9 for the control trace and 5.7 for the D47A trace. (*B*) Hill plot transformation of tension-pCa data. The Hill coefficients for points to the right of the pCa_{50} (n_1) are 2.2 and 1.6 for control and D47A fibers, respectively, and for points to the left of the pCa_{50} (n_2) are 6.3 (control) and 2.2 (D47A).

increase tension substantially. As further evidence that TnC extraction cannot account for the observed effects of the exchange protocol, the WT RLC exchange involved a protocol identical to that for D47A but did not produce the effects of D47A exchange on maximum tension and the tension-pCa relationship. Finally, the effects of D47A exchange were substantially reversed by reexchange of rabbit native RLC into the fibers.

These results present a compelling case that the effects of D47A incorporation on fiber properties are due to the presence of the mutant RLC in the fiber. Comparison of the results of D47A exchange with the effects of WT RLC exchange provides strong evidence that the decline in maximal tension is specifically due to the mutation in the Ca^{2+}/Mg^{2+} binding site. Although the effects are most easily interpreted as being specifically due to the elimination of divalent cation binding to the RLC, it is possible that the mutation at this site produces other effects in addition to those related to the ability of the mutant to bind divalent cations. Xie et al. (1994) have proposed that divalent cation binding to RLC in scallop muscle results in a conformational change in the RLC that affects its ability to bind to myosin. It is possible, then, that the mutant RLC binds to myosin incompletely or in a different orientation than the native RLC. However, as discussed above, our results indicate that the ability of D47A to bind to myosin was not impaired, because gel analysis revealed no decline in total RLC content after exchange and because the mechanical properties after exchange were not consistent with a reduced total RLC content. Also, Reinach et al. (1986) found no evidence that the ability of the D47A mutant to bind to scallop myosin was diminished compared with native RLC.

Divalent cation binding to RLC

Thus, the effects of D47A RLC on maximum tension and the tension-pCa relationship strongly suggest that divalent cation binding to the RLC of myosin plays an important role in modulating the generation of tension in skeletal muscle fibers. Presently, it is not known whether the observed effects of D47A incorporation are due to decreased binding of Ca^{2+}, of Mg^{2+}, or of either. Although this mutation of the RLC has been shown to reduce Ca^{2+} binding to near zero (Reinach et al., 1986), the effects of the mutation on Mg^{2+} binding have yet to be determined, although it seems likely that Mg^{2+} binding would be similarly reduced (Strynadka and James, 1989).

Previous results from this laboratory have suggested that Ca^{2+} binding to the RLC may modulate tension development in skeletal muscle fibers. Extraction of RLC increased both tension and the rate constant of tension development (k_{tr}) during submaximal activations but had no effect on tension or k_{tr} in maximally activated fibers (Hofmann et al., 1990; Metzger and Moss, 1992). In RLC-replete fibers, increased free $[Mg^{2+}]$ reduced k_{tr} at low levels of activation, and this effect was eliminated by extraction of RLC but not by partial extraction of TnC. From these results, it appeared that exchange of Mg^{2+} for Ca^{2+} at the divalent cation binding site on RLC slowed k_{tr} and led to the hypothesis (Metzger and Moss, 1992) that under normal conditions, the RLC represses the formation of force-generating cross-bridges presumably by slowing transitions between unattached states and attached, force-generating states. From earlier results, it appears that this repressive effect of RLC can be relieved by Ca^{2+} binding to RLC or by extracting RLC from the myosin head. Data from the present study extend this model in providing direct evidence that the RLC must have an intact divalent cation binding site for cross-bridges to assume force generating configurations. In addition, the observation that stiffness declined in parallel with tension indicates that cross-bridges with D47A RLC cannot assume strongly bound pre-force-generating states.

Possible mechanism of action of RLC on maximum tension

The mechanism by which divalent cation binding to RLC might influence tension generation is uncertain, but could involve either a change in the number of tension generating cross-bridges or a change in the force developed by each attached cross-bridge. For instance, binding of divalent cations to RLC might stabilize the structure of the myosin head during tension generation, an idea that is consistent with the proposal by Rayment et al. (1993) that the light chains stabilize the α-helical segment of myosin subfragment-1.

Results of this study strongly suggest, however, that replacement of endogenous RLC with D47A acts to reduce the number of force-generating cross-bridges and not the force per cross-bridge. This conclusion is evident in the data shown in Fig. 5, in which high-frequency stiffness, a measure of the number of attached cross-bridges (Ford et al., 1977), decreased in direct proportion to maximum tension in D47A-exchanged fibers. This finding implies that cross-bridges that contain RLC that lack the ability to bind divalent cations lose the ability to generate tension. Thus, the divalent cation binding site on RLC might have a regulatory function, e.g., modulation of the availability of cross-bridges for binding to actin. Our results do not eliminate the possibility that the binding site also has a structural role, because if D47A cross-bridges are unable to bind to actin, mechanical measurements would not probe the force-generating capabilities of these cross-bridges.

Effects of D47A on the tension-pCa relationship

The idea that the divalent cation binding site of RLC is involved in modulation of cross-bridge availability to actin is corroborated by effects of D47A on submaximally activated tension. Tension-pCa relationships from D47A-exchanged fibers exhibited both a decrease in the Ca^{2+} sensitivity of tension, evidenced by a right shift of pCa_{50} to higher $[Ca^{2+}]$, and a decrease in the apparent cooperativity of tension development, evidenced by smaller Hill coefficients (Shiner and Solaro, 1984). The decrease in Ca^{2+} sensitivity of tension

is consistent with earlier observations indicating that Ca^{2+} sensitivity is closely related to the maximum force-generating capability of a fiber (Sweitzer and Moss, 1990). Because cross-bridge attachment is known to cooperatively activate both Ca^{2+} and cross-bridge binding to the thin filament (Bremel and Weber, 1972; Greene and Eisenberg, 1980), the decrease in apparent cooperativity in D47-exchanged fibers is probably a direct result of a decrease in the number of cross-bridges available for attachment. However, our results do not exclude the possibility that reduced divalent cation binding to mutant RLC may have a direct effect on cooperativity of tension generation through some other mechanism.

CONCLUSIONS

Evidence presented here indicates that incorporation of a mutant RLC with much reduced affinity for divalent cations reduces the tension-generating capability of skinned skeletal muscle fibers during maximal and submaximal activations with Ca^{2+}. These effects do not appear to be due to gross alterations in the structure of the RLC, because it appears to bind readily to the myosin heavy chain. Control experiments in which wild-type chicken RLC and native rabbit RLC had little or no effect on tension-generating capabilities allow us to conclude that the effects of D47A incorporation are specifically due to the mutation of the divalent cation binding site. Measurements of fiber stiffness indicate that the tension decrease in D47A-containing fibers results from a reduction in the number of force-generating cross-bridges rather than a change in the force per cross-bridge. These results suggest that the RLC plays an important role in tension generation in vertebrate skeletal muscle, and the mechanism of modulation involves the divalent cation binding site.

This work was supported by grants from the National Institutes of Health to RLM (HL-25861) and to GMD (AR-08226), and by USDA 91-37206-6743 to MLG. We would like to thank Dr. James Graham for SDS-PAGE analysis of the fiber samples.

REFERENCES

Bremel, R. D., and A. Weber. 1972. Cooperation within the actin filament in vertebrate skeletal muscle. *Nature New Biol.* 238:97–101.

Brenner, B. 1986. The cross-bridge cycle in muscle. Mechanical, biochemical, and structural studies on skinned rabbit psoas fibers to characterize cross-bridge kinetics in muscle for correlation with the actomyosin-ATPase in solution *Basic Res. Cardiol.* 81:1–15.

Collins, J. H. 1976. Homology of myosin DTNB light chain with alkali light chains, troponin C, and parvalbumin. *Nature.* 259:699–700.

Diffee, G. M., M. L. Greaser, F. Reinach, and R. L. Moss. 1994. The effect of altered Ca^{2+} binding to myosin regulatory light chain on tension generation in skinned skeletal muscle fibers. *Biophys. J.* 66: 192*a*. (Abstr.)

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

Ford, L. E., A. F. Huxley, and R. M. Simmons. 1977. Tension responses to sudden length changes in stimulated frog muscle fibers near slack length. *J. Physiol.* 269:441–515.

Giulian, G. G., R. L. Moss, and M. L. Greaser. 1983. Improved methodology for analysis and quantitation of proteins on one-dimensional silver-stained slab gels. *Anal. Biochem.* 129:277–287.

Godt, R. E., and B. D. Lindley. 1982. Influence of temperature upon contractile activation and isometric force production in mechanically skinned muscle fibers of the frog. *J. Gen. Physiol.* 80:279–297.

Goodwin, E. B., L. A. Leinwand, and A. G. Szent-Gyorgi. 1990. Regulation of scallop myosin by mutant regulatory light chains. *J. Mol. Biol.* 216: 85–93.

Grabarek Z., J. Grabarek, P. C. Leavis, and J. Gergely. 1983. Cooperative binding to Ca^{2+}-specific sites of troponin C in regulated actin. *J. Biol. Chem.* 258:14098–14102.

Greaser, M. L., and J. Gergely. 1971. Reconstitution of troponin activity from three protein components. *J. Biol. Chem.* 246:4226–4233.

Greene, L. E., and E. Eisenberg. 1980. Cooperative binding of myosin subfragment-1 to the actin-troponin-tropomyosin complex. *Proc. Natl. Acad. Sci. USA.* 77:2616–2620.

Hofmann, P. A., H. C. Hartzell, and R. L. Moss. 1991. Alterations in Ca^{2+} sensitive tension due to partial extraction of C-protein from rat skinned cardiac myocytes and rabbit skeletal muscle fibers. *J. Gen. Physiol.* 97: 1141–1163.

Hofmann, P. A., J. M. Metzger, M. L. Greaser, and R. L. Moss. 1990. Effects of partial extraction of light chain 2 on the Ca^{2+} sensitivities of isometric tension, stiffness, and velocity of shortening in skinned skeletal muscle fibers. *J. Gen. Physiol.* 95:477–498.

Kwon, H., E. B. Goodwin, L. Nyitray, E. Berliner, E. O'Neall-Hennessey, F. D. Melandri, and A. G. Szent-Gyorgi. 1990. Isolation of the regulatory domain of scallop myosin: role of the essential light chain in calcium binding. *Proc. Natl. Acad. Sci. USA.* 87:4771–4770.

Lehman, W. H. 1978. Thick-filament linked calcium regulation in vertebrate striated muscle. *Nature.* 274:80–81.

Lowey, S., and D. Risby. 1971. Light chains from fast and slow myosins. *Nature.* 234:81–85.

Lowey, S., G. S. Waller, and K. M. Trybus. 1993. Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature.* 365:454–456.

Margossian, S. S., A. K. Bhan, and H. S. Slayter. 1983. Role of the regulatory light chains in skeletal muscle actomyosin ATPase and in mini-filament formation. *J. Biol. Chem.* 258:13359–13369.

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.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1982. Physiological effects accompanying the removal of myosin LC2 from skinned skeletal muscle fibers. *J. Biol. Chem.* 257:8588–8591.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1983. Effects of EDTA treatment upon the protein subunit composition and mechanical properties of mammalian skeletal muscle fibers. *J. Cell. Biol.* 96: 970–978.

Moss, R. L., G. G. Giulian, and M. L. Greaser. 1985. The effects of partial extraction of TnC upon the tension-pCa relationship in mammalian skeletal muscle. *J. Gen. Physiol.* 86:585–600.

Patel, J. R., G. M. Diffee, F. Reinach, and R. L. Moss. 1994. Kinetics of calcium binding to myosin light chain 2 in rabbit psoas fibers following flash photolysis of caged calcium. *Biophys. J.* 66:301*a*. (Abstr.)

Rayment, I., W. R. Rypniewski, K. Schmidt-Bäse, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelmann, G. Wesenberg, and H. M. Holden. 1993. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science.* 261:50–58

Reinach, F. C., K. Nagai, and J. Kendrick-Jones. 1986. Site-directed mutagenesis of the regulatory light-chain Ca^{2+}/Mg^{2+} binding site and its role in hybrid myosins. *Nature.* 322:80–83.

Shiner, J. S., and R. J. Solaro. 1984. The Hill coefficient for the Ca^{2+} activation of striated muscle contraction. *Biophys. J.* 46:541–543.

Simmons, R. M., and A. G. Szent-Gyorgi. 1978. Reversible loss of calcium control of tension in scallop striated muscle associated with the removal of regulatory light chain. *Nature.* 273:62–64.

Strynadka, N. C. J., and M. N. G. James. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu. Rev. Biochem.* 58: 951–998.

Sweitzer, N. K., and R. L. Moss. 1990. The effect of altered temperature on Ca^{2+}-sensitive force in permeabilized myocardium and skeletal muscle: evidence for force dependance of thin filament activation. *J. Gen. Physiol.* 96:1221–1245.

Wagner, P. D. 1982. Preparation and fractionation of myosin light chains and exchange of the essential light chain. *Methods Enzymol.* 85:72–81.

Wagner, P. D. 1984. Effect of skeletal muscle light chain 2 on the Ca^{2+}-sensitive interaction of myosin and heavy meromyosin with regulated actin. *Biochemistry.* 23:5950–5956.

Weeds, A. G., and S. Lowey. 1971. Substructure of the myosin molecule. II. The light chains of myosin. *J. Mol. Biol.* 61:701–725.

Xie, X., D. H. Harrison, I. Schlichting, R. M. Sweet, V. N. Kalabokis, A. G. Szent-Györgyi, and C. Cohen. 1994. Structure of the regulatory domain of scallop myosin at 2.8 Å resolution. *Nature.* 368: 306–312.