Acceleration of Stretch Activation in Murine Myocardium due to Phosphorylation of Myosin Regulatory Light Chain

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The regulatory light chains (RLCs) of vertebrate muscle myosins bind to the neck region of the heavy chain domain and are thought to play important structural roles in force transmission between the cross-bridge head and thick filament backbone. In vertebrate striated muscles, the RLCs are reversibly phosphorylated by a specific myosin light chain kinase (MLCK), and while phosphorylation has been shown to accelerate the kinetics of force development in skeletal muscle, the effects of RLC phosphorylation in cardiac muscle are not well understood. Here, we assessed the effects of RLC phosphorylation on force, and the kinetics of force development in myocardium was isolated in the presence of 2,3-butanedione monoxime (BDM) to dephosphorylate RLC, subsequently skinned, and then treated with MLCK to phosphorylate RLC. Since RLC phosphorylation may be an important determinant of stretch activation in myocardium, we recorded the force responses of skinned myocardium to sudden stretches of 1% of muscle length both before and after treatment with MLCK. MLCK increased RLC phosphorylation, increased the Ca^{2+} sensitivity of isometric force, reduced the steepness of the force–pCa relationship, and increased both Ca^{2+}-activated and Ca^{2+}-independent force. Sudden stretch of myocardium during an otherwise isometric contraction resulted in a concomitant increase in force that quickly decayed to a minimum and was followed by a delayed redevelopment of force, i.e., stretch activation, to levels greater than pre-stretch force. MLCK had profound effects on the stretch activation responses during maximal and submaximal activations: the amplitude and rate of force decay after stretch were significantly reduced, and the rate of delayed force recovery was accelerated and its amplitude reduced. These data show that RLC phosphorylation increases force and the rate of cross-bridge recruitment in murine myocardium, which would increase power generation in vivo and thereby enhance systolic function.

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INTRODUCTION

The myosin molecule in vertebrate striated muscle is a hexamer comprised of two heavy chains (~220 kD each) and two pairs of light chains (~20 kD each) (Sweeney et al., 1993). The heavy chains have distinctive α-helical regions in the head, neck, and tail domains, while the two globular heads at the N terminus of the molecule contain ATP and actin binding sites. The light chain subunits include both essential light chains (ELCs) and regulatory light chains (RLCs), which bind to the neck region of the heavy chain and are thought to mechanically stabilize the α-helical lever arm during force transmission (Rayment et al., 1993). Consistent with this idea, selective removal of RLC has been shown to change the structure of cardiac myosin by altering the length of its globular region (Margossian and Slater, 1987). Furthermore, partial extraction of RLC from skinned skeletal muscle fibers reversibly increased force at submaximal levels of activation (Moss et al., 1982; Hofmann et al., 1990) and abolished the Ca^{2+} dependence of the rate constant of force development (k_{tr}) after a release and restretch protocol (Patel et al., 1996).

In mammalian striated muscles, phosphorylation of RLC at residues near the N terminus by a Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) increases force development by inducing movement of the myosin head away from the thick filament backbone, which in turn increases the likelihood of its interaction with actin (Metzger et al., 1989; Levine et al., 1996; Yang et al., 1998). Previous work has shown that phosphorylation of RLC increases the Ca^{2+} sensitivity of force in skinned skeletal (Persechini et al., 1985; Metzger et al., 1989; Sweeney and Stull, 1990; Szczesna et al., 2002; Davis et al., 2002b) and cardiac muscles (Sweeney and Stull, 1986; Morano et al., 1985, 1988; Sanbe et al., 1999; Pi et al., 2003; Olsson et al., 2004) and accelerates the rate of force development (k_{tr}) in skinned skeletal muscle fibers at low levels of activation (Metzger et al., 1989; Sweeney and Stull, 1990). These results are consistent with the idea that phosphorylation increases the probability of interaction between myosin and actin and accelerates binding and/or the transition to force generating states.

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Abbreviations used in this paper: BDM, 2,3-butanedione monoxime; IEF, iso-electric focusing; MLCK, myosin light chain kinase; RLC, regulatory light chain.

The importance of RLC in cardiac function is evident from findings that mutations in RLC underlie some cases of hypertrophic cardiomyopathy (Poetter et al., 1996; Flavigny et al., 1998; Andresen et al., 2001; Kabaeva et al., 2002; Richard et al., 2003; Szczesna-Cordary et al., 2005). Furthermore, based on findings that the expression and activity of MLCK systematically vary through the thickness of the ventricular free walls, it has been proposed that the spatial pattern of RLC phosphorylation in the heart is an important determinant of cardiac function (Davis et al., 2001, 2002b). Consistent with this idea, the phosphorylation state of RLC in skeletal muscle fibers (Davis et al., 2002b) has been shown to alter both the amplitude and the rate of stretch activation responses recorded during submaximal activations with Ca^{2+}. Stretch activation is an intrinsic property of oscillatory muscles (Pringle, 1978), including myocardium (Steiger, 1977), and is thought to play an important role in cardiac contraction and relaxation (Vemuri et al., 1999; Epstein and Davis, 2003, 2006; Campbell and Chandra, 2006). Our recent study (Stelzer et al., 2006b) showed that the amplitude and kinetics of the response to stretch in myocardium vary with the level of activating Ca^{2+}, suggesting that the stretch-induced modulation of cardiac contraction varies on a beat-to-beat basis.

Based on studies using skeletal muscle fibers, it has been proposed that RLC phosphorylation modulates the work performed by the heart (Davis et al., 2001). The present study was undertaken to test this idea experimentally by examining the effects of RLC phosphorylation on the stretch activation response in murine myocardium and the sensitivity of these responses to Ca^{2+}. Our results show that RLC phosphorylation has profound effects on cardiac muscle contractility by increasing the Ca^{2+} sensitivity of force and significantly accelerating the stretch activation response. These data support the idea (Davis et al., 2001) that previously observed that regional differences in RLC phosphorylation in the heart play an important role in determining the amount and rate of force generation across the ventricular wall, thereby modulating systolic ejection.

MATERIALS AND METHODS

Solutions

Solution compositions were calculated using the computer program of Fabiato (Fabiato, 1988) and the stability constants listed by Godt and Lindley (1982) (corrected to pH 7.0 and 22°C). All solutions contained 100 mM *N,N*-bis(2 hydroxy-ethyl)-2-aminoethanesulfonic acid (BES), 15 mM creatine phosphate, 5 mM dithiothreitol, 1 mM free Mg^{2+}, and 4 mM MgATP. In addition, pCa 9.0 solution contained 7 mM EGTA and 0.02 mM $CaCl_2$, pCa 4.5 contained 7 mM EGTA and 7.01 mM $CaCl_2$, and preactivating solution contained 0.07 mM EGTA. Ionic strength of all solutions was adjusted to 180 mM with potassium propionate. Solutions containing different amounts of $[Ca^{2+}]_{free}$ (i.e., pCa 6.2 to 5.4) were prepared by mixing appropriate volumes of pCa 9.0 and pCa 4.5. Smooth muscle MLCK was prepared as previously described (Nagamoto and Yaki, 1984) and stored at −80°C until used in an experiment.

Skinned Myocardial Preparations

After i.p. injection of 5,000 U heparin/kg body weight, S129 mice of either sex (3–6 mo old) were anesthetized with inhaled isoflurane (15% isoflurane in mineral oil) in accordance with the animal care guidelines of the University of Wisconsin School of Medicine and Public Health. The hearts were excised and right and left ventricles were dissected free in Ringer's solution (120 mM NaCl, 19 mM $NaHCO_3$, 10 mM glucose, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM Na_2HPO_4, 1 mM $CaCl_2$; pH 7.4; 22°C) and pre-equilibrated with 95% O_2/5% CO_2. The tissue was pinned to the bottom of the dissecting dish and perfused with fresh Ringer's solution containing 30 mM 2,3-butanedione monoxime (BDM), a compound that acts as a chemical phosphatase (Wiggins et al., 1980) and has been shown to dephosphorylate RLC in myocardium (Turnbull et al., 2002; Olsson et al., 2004). Treatment of cardiac tissue with BDM was necessary in order to ensure that all preparations chosen for experiments will have a homogeneous RLC phosphorylation profile, as it has been previously shown that regional differences in RLC phosphorylation levels exist in murine hearts (Davis et al., 2001). After a 30-min incubation with BDM, heart tissue was frozen in liquid nitrogen and stored at −80°C until the day of the experiment.

On the day of the experiment, skinned myocardium was prepared by the thawing the frozen ventricles and homogenizing for ∼2 s in relaxing solution (100 mM KCl, 20 mM imidazole, 7 mM $MgCl_2$, 2 mM EGTA, and 4 mM MgATP; pH 7.0) using a Polytron homogenizer, which yielded multicellular preparations of 100–250 μm × 600–900 μm. The homogenate was centrifuged at 120 *g* for 1 min, and the resulting pellet was washed with fresh relaxing solution and resuspended in relaxing solution containing 250 μg saponin/ml and 1% Triton X-100. After 30 min, the skinned preparations were washed with fresh relaxing solution and were dispersed in ∼50 ml relaxing solution in a glass Petri dish. The dish was kept on ice except during the selection of individual preparations for mechanical experiments.

Apparatus and Experimental Protocol

Skinned preparations with well-defined edges were transferred from the Petri dish to a stainless steel experimental chamber (Stelzer et al., 2004) containing relaxing solution. The ends of each preparation were attached to the arms of a motor (model 312B; Aurora Scientific Inc.) and force transducer (model 403; Aurora Scientific Inc.), as described earlier (Stelzer et al., 2004). The chamber assembly was then placed on the stage of an inverted microscope (Carl Zeiss MicroImaging, Inc.) fitted with a 40× objective and a CCTV camera (model WV-BL600, Panasonic). Bitmap images of the preparations were acquired using an AGP 4X/2X graphics card and associated software (ATI Technologies Inc.) and were used to assess mean sarcomere length (SL) during the course of each experiment. Changes in force and motor position were sampled (16-bit resolution, DAP5216a, Microstar Laboratories) at rates ≥2.0 kHz using SLControl software developed in our laboratory (Campbell and Moss, 2003) and saved to computer files for later analysis. Fiber force during the experiments was also recorded on a digital oscilloscope (Nicolet Instrument Corporation).

At the start of each experiment, the preparation was stretched to a mean sarcomere length of ∼2.2 μm for measurements of steady-state Ca^{2+}-activated force, which was measured as the difference between total force and resting force at each pCa studied. The preparation was initially activated at pCa 4.5 to determine maximum Ca^{2+}-activated force (P_o). A force–pCa relationship was generated by activating cardiac preparations in solutions of

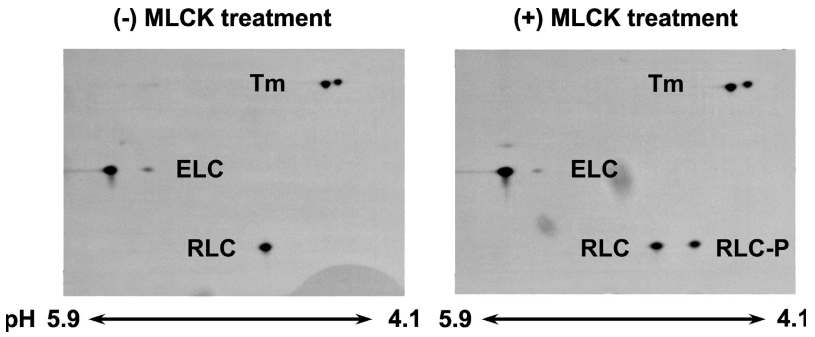

Figure 1. Myosin regulatory light chain (RLC) phosphorylation of murine skinned myocardium. Two-dimensional SDS-PAGE/IEF gels were used to determine levels of RLC phosphorylation in skinned myocardium. The gel on the left shows that BDM treatment resulted in no detectable RLC phosphorylation, while the gel on the right shows that subsequent treatment with MLCK phosphorylated a significant fraction of RLC. Densitometric scans of the two RLC spots in the gel on the right indicated that 39% of total RLC was phosphorylated. Other spots represent tropomyosin (Tm) and essential light chain (ELC).

varying pCa (6.2–4.5), and once steady tension had developed, the muscle was slackened to reduce force to zero. Normalized force–pCa relationships were generated by expressing submaximal force (P) at each pCa as a fraction of maximal force (P_o) measured in the same preparation at pCa 4.5, i.e., P/P_o. Apparent cooperativity of force development was inferred from the steepness of the force–pCa relationship, which was quantified using a Hill plot transformation of the data (Stelzer et al., 2004).

Force–pCa relationships were also generated using skinned cardiac preparations that were subjected to a protocol to phosphorylate RLC. These preparations were incubated for 10 min in preactivating solution containing 5 mM BDM (to inhibit force development); for 30 min in preactivating solution containing 5 mM BDM, 67 μM $CaCl_2$, 6 μM calmodulin, and 0.4 μM smooth muscle MLCK (to phosphorylate RLC); and then for 30 min (three solution changes) in solution of pCa 9.0 to wash out MLCK (Olsson et al., 2004). BDM acts as a phosphatase in the intact heart but not in isolated skinned myocardium (Olsson et al., 2004). Control experiments performed in the present study and in previous studies (Olsson et al., 2004) have shown that incubation of skinned cardiac fibers with BDM alone does not alter RLC phosphorylation levels or contractile function.

For stretch activation experiments, sarcomere length was initially set at ∼2.2 μm and preparations were activated at pCa 4.5 to determine maximal Ca^{2+}-activated force and pCa 9.0 to determine Ca^{2+}-independent resting force. Stretch activation responses were first assessed at three levels of activation in preparations in which RLC was not phosphorylated, i.e., in solutions yielding maximum force (pCa 4.5), ∼50% of maximum force (pCa ∼5.75), and ∼25% of maximum force (pCa ∼5.85). Once steady-state force was reached, a rapid stretch of 1% of preparation length (L_o) was imposed and held for 5 s before returning the preparation to solution of pCa 9.0. This protocol was then repeated on the same fibers after incubation with MLCK, as described above.

The stretch activation variables were measured as previously described (Stelzer et al., 2006b). All amplitudes were normalized to prestretch Ca^{2+}-activated isometric force to allow comparisons between different levels of activation. Amplitudes were measured as follows: P_1, measured from prestretch steady-state force to the peak of phase 1, P_2, measured from prestretch isometric force to the minimum force at the end of phase 2, P_3, measured from prestretch isometric force to the peak of delayed force in phase 3, and P_{df}, the difference between P_3 and P_2. Apparent rate constants were derived for phase 2 (k_{rel}, s^{-1}) and for phase 3 (k_{df}, s^{-1}) from the point of force reuptake after phase 2 to the completion of delayed force development.

At the conclusion of the mechanical experiment, the preparation was cut free at the points of attachment and placed in rehydration/sample buffer (Bio-Rad Laboratories). The samples were then stored at −80°C until subsequent analysis of RLC phosphorylation state using two-dimensional gel electrophoresis in a mini gel system (Bio-Rad Laboratories) as previously described (Olsson et al., 2004). In brief, the first dimensional iso-electric focusing (IEF) tube gels containing 8 mM urea, 4% acrylamide-bisacrylamide (30% acrylamide/bisacrylamide solution; Bio-Rad Laboratories), 2% Triton X-100, 2% ampholyte (pH 4.1–5.9; Bio-Rad Laboratories), 0.02% ammonium persulfate, and 0.2% TEMED were prefocused first at 200 V for 15 min and then at 400 V for 15 min. The samples were then loaded onto the gels and electrofocused first at 500 V for 20 min and then at 750 V for 4 h 40 min. The IEF tube gels were ejected onto a 12.5% Tris-HCL Criterion Precast gel (Bio-Rad Laboratories) and electrophoresed at 150 V for 1 h 30 min. The gels were then silver stained at room temperature using published methods (Shevchenko et al., 1996) with minor modifications, as follows: the gels were (a) incubated in fixing solution containing 50% methanol and 10% acetic acid for 20 min, (b) washed with distilled water for 20 min (4× water change), (c) incubated in 0.01% sodium thiosulfate solution for 1.5 min and then rinsed 4× with distilled water, (d) incubated in 0.09% silver nitrate solution for 20 min and then rinsed 4× with distilled water, (e) incubated in developing solution containing 0.0004% sodium thiosulfate, 2% potassium carbonate, and 0.0068% formaldehyde until proteins were visible and then rinsed 4× with distilled water, (f) incubated in destaining solution containing 10% methanol and 10% acetic acid for 20 min, rinsed 4× in distilled water, and finally rinsed (with slow rotation) overnight in distilled water. The gels were then dried between two sheets of cellophane overnight, and the percent RLC phosphorylation was quantified using UVP BioImaging System and LaserPix software (Bio-Rad Laboratories).

Data Analysis

Cross-sectional areas of skinned preparations were calculated by assuming that the preparations were cylindrical and by equating the width, measured from video images of the mounted preparations, to diameter. Each submaximal Ca^{2+}-activated force (P) was expressed as a fraction of the maximum Ca^{2+}-activated force (P_o) generated by the same preparation at pCa 4.5, i.e., P/P_o. To determine the Ca^{2+} sensitivity of isometric force (pCa_{50}), the force–pCa data were fitted with the Hill equation: $P/P_o = [Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)]$, where n is the slope (Hill coefficient) and k is the Ca^{2+} concentration required for half-maximal activation.

Analysis of stretch activation data was performed as previously described (Stelzer et al., 2006b). In brief, rate constants of force decay (k_{rel}) were obtained by fitting a single exponential to the time course of decay, i.e., $y = a\,(1 - \exp(-k_1 \cdot x))$, where "$a$" is the amplitude of the single exponential phase and k_1 is the rate constant of decay. Rate constants of delayed force development in phase 3 were estimated either with a double exponential fit, $y = a \cdot \exp(-k_1 \cdot x) + b \cdot \exp(-k_2 \cdot x)$, where "$a$" is the amplitude of the first exponential phase rising with rate constant k_1, and "b" is the amplitude of the second exponential phase rising with rate constant k_2, or were estimated as a single composite rate constant

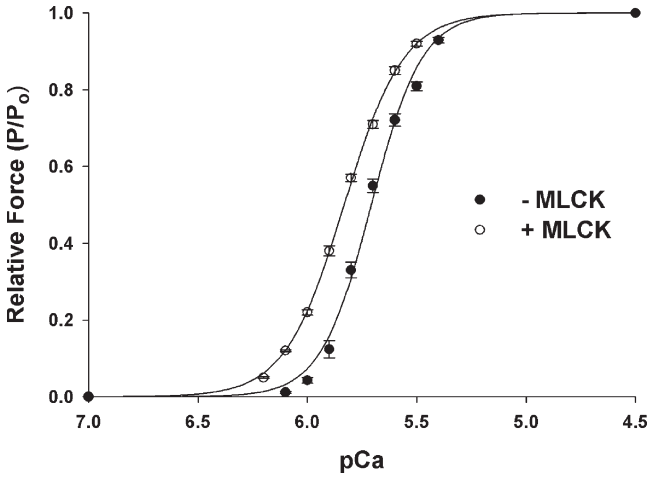

Figure 2. Effect of RLC phosphorylation on force-pCa relationships. RLC phosphorylation increased the Ca^{2+} sensitivity force and decreased cooperative activation of force development: fitted values for n_H and pCa_{50} were 3.79 ± 0.23 and pCa 5.71 ± 0.01 for untreated myocardium (−MLCK ●, $n = 10$) and 3.22 ± 0.26 and pCa 5.83 ± 0.01 for MLCK-treated myocardium (+MLCK ○, $n = 10$). Data points are means ± SEM. Forces measured at submaximal free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) were expressed relative to the maximal force obtained at pCa 4.5. The smooth lines were fit using the Hill equation: $P/P_o = [Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)]$, where P is the force measured at submaximal $[Ca^{2+}]_{free}$, P_o is the force measured at maximal $[Ca^{2+}]_{free}$ (pCa 4.5), n_H is the Hill coefficient, and k is the Ca^{2+} concentration required for half-maximal activation.

by linear transformation of the half-time of force redevelopment, i.e., ($k_{df} = -\ln 0.5 \times [t_{1/2}]^{-1}$) (Stelzer et al., 2006b).

All data are reported as means ± SEM. Statistical analysis was done using a repeated measures analysis of variance (ANOVA) and a Tukey post-hoc test where appropriate, and P values <0.05 were taken as indicating significant differences.

RESULTS

Effect of RLC Phosphorylation on Ca^{2+} Sensitivity of Force

The preparations used in this study were isolated from murine hearts that were first incubated in the presence of BDM in order to dephosphorylate RLC and thereby establish a uniformly low baseline level of RLC phosphorylation before mechanical measurements. Fig. 1 A is a two-dimensional (PAGE vs. IEF) gel of a skinned myocardial preparation treated in this way, demonstrating that this protocol was effective in reducing RLC phosphorylation in skinned cardiac preparations to near zero, i.e., no RLC phosphorylation was detected with our BioImaging system. Subsequent treatment of these preparations with MLCK, as described in Materials and methods, resulted in phosphorylation of RLC to an average of 38 ± 3% ($n = 7$) of total light chain, as shown in the two-dimensional gel in Fig. 1 B. This level of phosphorylation is close to values that have been reported for living myocardium (Morano, 1999; van der Velden et al., 2003) and also to values reported previously in skinned myocardium treated with MLCK (Sanbe et al., 1999; Olsson et al., 2004).

TABLE I

Effect of MLCK Treatment on Steady-state Contractile Properties of Murine Myocardium

Group	F_{max}	F_{min}	pCa_{50}	n_H
	mN/mm²	*mN/mm²*		
−MLCK	15.5 ± 0.8	0.7 ± 0.2	5.71 ± 0.01	3.79 ± 0.23
+MLCK	21.7 ± 1.3[a]	1.4 ± 0.4[a]	5.83 ± 0.01[a]	3.22 ± 0.26[a]

Data are means ± SEM from 10 preparations. F_{max}, maximum Ca^{2+}-activated force at pCa 4.5; F_{min}, Ca^{2+}-independent force at pCa 9.0; pCa_{50}, pCa required for half-maximal force; n_H, Hill coefficient for force–pCa relationship.

[a]Significantly different from data obtained −MLCK, $P < 0.05$.

Treatment of the skinned preparations with MLCK increased maximal Ca^{2+}-activated (pCa 4.5) force by ∼40% and increased Ca^{2+}-independent (pCa 9.0) resting force by ∼100% (Table I). Force–pCa relationships measured in skinned myocardium both before and after MLCK treatment were sigmoidal and were fit well with the Hill equation (Fig. 2). Phosphorylation of RLC increased the Ca^{2+} sensitivity of force, i.e., pCa_{50} increased from 5.71 ± 0.01 to 5.83 ± 0.01 ($P < 0.01$, Table I) after MLCK treatment, thus reducing the Ca^{2+} concentration required to evoke a given submaximal force. The steepness of the force–pCa relationship, which is an indicator of the apparent cooperativity of force development, was markedly reduced after MLCK treatment, in that the Hill coefficient (n_H) decreased from 3.79 ± 0.23 before treatment to 3.22 ± 0.26 after treatment ($P < 0.01$, Table I).

Effects of RLC Phosphorylation on the Stretch Activation Response of Murine Skinned Myocardium

The records in Fig. 3 exemplify the stretch activation response to a 1% increase in preparation length at an activation level of ∼50% of maximal and in the absence of RLC phosphorylation. The multiphasic response to stretch of an otherwise isometrically contracting preparation starts with an increase in force (phase 1) coincident with the stretch, which is followed by a rapid decay in force (phase 2), which reaches a minimum (P_2) before force recovers (phase 3, delayed force rise) to a level (P_3) greater than the prestretch isometric force and then eventually decays back to the prestretch level.

RLC phosphorylation resulted in significant changes to the stretch activation response of skinned myocardium, as shown in the records of Fig. 4 in which a stretch of 1% of initial muscle length was imposed during an activation that was ∼50% maximal (pCa 5.75). RLC phosphorylation evoked a large increase in prestretch isometric force and altered the stretch activation response (Fig. 4). To allow direct comparisons of the effects of RLC phosphorylation on stretch activation

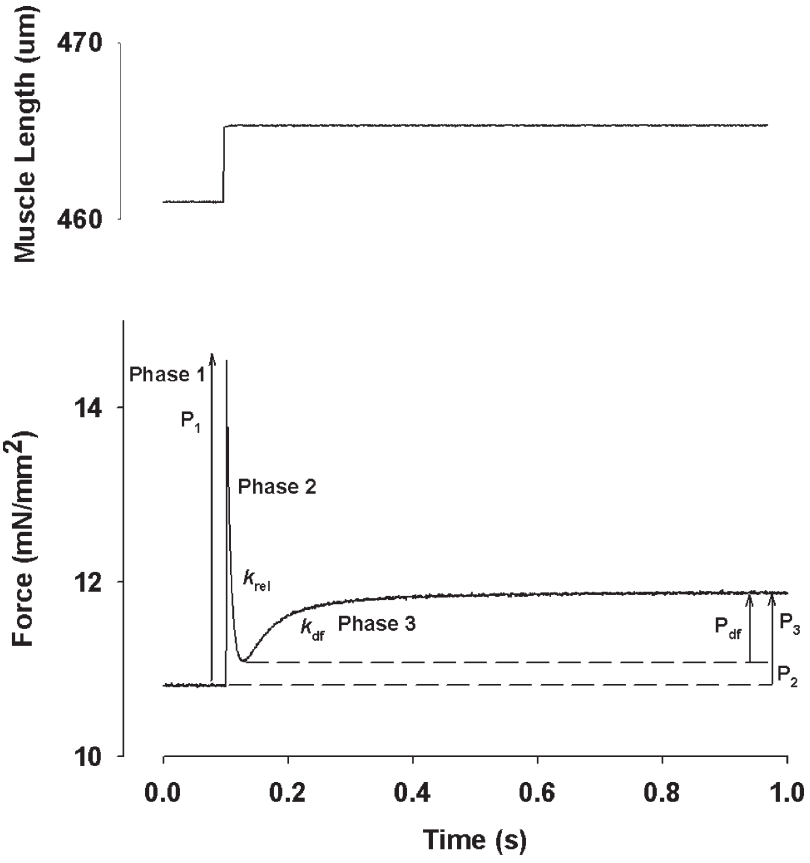

Figure 3. Stretch activation response in murine myocardium. The force transient shown (bottom panel) is typical of the stretch activation responses of WT myocardium after a stretch of 1% of muscle length (top panel). Once a steady-state isometric force of ~50% of maximal was achieved in the presence of Ca^{2+}, the muscle was stretched and then held at the longer length, as described in Materials and Methods. The recorded variables are labeled on the force record and described in the text.

variables, traces obtained at different levels of Ca^{2+} activation were normalized to prestretch isometric force (Fig. 5), which is expressed as a "zero" baseline. As reported previously (Stelzer et al., 2006b), increases in Ca^{2+} activation accelerated the overall rate of the stretch activation response and reduced its amplitude (normalized to prestretch isometric force) (Stelzer et al., 2006b). After RLC phosphorylation, the amplitude of the stretch activation response (phase 3) at each level of activation was reduced and delayed force reached a maximum more quickly, with the most prominent effects occurring at submaximal levels of activation (Fig. 5). Superimposing normalized stretch activation traces obtained from the same fiber before (−MLCK) and following (+MLCK) RLC phosphorylation (Fig. 6 A) clearly shows these differences. We also examined the effect of RLC phosphorylation on the stretch activation response as a function of relative maximal force, i.e., P/P_o. The trace shown (Fig. 6 B) for the RLC phosphorylated preparation was obtained using a decreased $[Ca^{2+}]$ compared with the RLC nonphosphorylated preparation; however, it can be seen that the stretch activation response was still accelerated after RLC phosphorylation as when plotted at the same $[Ca^{2+}]$ (Fig. 6 A). The amplitude (P_1) of phase 1 increased as a function of increasing prestretch isometric force and did not differ significantly with RLC phosphorylation (unpublished data), at least within the frequency response of our force transducer.

In both the presence and absence of RLC phosphorylation, reducing the level of prestretch activation by reducing the $[Ca^{2+}]$ slightly accelerated the rapid force decay in phase 2 (k_{rel}). Phase 2 is thought to represent detachment of cross-bridges that are strained by stretch and their rapid replacement by unstrained cross-bridges (Davis and Rodgers, 1995; Piazzesi et al., 1997) and was substantially slower at all levels of activation after RLC phosphorylation (Fig. 7). This result is consistent with earlier reports (Patel et al., 1998; Davis et al., 2002b) that RLC phosphorylation slowed the rate of relaxation in skinned skeletal fibers. Furthermore, RLC phosphorylation significantly reduced the amount of force decay during phase 2 (Figs. 6 and 7), such that delayed force recovery (phase 3) began from a higher force (P_2) and the overall amplitude of the phase 3 delayed force transient (i.e., trough to peak excursion, P_{df}) was significantly reduced (Fig. 8).

The delayed recruitment of cross-bridges due to stretch is highly dependent on the level of prestretch force (Linari et al., 2004; Stelzer et al., 2006b) and determines the amplitude (P_{df}) of phase 3. Both before and after phosphorylation of RLC, P_{df} was inversely proportional to activation level (Stelzer et al., 2006b), such that relatively fewer cross-bridges were recruited by

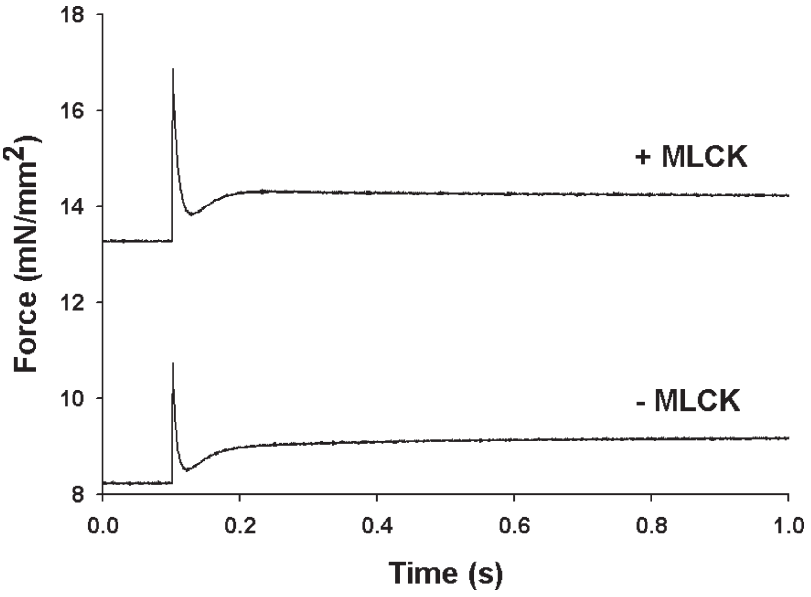

Figure 4. Effect of RLC phosphorylation on the force transients produced by stretch. Force responses of skinned myocardium to a stretch of 1% of muscle length applied during isometric contractions (~50% of maximal, pCa 5.75) were recorded in an untreated preparation (−MLCK, bottom trace) and in the same preparation after phosphorylation of RLC (+MLCK, top trace). RLC phosphorylation increased the Ca^{2+}-activated isometric force (from 8.3 mN/mm^2 to 13.4 mN/mm^2) and accelerated the overall rate of the stretch activation response.

stretch as the level of activation was increased. RLC phosphorylation decreased the amplitude of P_{df} at each level of activation studied (Fig. 8), primarily as a consequence of the effects of phosphorylation to increase P_2, the baseline from which P_{df} was measured.

Delayed force development (phase 3) after stretch is generally thought to involve the recruitment of cross-bridges into strongly bound states (Lombardi and Piazzesi., 1990; Piazzesi et al., 1997; Campbell et al., 2004), and, as previously reported (Linari et al., 2004; Stelzer et al., 2006b), does not occur as a simple exponential process and is therefore not well fit with a single exponential equation, especially at low levels of activation. To facilitate comparisons of phase 3 rates of force development at different levels of activation, a composite apparent rate constant (k_{df}) of delayed force development was calculated from the half-time of force development. Consistent with earlier reports (Stelzer et al., 2006a,b), k_{df} in murine myocardium was accelerated by increasing the level of activation (Table II; Fig. 8), which was also the case after RLC phosphorylation. However, RLC phosphorylation accelerated k_{df} at all levels of activation, with the greatest effects observed at low levels of activation (Fig. 8; Table II).

The kinetics of delayed force development were also analyzed with a double exponential fit, which yielded fast and slow rate constants (k_1 and k_2) and their corresponding amplitudes (a and b), as shown in Table II. Prior to MLCK treatment, decreasing the level of activation progressively increased the amplitude (b) of the slower rate process (thought to manifest cooperative recruitment of cross-bridges) and decreased its apparent rate (k_2) (Stelzer et al., 2006a,b). After MLCK treatment, delayed force development occurred as a single exponential process at all levels of activation (Table II), such that the slower rate process (k_2) was absent. RLC phosphorylation accelerated force development at each level of activation (Fig. 8; Table II), but again, these effects were greatest at the lowest levels of activation studied (Table II). Since the slower phase of delayed force development appears to manifest cooperative recruitment of cross-bridges to force generating states, which is most prominent at low levels of activation (Moss, et al., 2004), RLC phosphorylation presumably either accelerates or eliminates this recruitment process.

DISCUSSION

Effect of RLC Phosphorylation on Isometric Force and the Ca^{2+} Sensitivity of Force

A consistent observation in previous studies is that phosphorylation of RLC in both skeletal (Persechini et al., 1985; Metzger et al., 1989; Sweeney and Stull, 1990; Szczesna et al., 2002; Davis et al., 2002b) and cardiac (Sweeney and Stull, 1986; Morano et al., 1985, 1988; Sanbe et al., 1999; Pi et al., 2003; Olsson et al., 2004) muscles causes an increase in the Ca^{2+} sensitivity of force, which was also observed here (Fig. 2). Such effects are presumably the result of increased probability of cross-bridge binding to actin as a result of phosphorylation (Metzger et al., 1989; Sweeney and Stull, 1990), most likely due to increased radial dispersion of the myosin heads (Levine et al., 1998; Morano, 1999). RLC phosphorylation increased both resting force at pCa 9.0 and maximal force at pCa 4.5, while decreasing the overall steepness of the force–pCa relationship, suggesting an increase in cross-bridge binding both in the absence and presence of Ca^{2+}. Phosphorylation-dependent increases in maximal force have been observed by

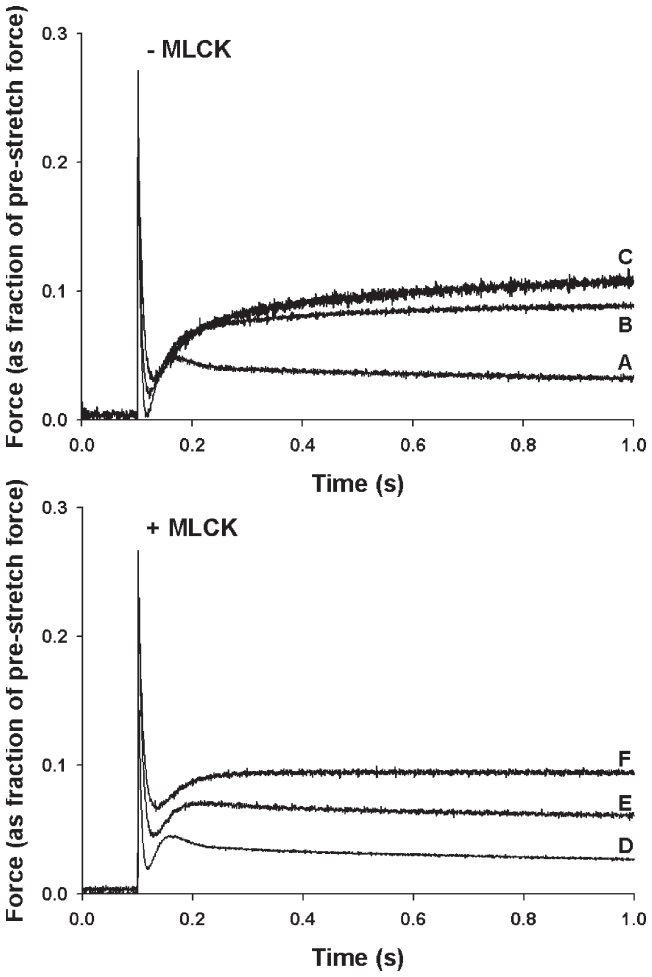

Figure 5. Effect of RLC phosphorylation on stretch activation responses of murine skinned myocardium at various levels of Ca^{2+} activation. The force responses of murine myocardium to a sudden stretch of 1% of muscle length were recorded before MLCK treatment (−MLCK, top) and after MLCK treatment (+MLCK, bottom). In each case, the force responses were normalized to the prestretch isometric force (corresponding to the zero baseline) recorded at the same level of Ca^{2+} activation, i.e., pCa 4.5 in A and D, pCa 5.75 in B and E, and pCa 5.85 in C and F.

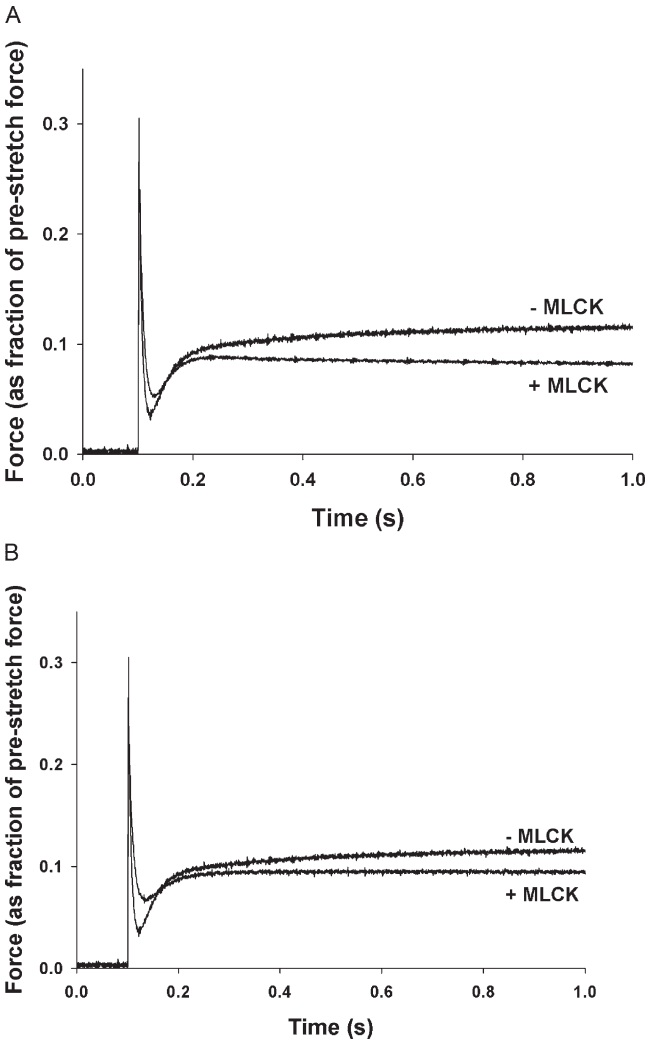

Figure 6. Effect of RLC phosphorylation on the stretch activation response of murine skinned myocardium. (A) Force responses to a stretch of 1% of muscle length were recorded at pCa 5.75 before and after treatment of myocardium with MLCK. All transients are normalized to prestretch force, which corresponds to the zero baseline. Apparent rate constants of force decay (k_{rel}) and delayed force development (k_{df}) were 224 s^{-1} and 17.2 s^{-1} before MLCK treatment and 178 s^{-1} and 32.7 s^{-1} after MLCK treatment, respectively. (B) Force responses to a stretch of 1% of muscle length were recorded at equivalent activation levels (i.e., P/P_o ~50% of maximal) before and after treatment of myocardium with MLCK. Nonphosphorylated trace is the same as in A, and apparent rate constants of force decay (k_{rel}) and delayed force development (k_{df}) were 182 s^{-1} and 23.2 s^{-1} after MLCK treatment, respectively.

some investigators (Godt and Nosek, 1989; Szczesna et al., 2002; Olsson et al., 2004) but not others (Sweeney and Stull, 1986; Metzger et al., 1989; Sweeney et al., 1993). The reasons for these discrepancies are not known but may involve differences in the basal levels of RLC phosphorylation in the various experimental preparations used.

The effects of RLC phosphorylation to increase force at all levels of activation can be explained by increased proximity of the myosin heads to actin, as discussed above, which would increase the probability of binding and thereby increase force. The effects of phosphorylation were most pronounced at low levels of activation, where there are relatively few strongly bound cross-bridges and the activation of force exhibits greatest cooperativity (for review see Moss et al., 2004). In these cases, the greater proximity of myosin to the thin filaments would facilitate cooperative binding of cross-bridges to actin, which would be manifested as the observed increases in force at low [Ca^{2+}] and the decrease in the steepness of the force–pCa relationship.

The observation that RLC phosphorylation increased resting force in skinned myocardium can also be

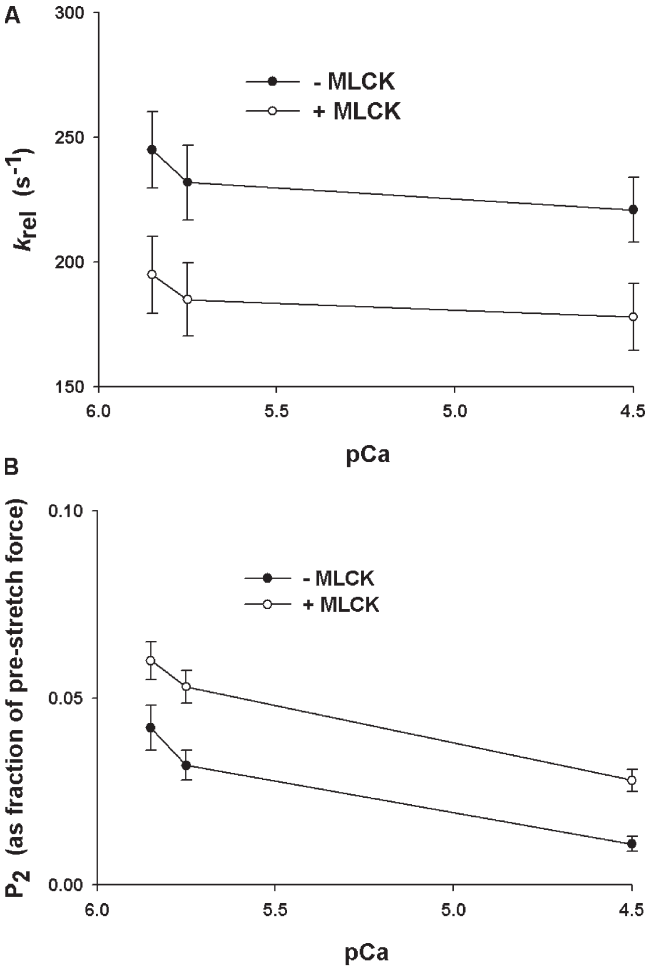

Figure 7. Effect of RLC phosphorylation on k_{rel} and P_2 at different levels of Ca^{2+} activation. (A) k_{rel} values and (B) P_2 values normalized to prestretch isometric force are plotted as a function of pCa in skinned myocardium before treatment (filled circles, $n = 10$) and after treatment (open circles, $n = 10$) with MLCK. The data shown were obtained from force responses to stretches of 1% of muscle length. Data are means ± SEM.

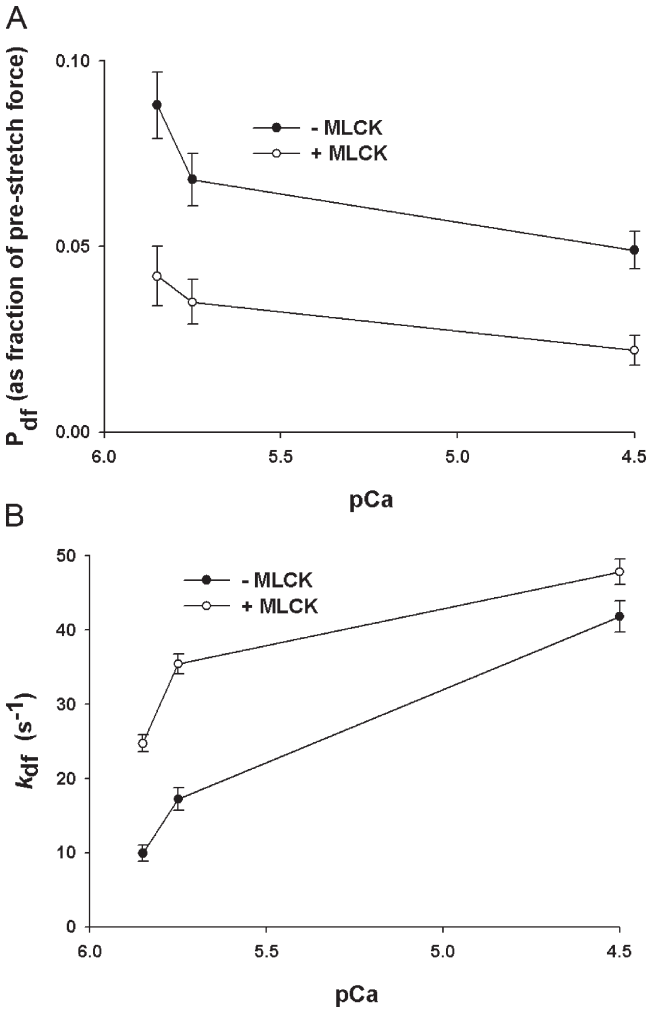

Figure 8. Effect of RLC phosphorylation on P_{df} and k_{df} at different levels of Ca^{2+} activation. (A) P_{df} values normalized to prestretch isometric force and (B) k_{df} values are plotted as a function of pCa in skinned myocardium both before treatment (filled circles, $n = 10$) and after treatment with MLCK (open circles, $n = 10$). The data shown were obtained from force responses to stretches of 1% of muscle length. Data are means ± SEM.

explained on the basis of an increased probability of cross-bridge binding. The increase in resting force in cardiac (but not in skeletal) muscle presumably manifests the significantly greater thin filament–activating effects of strong binding cross-bridges in cardiac muscle (Fitzsimons et al., 2001b).

Effects of RLC Phosphorylation on Stretch Activation of Murine Myocardium

Stretch activation is a feature of all oscillatory muscles (Pringle, 1978; Steiger, 1977), and in mammals it appears that this property of myocardium generates additional power to help propel systolic ejection (Poetter et al., 1996; Vemuri et al., 1999; Davis et al., 2001). Here we show that increased phosphorylation of RLC significantly accelerates the overall rate of the stretch activation response in murine ventricular myocardium.

When contracting muscle is suddenly stretched to a new isometric length, the resulting force transient consists of three distinct phases: an initial increase force (phase 1) coincident with the stretch that is presumably due to increased strain of attached cross-bridges (Huxley and Simmons, 1971), a subsequent rapid decay in force (phase 2) as highly strained cross-bridges are forcibly detached, and a delayed development of force (phase 3) to levels greater than the prestretch force due to attachment of new cross-bridges (Davis and Rodgers, 1995; Piazzesi et al., 1997). Ultimately, force declines to a level appropriate to the new muscle length. Similar to previous findings in slow-twitch skeletal muscle fibers (Davis et al., 2002a, b), phosphorylation of RLC decreased the extent, i.e., greater P_2 (Figs. 5–7), and slowed the rate of force decay (k_{rel}) (Fig. 7) in phase 2

TABLE II

Effect of MLCK Treatment on the Ca^{2+} Activation Dependence of the Phase 3 Delayed Force Increase in Murine Myocardium

Group	pCa	k_{df}	a	k_1	b	k_2
		s^{-1}		s^{-1}		s^{-1}
−MLCK	4.5	41.8 ± 2.1	1.00	41.0 ± 2.4	–	–
+MLCK		47.8 ± 1.7[a]	1.00	48.9 ± 2.5[a]	–	–
−MLCK	5.75	17.2 ± 1.5	0.62 ± 0.03	28.5 ± 1.8	0.38 ± 0.03	4.0 ± 0.7
+MLCK		35.4 ± 1.3[a]	1.00[a]	36.1 ± 1.7[a]	–	–
−MLCK	5.85	9.9 ± 1.1	0.48 ± 0.03	20.5 ± 1.6	0.52 ± 0.03	2.1 ± 0.4
+MLCK		24.7 ± 1.1[a]	1.00[a]	25.3 ± 1.4[a]	–	–

Data are means ± SEM, from 10 preparations subjected to a sudden stretch of 1% of muscle length. Apparent rate constants for the phase 3 delayed force increase were obtained either from conversion of half times of force recovery (k_{df}) or by fitting a double exponential to each record, $y = a \cdot \exp(-k_1 \cdot x) + b \cdot \exp(-k_2 \cdot x)$, where "*a*" and "*b*" are the amplitudes of the fast and slow phases, respectively.

[a]Significantly different from value obtained −MLCK, $P < 0.05$.

of the force transients in skinned myocardium. It is likely that an increase in probability of cross-bridge attachment to actin accounts for the slowing of k_{rel} and the increase in P_2 after RLC phosphorylation. Such a mechanism would result in faster cross-bridge attachment even during the phase of force decay, such that the rate of decay would be slowed. In this case, changes in the rates of force decay (k_{rel}) and force recovery (k_{df}) may govern the amplitude of P_2, which is the transition point at which the cross-bridge recruitment phase begins to dominate the cross-bridge detachment phase (Campbell et al., 2004). Because phosphorylation of RLC decreases the rate of force decay and simultaneously accelerates force recovery, the difference in rates of the two phases decreases, which increases the value of P_2.

A critical feature of the stretch activation response is delayed force development, which has been associated with the generation of power during contraction (Kawai et al., 1993). In this regard, mutations in the regulatory domain of myosin, which binds the essential and regulatory light chains, alter the stretch activation responses and power output of insect muscles (Tohtong et al., 1995) and mouse myocardium (Vemuri et al., 1999). In the context of the present study, the effects of RLC phosphorylation on contraction could be manifested as changes in the amount of force produced at a given $[Ca^{2+}]$, in the numbers of additional cross-bridges recruited by stretch (P_3), a change in the rate of delayed force development (k_{df}), or both.

The amplitude (P_3) of the delayed force transient normalized to prestretch force is inversely related to level of activation, i.e., it is smaller at higher levels of activation (Linari et al., 2004; Stelzer et al., 2006b), presumably because there are fewer unbound cross-bridges available for recruitment to force-generating states. In this study, P_3 was slightly reduced by RLC phosphorylation (Fig. 6), suggesting that fewer cross-bridges were recruited as a result of stretch. This result can be explained by the fact that phosphorylation increases the Ca^{2+} sensitivity of force, i.e., increases the number of cross-bridges bound at a given level of Ca^{2+} activation, so that at each level of activation fewer cross-bridges are available for recruitment due to stretch.

While the decrease in amplitude of delayed force with RLC phosphorylation would by itself depress contractile function, the dramatic acceleration of delayed force development observed at all levels of activation would be expected to enhance contractile function. The increased proximity of myosin heads with actin after RLC phosphorylation (Sweeney et al., 1994; Levine et al., 1998) would increase the probability of cross-bridge interaction with actin and thereby accelerate the rate of force development. We have previously suggested (Stelzer et al., 2006b) that the activation dependence of the rate constant of delayed force development (k_{df}) in murine myocardium is due in part to a decrease in the cooperative recruitment of cross-bridges at high levels of activation because fewer cross-bridges are available for recruitment in response to stretch. This would have the effect of accelerating k_{df} at these levels of activation (Moss et al., 2004). Conversely, at low levels of activation, a relatively small fraction of available cross-bridges is strongly bound to actin, leaving a larger number of myosin heads for recruitment upon stretch, which would slow the overall rate of force development (Campbell, 1997). Thus, the greater acceleration of delayed force development with RLC phosphorylation at submaximal $[Ca^{2+}]$ is presumably due to either an acceleration of cooperative recruitment of cross-bridges by stretch or a reduction in the amplitude of this process. Either mechanism is consistent with the apparent elimination of the slower phase of delayed force development due to RLC phosphorylation (Table II).

The effect of RLC phosphorylation on k_{df} was most prominent at low levels of activation where large accelerations in the rates of delayed force development were observed, suggesting that cooperative activation or transitions of cross-bridges to strongly bound force-generating states or both were accelerated. As Ca^{2+} activation

was increased, the effects of RLC phosphorylation to accelerate force development became less pronounced but were still significant even at maximal levels of Ca^{2+} activation where relatively little cooperative recruitment of cross-bridges would be expected (Fig. 8). However, even at maximal activation, it seems likely that some unbound cross-bridges are still available for recruitment, since incubating skinned skeletal or cardiac muscles with a nonforce-generating, derivative of myosin S1 (NEM-S1) can produce rates of force development that are faster than those recorded at saturating Ca^{2+} in the absence of NEM-S1 (Fitzsimons et al., 2001a,b). Increased levels of Mg ATPase activity at maximal Ca^{2+} activations have also been observed with MLCK-mediated RLC phosphorylation of murine skinned myocardium (Sanbe et al., 1999). Thus, the present results indicate that the kinetics of cross-bridge cycling measured during maximal Ca^{2+} activations are not truly maximal since the overall rate of the stretch activation response can be accelerated by RLC phosphorylation. As discussed earlier, the increase in maximal force due to RLC phosphorylation indicates that additional cross-bridges are bound, which could accelerate or eliminate cooperative recruitment of cross-bridges to force-generating states.

Phosphorylation of RLC appears to affect the stretch activation response differently across muscle types and species. For example, mutating the phosphorylation sites in RLC of *Drosophila* indirect flight muscle from serines to nonphosphorylatable alanines decreased oscillatory power (Tohtong et al., 1995; Dickinson et al., 1997) by decreasing the amplitude of stretch activation but without altering cross-bridge kinetic rates. In contrast, the amplitude (P_3) of stretch activation in mammalian slow skeletal fibers (Davis et al., 2002b) and murine myocardium (present study) is decreased by RLC phosphorylation, while the rate of force development (k_{df}) is slightly increased in slow skeletal muscle and dramatically increased in myocardium. Despite these differences in the effects of RLC phosphorylation on stretch activation amplitude and kinetics, an interpretation common to the above studies is that RLC phosphorylation slows cross-bridge detachment and/or facilitates cross-bridge attachment so that more cross-bridges contribute to force development.

With regard to possible direct effects of RLC phosphorylation on steps in the cross-bridge cycle, Davis et al. (2002b) have suggested that actin-induced phosphate release from the weakly bound state is accelerated, thereby increasing the number of cross-bridges entering the contractile cycle. This idea is based on their observation that RLC phosphorylation reverses the accumulation of cross-bridges in weakly bound states due to treatment of skeletal muscle fibers with 2,3-butanedione monoxime (BDM). Thus, RLC phosphorylation speeds the transitions between weakly bound or low force states to strongly bound high force states (accelerates k_{df}), and slows the transition from the high force to the weakly bound states (decelerates k_{rel}), thereby favoring the strongly bound state and force generation. The decreased amplitudes of force decay (i.e., increased P_2) and of the peak-to-trough excursion of phase 3 (P_{df}) observed in the present study are consistent with this idea.

Possible Roles of RLC Phosphorylation in Cardiac Function

Previous studies show that a gradient of RLC phosphorylation exists in the murine heart (Davis et al., 2001; Davis et al., 2002a), such that the epicardium is hyperphosphorylated and the endocardium is hypophosphorylated. The greater torsional strain of the endocardium as compared with the epicardium during systole (Bogaert and Rademakers, 2001) suggests that regional differences in RLC phosphorylation control the timing of force production across the ventricular wall. RLC phosphorylation enhances cardiac contraction by mediating a substantial increase in the ability of myocardium to produce force at a given level of Ca^{2+}, thereby increasing the amount of work produced during each beat. Based on our results and as predicted by Davis et al. (2001), epicardial myocardium with higher levels of RLC phosphorylation would produce more force and exhibit accelerated stretch activation with diminished amplitude compared with endocardial myocardium. Therefore, while endocardial fibers are arranged in a right-handed helical formation and epicardial fibers in an opposing left-handed helical formation (Streeter et al., 1969), ventricular torsional twist during systole proceeds in the direction of the epicardial fibers (Epstein and Davis, 2006) such that the endocardium is forcibly strained by epicardial fibers to produce delayed stretch activation of the endocardium. Indeed, recent evidence shows that despite being activated first, the endocardium is actively stretched by the epicardium later in systole (Nickerson et al., 2005; Buckberg et al., 2006). Thus, the phosphorylation state of RLC is a determinant of the stretch activation response of myocardium and thereby appears to contribute to work production during ventricular systole. Furthermore, the findings that RLC phosphorylation levels are decreased in end-stage human heart failure (van der Velden et al., 2003) suggest that the timing of force generation and work production is disrupted and contributes to depressed systolic function in this disease.

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