

Activation Dependence of Stretch Activation in Mouse Skinned Myocardium: Implications for Ventricular Function

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Recent evidence suggests that ventricular ejection is partly powered by a delayed development of force, i.e., stretch activation, in regions of the ventricular wall due to stretch resulting from torsional twist of the ventricle around the apex-to-base axis. Given the potential importance of stretch activation in cardiac function, we characterized the stretch activation response and its Ca^{2+} dependence in murine skinned myocardium at 22°C in solutions of varying Ca^{2+} concentrations. Stretch activation was induced by suddenly imposing a stretch of 0.5–2.5% of initial length to the isometrically contracting muscle and then holding the muscle at the new length. The force response to stretch was multiphasic: force initially increased in proportion to the amount of stretch, reached a peak, and then declined to a minimum before redeveloping to a new steady level. This last phase of the response is the delayed force characteristic of myocardial stretch activation and is presumably due to increased attachment of cross-bridges as a consequence of stretch. The amplitude and rate of stretch activation varied with Ca^{2+} concentration and more specifically with the level of isometric force prior to the stretch. Since myocardial force is regulated both by Ca^{2+} binding to troponin-C and cross-bridge binding to thin filaments, we explored the role of cross-bridge binding in the stretch activation response using NEM-S1, a strong-binding, non-force-generating derivative of myosin subfragment 1. NEM-S1 treatment at submaximal Ca^{2+} -activated isometric forces significantly accelerated the rate of the stretch activation response and reduced its amplitude. These data show that the rate and amplitude of myocardial stretch activation vary with the level of activation and that stretch activation involves cooperative binding of cross-bridges to the thin filament. Such a mechanism would contribute to increased systolic ejection in response to increased delivery of activator Ca^{2+} during excitation–contraction coupling.

INTRODUCTION

Force generation in myocardium is a critical determinant of the rate and extent of systolic ejection during the cardiac cycle and is thus closely regulated on a beat-to-beat basis by factors and mechanisms that are both extrinsic (hormones and autonomic nerves) and intrinsic (Frank-Starling mechanism) to the myocardial cell. Work by Davis et al. (2001) suggests that force generation actually varies through the thickness of the ventricular wall, such that the epicardium develops more force than the endocardium, perhaps due to variations in myofibrillar protein phosphorylation. Such a differential in strength of contraction predicts that the epicardium would stretch the endocardium during the ventricular torsional twist that takes place during systole. The resulting stretch activation (Davis et al., 2001) of the endocardium could contribute significantly to the power required to drive systolic ejection. Because of the potential physiological importance of stretch activation, the present study characterized the activation dependence of stretch activation in myocardium and investigated the mechanism(s) underlying this phenomenon.

Stretching an active muscle to a new isometric length results in a multiphase force response, beginning with

an increase in force that is coincident with the stretch and is presumably an elastic response mediated by attached cross-bridges. Force then rapidly decays to near prestretch levels due to detachment of strained cross-bridges (Davis and Rodgers, 1995; Piazzesi et al., 1997), followed by redevelopment of force due to cross-bridge attachment and transitions to force-generating states (Lombardi et al., 1995; Dobbie, et al., 1998) and establishment of a new steady state. The delayed development of force, or stretch activation, is evident in all oscillatory muscles (Pringle, 1978), including myocardium (Steiger, 1977), and has been the subject of recent investigations directed to elucidation of the mechanisms of force generation.

It has been suggested that the delayed force response of muscle to stretch is mediated by recruitment of weakly bound nonforce generating cross-bridges into strong-binding force-generating states (Getz et al., 1998; Linari et al., 2004a). It is also possible that cross-bridges are cooperatively recruited into force-generating states after stretch, similar to the mechanism that was proposed earlier to account for the activation dependence

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Abbreviations used in this paper: RLC, regulatory light chain; Tm, tropomyosin; TnC, troponin C.

of the rate constant of force redevelopment (k_{tr}) after release and restretch of skinned skeletal (Fitzsimons et al., 2001a) and cardiac muscles (Fitzsimons et al., 2001b; Stelzer et al., 2004). The results of the present study show that stretch activation in myocardium varies with the level of isometric force (or number of strongly bound cross-bridges) before stretch and involves cooperative recruitment of cross-bridges into strongly bound force-generating states.

MATERIALS AND METHODS

Solutions

Solution compositions were calculated using the computer program of Fabiato (1988) and the stability constants listed by Godt and Lindley (Godt and Lindley, 1982) (corrected to pH 7.0 and 22°C). All solutions contained (in mM) 100 *N,N*-bis(2-hydroxy-ethyl)-2-aminoethanesulfonic acid (BES), 15 creatine phosphate, 5 dithiothreitol, 1 free Mg^{2+} , and 4 MgATP. pCa 9.0 solution contained 7 EGTA, and 0.02 $CaCl_2$, pCa 4.5 contained 7 EGTA and 7.01 $CaCl_2$, and preactivating solution contained 0.07 EGTA. Ionic strength of all solutions was adjusted to 180 mM with potassium propionate. Solutions containing different amounts of $[Ca^{2+}]_{free}$ were prepared by mixing volumes of pCa 9.0 and pCa 4.5.

Skinned Myocardial Preparations

Skinned ventricular myocardium was prepared as previously described (Patel et al., 2001). After i.p. injection of 5,000 U heparin/kg body weight, S129 wild-type mice (3–6 mo old) were anesthetized with inhaled isoflurane (15% isoflurane in mineral oil) in accordance with institutional animal care guidelines. The hearts were excised and right and left ventricles were dissected in room temperature relaxing solution (in mM: 100 KCl, 20 imidazole, 7 $MgCl_2$, 2 EGTA, and 4 MgATP; pH 7.0) and then rapidly frozen in liquid nitrogen. To prepare skinned myocardium, the frozen ventricles were thawed and homogenized for ~2 s in relaxing solution using a Polytron, which yielded multicellular preparations of 100–250 $\mu m \times 600$ –900 μm . The homogenate was centrifuged at 120 *g* for 1 min, and the pellet was washed with fresh relaxing solution and resuspended in relaxing solution containing 250 $\mu g/ml$ saponin and 1% Triton X-100. After 30 min, the skinned preparations were washed with fresh relaxing solution and then dispersed in relaxing solution in a glass dish. The dish was kept on ice except during the selection of preparations for mechanical experiments.

Apparatus and Experimental Protocol

Skinned preparations with well-defined, unbranched edges were transferred from the Petri dish to a stainless steel experimental chamber (Stelzer et al., 2004) containing relaxing solution. The ends of the preparation were attached to the arms of a motor (model 312B, Aurora Scientific) and force transducer (model 403, Aurora Scientific) as described earlier (Stelzer et al., 2004). The chamber assembly was placed on the stage of an inverted microscope fitted with a 40 \times 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) and were used to assess mean sarcomere length (SL) during each experiment. Changes in force and motor position were sampled (16-bit resolution, DAP5216a, Microstar Laboratories) at rates ≥ 2.0 kHz using SLControl software (Campbell and Moss, 2003) and saved to computer files for later analysis.

Preparation length was set to a sarcomere length of $\sim 2.12 \mu m$ for measuring initial isometric force and for subsequent imposition of stretch. The preparations were first activated at pCa 4.5 to establish maximal force (P_o). Ca^{2+} -activated force (P) at each pCa was determined as the difference between total force in the activating solution and resting force measured in solution of pCa 9.0. Fibers were activated in a series of [pCa] yielding a range of forces ($P/P_o = 0.12$ –1.00), when the fiber reached steady-state force a series of instantaneous stretches ranging from 0.5 to 2.5% of fiber length (L_o) were imposed and each held for 5 s before returning the fiber to pCa 9.0. In these experiments, high speed stretches (complete in ~ 2 ms) were used to minimize the changes in cross-bridge populations during the stretch itself, so that the stretch activation response observed was presumably due to the elastic properties of the cross-bridges present before the stretch was imposed since cross-bridge cycling and recruitment would be minimal under our experimental conditions.

The stretch activation variables measured are shown in Fig. 1. All amplitudes (A) were normalized to prestretch 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 steady-state force to the minimum force decay; P_3 , measured from prestretch steady-state force to the peak value of delayed force; and P_{diff} , difference between P_3 and P_2 . Apparent rate constants were derived for phase 2 (s^{-1} , k_{rel}) from the force decay after the peak of phase 1, and for phase 3 (s^{-1} , k_{diff}) from the point of force reuptake after phase 2 to the completion of delayed force development. For experiments using NEM-S1 each fiber was initially incubated for 15 min in solution of pCa 9.0 containing 0.5 or 1.0 μM NEM-S1 (Fitzsimons et al., 2001a) before proceeding with stretch activation measurements.

At the conclusion of the mechanical experiment, the cardiac fiber was cut free at the points of attachment, placed in rehydration/sample buffer (Bio-Rad Laboratories), and then stored at $-80^\circ C$. Since it has been previously shown that the phosphorylation state of myofibrillar proteins, in particular, phosphorylation of the regulatory light chain (RLC) (Davis et al., 2002), can affect the amplitudes and apparent rate constants of the stretch activation response, we quantified the basal level of RLC phosphorylation in the experimental samples by two-dimensional gel electrophoresis using a mini gel system (Bio-Rad Laboratories), as previously described (Olsson et al., 2004). Two-dimensional gel electrophoresis analysis revealed that the basal level of RLC phosphorylation in cardiac preparations was relatively low (4–10% phosphorylated) and there was little variation in the basal levels of RLC phosphorylation between cardiac preparations, suggesting that in the present study variations in the level of RLC phosphorylation did not significantly influence the stretch activation response.

Data Analysis

Cross-sectional areas of skinned preparations were calculated by assuming that the preparations were cylindrical and by measuring width of the mounted preparation. Submaximal Ca^{2+} -activated force (P) was expressed as a fraction of the force (P_o) generated at pCa 4.5, i.e., P/P_o . Rate constants of force decay were obtained by fitting a single exponential to the time course of decay, i.e., $y = a(1 - \exp(-k_1 \times x))$, 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 were either obtained by a double exponential fit, $y = a \times \exp(-k_1 \times x) + b \times \exp(-k_2 \times x)$, where ‘ a ’ is the amplitude of the first exponential phase that rises with rate constant k_1 and ‘ b ’ is the amplitude of the second exponential phase rising with rate constant of k_2 , or were estimated by linear transformation of the half-time of force redevelopment, i.e., $[k_1 = -\ln 0.5 \times (t_{1/2})^{-1}]$.

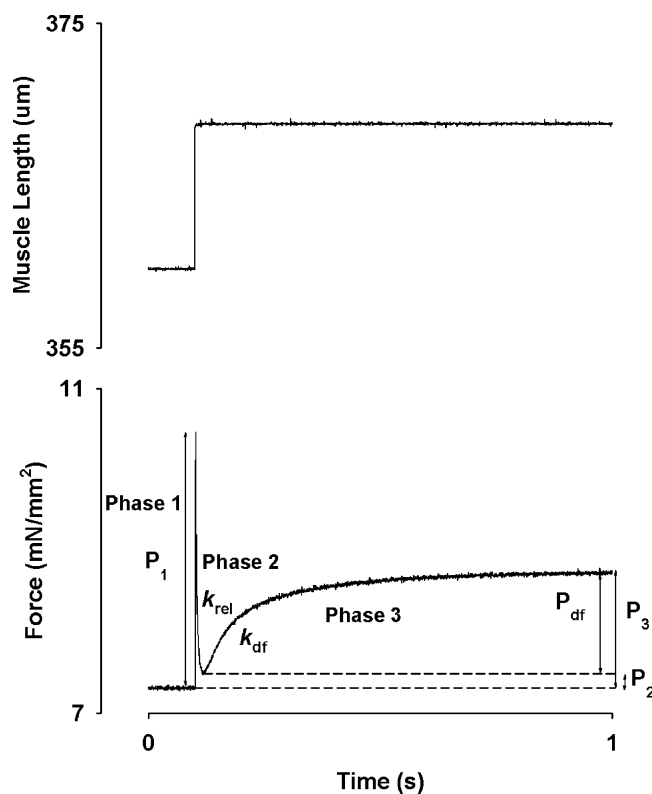


Figure 1. Stretch activation response in murine myocardium. The records of force (bottom) and length (top) exemplify stretch activation of myocardium in response to a stretch of 2.5% of muscle length. Once steady-state isometric force was achieved, the muscle was stretched and then held at the stretched length for 5 s. The recorded variables are labeled on the lower record and described in the text. Prestretch isometric force (the initial 0.1 s in this trace) was $\sim 50\%$ of maximal.

All data are reported as means \pm SEM. Comparisons of stretch activation variables at different levels of activation and the effects of NEM-S1 at the same level of activation were done with a one-way analysis of variance (ANOVA) with a Tukey post-hoc test for significance ($P < 0.05$). When appropriate (test of sample normality failed), the Kruskal-Wallis one-way ANOVA on Ranks was performed (significance set at $P < 0.01$).

RESULTS

Stretch Activation Response in Skinned Myocardium

The stretch activation response in mouse skinned myocardium is illustrated in Fig. 1, in which a stretch of 2.5% of initial length was imposed on a muscle generating Ca^{2+} -activated force that was $\sim 50\%$ maximal, where the stretch activation response is particularly prominent. The response to stretch is multiphasic (Fig. 1); there is an initial increase in force coincident with the stretch (phase 1), presumably due to stretch of attached cross-bridges, followed by a rapid decay in force (phase 2) to a minimum, and finally a delayed increase in force (phase 3, stretch activation), which reaches a plateau but then decays back to the original

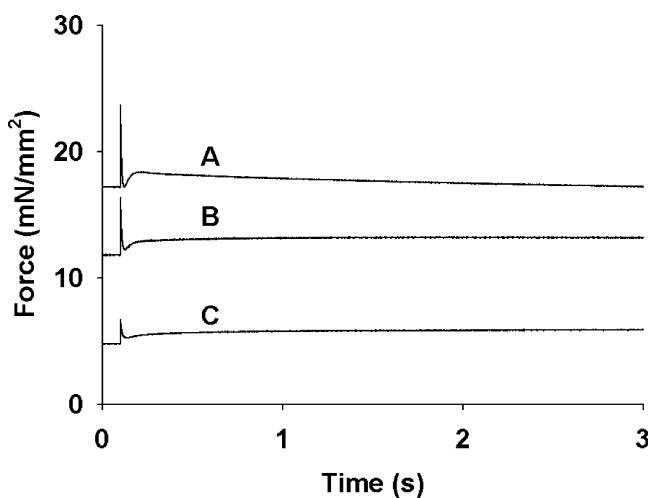


Figure 2. Stretch activation responses of a myocardial preparation at various levels of activation. Stretch of 1% of muscle length was applied during a maximal activation (A) maximal force, $P/P_0 = 1.00$, intermediate-level activation (B), $P/P_0 = 0.62$, and low-level activation (C), $P/P_0 = 0.28$.

isometric force over a period of several seconds. The amplitude of phase 1 relative to the prestretch isometric force is indicated as P_1 , the minimum force at the end of phase 2 is P_2 , and the amplitude of phase 3 is P_3 . It is important to note that since our data sampling rate is limited, it is likely that values of P_1 were underestimated, but characterization of P_1 was not the primary aim of this work.

The amplitude of phase 1 (P_1) increased with level of $[\text{Ca}^{2+}]$ activation and was related to the level of prestretch isometric force (Fig. 2), which is consistent with the idea that phase 1 is due to stretch of attached cross-bridges. When P_1 was normalized to prestretch force, the amplitude of phase 1 was greatest at low levels of activation and increased with degree of stretch (Fig. 3). The amplitude of P_2 measured relative to the prestretch isometric force was sometimes positive (as in Fig. 1) and sometimes negative, indicating that the phase 2 decay could reach forces below isometric, but in either case, P_2 was relatively small at all levels of activation. The amplitudes of P_1 and P_2 were not corrected for resting force, which in each case was $< 5\%$ of the maximum active force at pCa 4.5.

Phase 2 is thought to manifest the dynamic detachment of cross-bridges that are strained by stretch and their rapid replacement by unstrained cross-bridges (Piazzesi et al., 1997). The apparent rate constant of force decay (k_{rel}) was just slightly faster at the lowest and highest levels of activation but overall was not activation dependent (Fig. 4). However, k_{rel} was accelerated by increasing the degree of stretch (Fig. 5), which presumably is an effect of greater strain to accelerate cross-bridge detachment.

P_3 , which is a measure of cross-bridge recruitment (Linari et al., 2004a), and the amplitude of phase 3

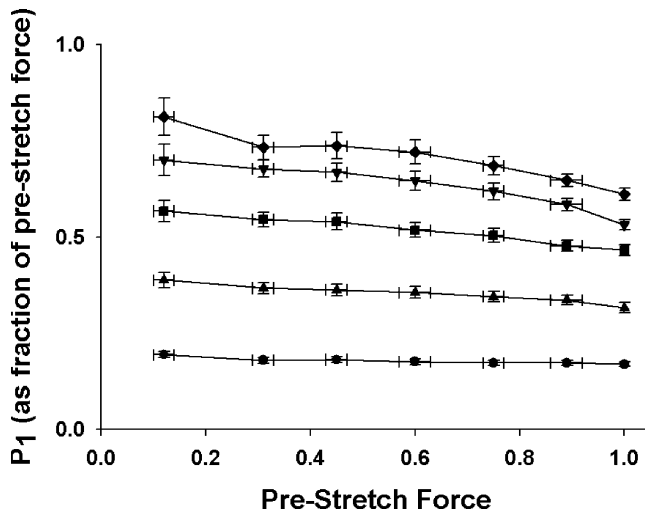


Figure 3. Effects of activation and size of stretch on P_1 . P_1 values normalized to prestretch isometric force at different levels of Ca^{2+} activation ($P/P_o = 0.12-1.00$). Stretch lengths expressed as percent muscle length were 0.5% (circles), 1.0% (upright triangles), 1.5% (squares), 2.0% (inverted triangles), and 2.5% (diamonds).

(P_{df}) both increased as the level of activation was increased, i.e., the post-stretch recruitment of cross-bridges increased as a function of prestretch isometric force. When P_3 was normalized to prestretch isometric force (Fig. 6), stretch activation actually decreased as a function of increased levels of Ca^{2+} activation in that proportionately fewer cross-bridges were recruited by stretch at higher levels of activation. Increasing the size of stretch increased both P_3 and P_{df} (Fig. 7), indicating that more cross-bridges were recruited as the size of

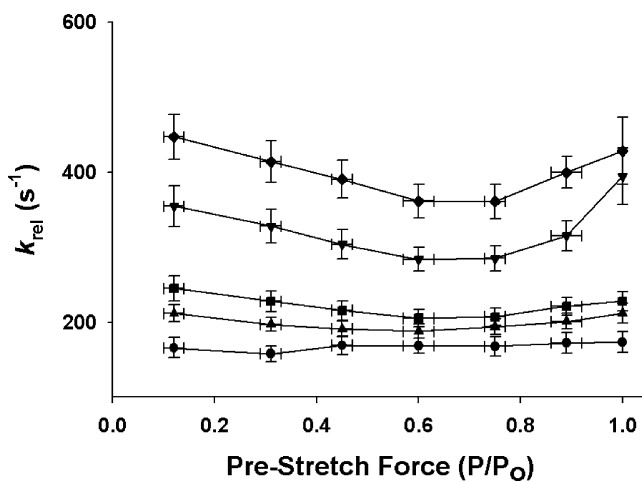


Figure 4. Effect of level of activation and stretch length on k_{rel} . k_{rel} values were normalized to prestretch isometric force at different levels of Ca^{2+} activation ($P/P_o = 0.12-1.00$). Stretch lengths expressed as percent muscle length were 0.5% (circles), 1.0% (upright triangles), 1.5% (squares), 2.0% (inverted triangles), and 2.5% (diamonds).

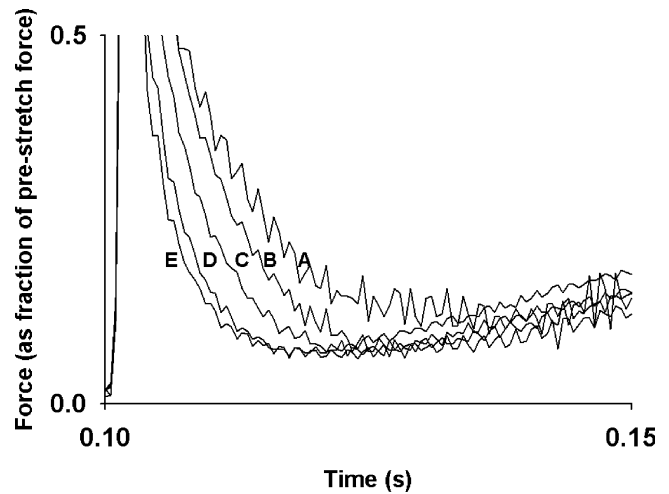


Figure 5. Effect of stretch length on k_{rel} . Stretch lengths were (A) 0.5%, (B) 1.0%, (C) 1.5%, (D) 2.0%, and (E) 2.5% of muscle length, and activation level in each case was $\sim 50\%$ of maximal. Apparent rate constants of force decay were (A) 192 s^{-1} , (B) 207 s^{-1} , (C) 278 s^{-1} , (D) 359 s^{-1} , and (E) 399 s^{-1} .

stretch was increased. As previously reported in insect flight muscles (Linari et al., 2004a), the stretch activation response (phase 3) does not occur as a simple exponential process but instead is a multiexponential process with distinguishable fast and slow phases. The response was biphasic at submaximal levels of activation and was fit well with a double exponential equation, exhibiting an initial fast rate of force development and a clearly discernable slower rate. As activation neared maximal, the force transient exhibited only

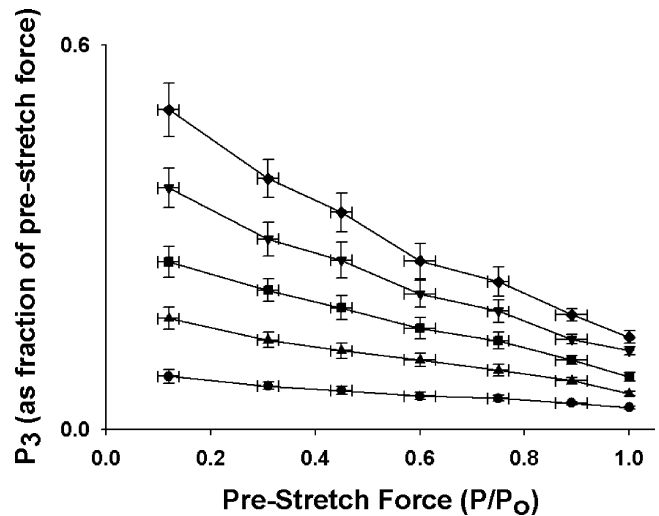


Figure 6. Effect of level of activation and stretch length on P_3 . P_3 values were normalized to prestretch isometric force at different levels of Ca^{2+} activation ($P/P_o = 0.12-1.00$). Stretch lengths expressed as percent muscle length were 0.5% (circles), 1.0% (upright triangles), 1.5% (squares), 2.0% (inverted triangles), and 2.5% (diamonds).

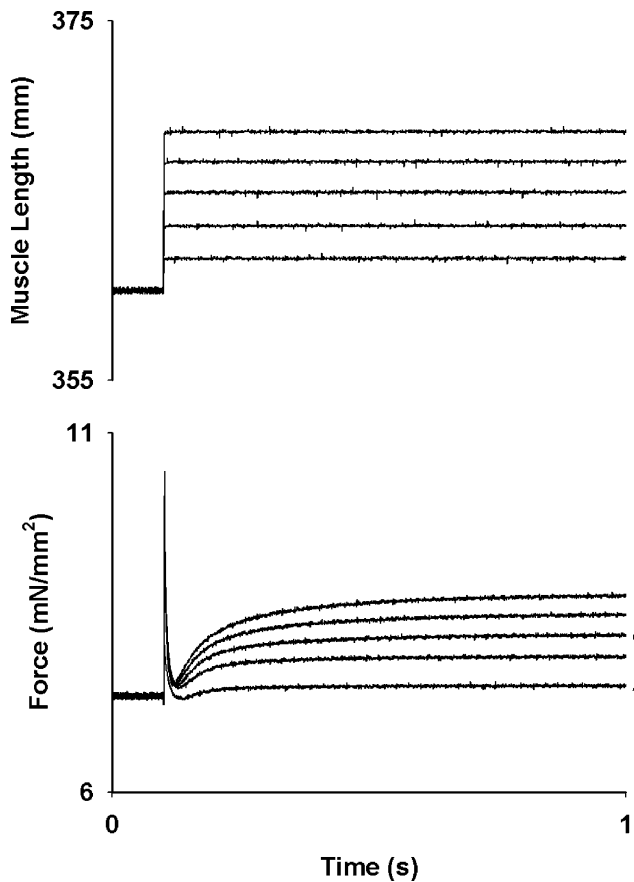


Figure 7. Effect of stretch length on stretch activation variables. Stretch activation responses of ventricular myocardium were recorded at activation levels of $\sim 50\%$ of maximal. Top, motor position changes when the preparation was stretched (A) 0.5%, (B) 1.0%, (C) 1.5%, (D) 2.0%, and (E) 2.5% of initial muscle length. Bottom, corresponding stretch activation responses recorded in absolute units. k_{df} values in this fiber were (A) 24.3 s^{-1} , (B) 20.6 s^{-1} , (C) 16.7 s^{-1} , (D) 12.5 s^{-1} , and (E) 10.9 s^{-1} .

a single rate, as records were well fit with a single exponential equation. As an initial approach to analyzing this data, apparent rate constants were derived from the half-times of force development (Linari et al., 2004a; Stelzer et al., 2004), which allowed comparisons of recovery rates at all levels of activation and is also consistent with methods used by other investigators in this field.

The rate of delayed force development in phase 3 of the stretch activation response is generally thought to involve the cooperative recruitment of cross-bridges into strongly bound states (Lombardi and Piazzesi, 1990; Piazzesi et al., 1997; Campbell et al., 2004). In the present study, increases in level of activation resulted in an acceleration of delayed force development. This is evident both in Fig. 8, which presents typical force traces obtained in response to stretches of 1% of muscle length at three different levels of activation, and in Fig. 9, which presents summary relationships between k_{df} and

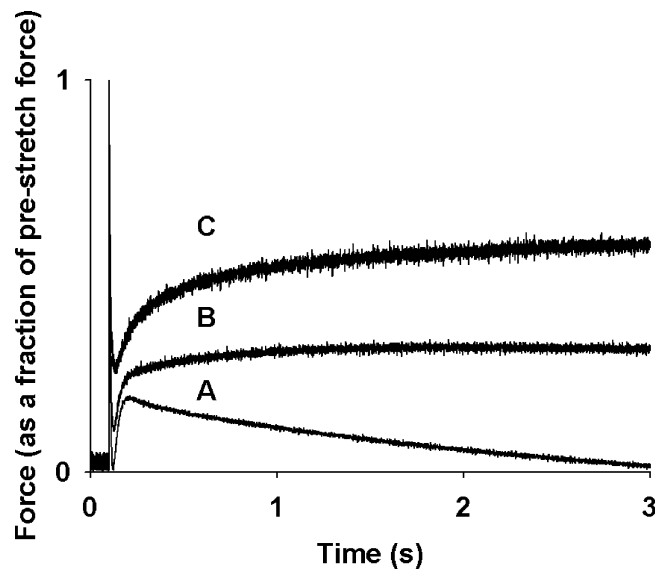


Figure 8. Stretch activation responses in skinned myocardium at different levels of activation. Stretch activation response to a 1% stretch at different levels of activation normalized to prestretch isometric force in the same fiber. The levels of activation were (A) maximal, $P/P_o = 1.00$, (B) intermediate, $P/P_o = 0.42$, and (C) low, $P/P_o = 0.13$. In this example the calculated rate constants of force development were (A) 37.2 s^{-1} , (B) 14.8 s^{-1} , and (C) 3.9 s^{-1} , and the rate constants of force relaxation were (A) 244 s^{-1} , (B) 226 s^{-1} , and (C) 221 s^{-1} .

isometric force (as a fraction of maximum) generated before the stretch was imposed. Fig. 9 also shows that the normalized amplitude of phase 3 is reduced at higher levels of activation, reinforcing an earlier observation (Fig. 6). The activation dependencies of the rate

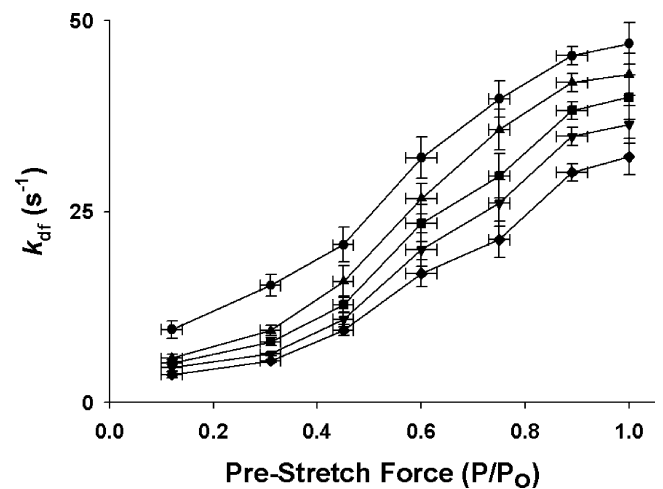


Figure 9. Effect of level of activation and stretch length on k_{df} . k_{df} values were normalized to prestretch isometric force at each level of activation ($P/P_o = 0.12-1.00$). Stretch lengths expressed as percent muscle length were 0.5% (circles), 1.0% (upright triangles), 1.5% (squares), 2.0% (inverted triangles), and 2.5% (diamonds).

and amplitude of the delayed force response are consistent with the idea that cooperative recruitment of cross-bridges into strong-binding states plays an important role in stretch activation. As the level of activation increases, the number of cross-bridges bound to the thin filament also increases, so that fewer are available for recruitment when the muscle is stretched, thereby reducing the amplitude of stretch activation and increasing its rate. On the other hand, the observed decrease in the rate of delayed force development with increasing amounts of stretch (Fig. 9) may manifest a cooperative inactivation of the thin filament due to increased cross-bridge detachment with greater stretch. Alternatively, the increased number of detached cross-bridges might result in the accumulation of weakly bound cross-bridges in the early states of force generation, transiently shifting the equilibrium distribution farther from the power stroke (Lombardi and Piazzesi, 1990; Piazzesi et al., 1992).

The data at submaximal activations was further analyzed by fitting a double exponential to the time course of delayed force development, which was required to adequately fit data obtained at steady isometric forces of $\sim 0.75 P_o$ and below (Table I). From this analysis, it is evident that the slow component of delayed force development comprises a progressively greater fraction of delayed force recovery as activation is reduced, reaching a maximum of $\sim 40\%$ of the response at the lowest levels of activation employed, i.e., $0.12 P_o$. The kinetics of both the fast and slow phases of force recovery were activation dependent in that both k_1 and k_2 decreased when activation was reduced.

When stretch length was increased from 0.5 to 1.0 to 2.0% of muscle length, k_{df} progressively decreased at all levels of activation (Table I). At submaximal levels of activation, both k_1 and k_2 decreased as a function of increasing stretch length, and there was a progressive increase in the relative amplitude of the slow phase of delayed force development.

Effect of NEM-S1 on the Stretch Activation Response

To explore the role(s) of cooperativity in cross-bridge binding in the stretch activation response, skinned myocardial preparations were incubated with NEM-S1 before stretch activation measurements. NEM-S1 is a strong binding derivative of myosin that has been used previously to assess the effects of cross-bridge binding on the regulation of force in myocardium (Fitzsimons, et al., 2001a), since it binds strongly and essentially irreversibly to the thin filament and does not generate force. The present experiments were done at submaximal Ca^{2+} concentrations ($<50\%$ of maximal force), since myocardium shows greatest sensitivity to the activating effects of strong-binding cross-bridges at low levels of activation (Moss et al., 2004). Since NEM-S1 increases submaximal force, the free Ca^{2+} concentra-

tion was reduced in the presence of NEM-S1 to ensure that prestretch forces were similar in the presence and absence of NEM-S1. Also, relatively low concentrations of NEM-S1 (0.5 or 1.0 μM) were used to avoid large changes in Ca^{2+} sensitivity of force or increases in resting force (Fitzsimons et al., 2001a).

Treatment of skinned myocardium with 0.5 or 1.0 μM NEM-S1 significantly reduced the amplitude of stretch activation in response to a stretch of 1% of muscle length: both P_3 and the amplitude of the force transient (P_{df}) were decreased by NEM-S1 at levels of activation yielding prestretch isometric forces of $\sim 20\%$ and $\sim 40\%$ of maximal (Table II). Qualitatively, NEM-S1 induced a change in shape of the stretch activation response at low levels of activation ($P/P_o \sim 0.2$ or ~ 0.4) so that response was more similar to that at high levels of activation (Fig. 10), the slow phase of the transient was diminished, k_{df} was accelerated as a result, and the amplitude of the response was reduced. The acceleration of k_{df} by NEM-S1 was dramatic at both levels of activation used in these experiments (Table II).

Further analysis of delayed force development (Table II) revealed that both the fast (k_1) and slow (k_2) phases of force development were accelerated by NEM-S1 at both levels of activation studied, and this effect was greater at 1.0 than at 0.5 μM NEM-S1. This suggests that cooperative recruitment of cross-bridges slows both phases of force development in the delayed force response. In addition, NEM-S1 reduced the amplitude of the slower phase of force development, so that the overall force response was dominated by the kinetics of the faster phase. This result is consistent with the idea that the slower phase of force development is mainly due to cooperative recruitment of cross-bridges, which serves to slow the overall delayed force response.

The apparent rate constant of force decay (k_{rel}) in phase 2 was unchanged by NEM-S1 at either level of activation compared with control values, which is summarized in Table III and is also evident in the records shown in Fig. 10. Thus, the kinetics of this phase of the force transient appear to be dominated by strain-induced cross-bridge detachment rather than collapse of cooperative activation of the thin filament subsequent to cross-bridge detachment.

DISCUSSION

Activation Dependence of the Force Response to Stretch in Murine Myocardium

Stretch of activated myocardium to a new isometric length evokes a multiphasic force response that has been attributed both to changes in numbers of cross-bridges bound to actin (Linari et al., 2004b) and to transitions of cross-bridges between attached states (Getz et al., 1998). The relative sliding of thick and thin

TABLE I
Activation Dependence of the Phase 3 Delayed Force Rise Following Stretches of Different Amplitudes

Activation level (P/P _o)	Stretch length (%ML)	k_{df}	a	k_1	b	k_2
		s ⁻¹		s ⁻¹		s ⁻¹
1.00 ± 0.00	0.5	47.0 ± 2.7	1.00	46.8 ± 3.7	-	-
	1.0	43.0 ± 2.8	1.00	44.1 ± 3.7	-	-
	2.0	36.4 ± 2.4	1.00	37.3 ± 3.4	-	-
0.89 ± 0.03	0.5	45.4 ± 1.2	1.00	45.9 ± 2.7	-	-
	1.0	41.9 ± 1.2	1.00	42.4 ± 2.6	-	-
	2.0	34.9 ± 1.2	1.00	35.3 ± 2.2	-	-
0.75 ± 0.02	0.5	40.0 ± 2.4	0.91 ± 0.04	42.1 ± 3.2	0.09 ± 0.04	9.9 ± 1.4
	1.0	35.7 ± 2.7	0.78 ± 0.04	37.0 ± 3.3	0.22 ± 0.04	7.6 ± 1.3
	2.0	26.1 ± 3.1	0.73 ± 0.04	32.5 ± 3.1	0.27 ± 0.04	5.9 ± 0.9
0.60 ± 0.03	0.5	32.1 ± 2.7	0.81 ± 0.03	39.3 ± 3.3	0.19 ± 0.02	8.5 ± 1.3
	1.0	26.7 ± 2.0	0.69 ± 0.03	33.3 ± 3.2	0.31 ± 0.03	6.6 ± 1.2
	2.0	20.0 ± 2.2	0.65 ± 0.03	26.7 ± 3.3	0.35 ± 0.03	4.7 ± 0.8
0.45 ± 0.02	0.5	20.7 ± 2.3	0.72 ± 0.03	31.9 ± 1.8	0.28 ± 0.02	4.6 ± 0.8
	1.0	15.9 ± 2.1	0.59 ± 0.02	24.6 ± 1.6	0.41 ± 0.02	3.2 ± 0.7
	2.0	10.9 ± 1.0	0.55 ± 0.03	17.9 ± 1.9	0.45 ± 0.03	3.0 ± 0.6
0.31 ± 0.02	0.5	15.4 ± 1.4	0.67 ± 0.03	24.8 ± 1.5	0.33 ± 0.03	3.0 ± 0.4
	1.0	9.5 ± 0.6	0.53 ± 0.03	17.5 ± 1.1	0.47 ± 0.03	2.1 ± 0.4
	2.0	6.4 ± 0.3	0.50 ± 0.03	13.5 ± 1.1	0.50 ± 0.03	1.9 ± 0.4
0.12 ± 0.02	0.5	9.6 ± 1.1	0.59 ± 0.03	17.6 ± 1.0	0.41 ± 0.03	2.2 ± 0.3
	1.0	5.8 ± 0.5	0.54 ± 0.03	13.0 ± 0.9	0.46 ± 0.03	1.4 ± 0.2
	2.0	4.6 ± 0.4	0.46 ± 0.03	9.6 ± 0.7	0.54 ± 0.03	1.2 ± 0.2

Data are means ± SEM, from 10 cardiac preparations. Data is presented for stretch lengths of 0.5, 1.0, and 2.0% of muscle length (ML). The apparent rate constants for the phase 3 delayed force transient were obtained either from conversion of half-times of force recovery (k_{df}), or by fitting a double exponential to each record, $y = a \times \exp(-k_1 \times x) + b \times \exp(-k_2 \times x)$, where "a" is the amplitude of the first exponential phase that rises with rate constant k_1 and "b" is the amplitude of the second exponential phase that rises with rate constant k_2 , as explained in the text.

filaments due to stretch increases the strain of attached cross-bridges and also changes the spatial register of some unbound myosin heads relative to actin, thereby increasing the likelihood that these heads will bind actin and generate force. Coincident with stretch, force increases in proportion to the amount of applied stretch (Figs. 1 and 3), which is presumably a manifestation of increased strain of cross-bridges already bound to actin (Huxley and Simmons, 1971). This is followed by a relatively rapid decay or relaxation of force that has been attributed to detachment of strained cross-bridges and reattachment of unstrained cross-bridges (Davis and Rodgers, 1995; Piazzesi et al., 1997), with a net reduction in the total number bound. The subsequent delayed rise in force is the hallmark of stretch activation and is due to an increase in the number of force-generating cross-bridges arising from either a stretch-dependent realignment of cross-bridges with actin, based on the misregistration of the myosin-actin repeats (Wray, 1979), or cooperative recruitment of cross-bridges into force-generating states (Linari, et al., 2004a; Campbell et al., 2004).

The minimum force and kinetics of the phase 2 force decay, defined by P_2 and k_{rel} , did not show strong activation dependence in myocardium (Figs. 4 and 5), since

both variables changed relatively little over large ranges of activation. Sudden stretch of an isometrically contracting muscle strains attached cross-bridges, leading to rapid detachment and relaxation of force. According to Piazzesi, et al. (1997), the force minimum (P_2) after stretch is determined not only by detachment but also by the attachment of new unstrained cross-bridges that replace strain-detached cross-bridges, a process that is thought to occur concurrently (and not sequentially) with strain-induced detachment. In the present study, P_2 tended to be lowest (as a fraction of prestretch force) during maximal activations and greatest in low-level activations, suggesting that stretch disrupts a greater fraction of force-generating cross-bridges in the high activation state. k_{rel} is much greater than k_{df} and dominates phase 2 kinetics such that even the faster k_{df} at high levels of activation is not able to offset the decrease in force due to rapid detachment of cross-bridges, resulting in greater phase 2 relaxation of force. Increasing the amount of stretch at each level of activation caused a significant increase in k_{rel} , most likely due to increased cross-bridge strain, but increased P_2 due to increased cross-bridge binding as a consequence of increased availability of thin filament sites for myosin binding.

TABLE II
Effect of NEM-S1 on the Phase 3 Delayed Force Development Rise in Response to a 1% Stretch

Group	Activation Level (P/P _o)	P ₃ (P ₃ /P _o)	P _{df} (P _{df} /P _o)	k _{df}	a	k ₁	b	k ₂
				s ⁻¹		s ⁻¹		s ⁻¹
Control (13) +0.5 μM	0.19 ± 0.02	0.161 ± 0.013	0.122 ± 0.012	6.9 ± 1.2	0.49 ± 0.02	15.4 ± 0.8	0.51 ± 0.02	1.7 ± 0.4
NEM-S1 (13) +1.0 μM	0.18 ± 0.02	0.121 ± 0.012 ^a	0.085 ± 0.006 ^a	11.7 ± 1.5 ^a	0.66 ± 0.03 ^a	17.8 ± 1.7	0.34 ± 0.03 ^a	3.6 ± 0.9 ^a
NEM-S1 (8)	0.20 ± 0.03	0.087 ± 0.012 ^b	0.067 ± 0.009 ^b	23.4 ± 2.6 ^b	0.84 ± 0.05 ^b	24.9 ± 2.4 ^b	0.16 ± 0.03 ^b	5.9 ± 1.1 ^b
Control (13) +0.5 μM	0.41 ± 0.02	0.133 ± 0.011	0.104 ± 0.011	12.0 ± 1.7	0.57 ± 0.02	23.0 ± 1.2	0.43 ± 0.02	2.9 ± 0.7
NEM-S1 (13) +1.0 μM	0.44 ± 0.03	0.104 ± 0.017	0.084 ± 0.008	14.5 ± 1.8	0.60 ± 0.03	24.1 ± 2.3	0.40 ± 0.03	3.8 ± 0.8
NEM-S1 (8)	0.36 ± 0.04	0.075 ± 0.021 ^c	0.058 ± 0.005 ^d	20.3 ± 2.7 ^c	0.73 ± 0.04 ^c	26.7 ± 2.6	0.27 ± 0.03 ^c	5.2 ± 0.8 ^c

Data are means ± SEM, with number of experiments performed in each case in parentheses. The apparent rate constants for phase 3 delayed force transient were obtained either from conversion of half-times of force recovery (k_{df}), or by fitting a double exponential to each record, $y = a \times \exp(-k_1 \times x) + b \times \exp(-k_2 \times x)$, where "a" is the amplitude of the first exponential phase that rises with rate constant k_1 and "b" is the amplitude of the second exponential phase that rises with rate constant k_2 , as explained in the text.

^aSignificantly different from control at low level of activation ($P < 0.05$).

^bSignificantly different from control and 0.5 μM NEM-S1 at low level of activation ($P < 0.05$).

^cSignificantly different from control at intermediate level of activation ($P < 0.05$).

^dSignificantly different from control and 0.5 μM NEM-S1 at intermediate level of activation ($P < 0.05$).

The primary focus of the present study is the delayed rise in force and particularly the mechanisms of its activation dependence. Stretch activation of mouse skinned myocardium varied with the level of Ca²⁺ activation, shown in Fig. 2 for steady prestretch forces between 0.12 and 1.00 P_o. Similar to the case in insect flight muscle (Linari, et al., 2004a), stretch of myocardium at levels of activation much below 0.12 P_o did not produce a measurable stretch activation response, at least within the resolution of the force transducer used. As activation was increased in the range 0.12–1.00 P_o, the absolute amplitude of stretch activation increased; however, the amplitude of stretch activation normalized to the prestretch force actually decreased over the same range of activations. This reduction in stretch activation as a fraction of prestretch force is a manifestation of the higher state of activation of the thin filament when [Ca²⁺] is high, which would presumably limit the amount of increased activation and recruitment of additional cross-bridges that can be achieved with stretch.

The rate constant of delayed force rise, i.e., k_{df} , was observed to increase as a function of increasing prestretch force (Fig. 9; Table I), which was varied by adjusting [Ca²⁺]_{free}, while the normalized amplitudes of phase 3, P₃ (Fig. 6) and P_{df} (not depicted), were inversely proportional to prestretch force, similar to the relationships previously observed in insect flight muscles (Linari et al., 2004a; Agianian et al., 2004). The Ca²⁺ sensitivity of k_{df} observed in this study is similar to the Ca²⁺ sensitivity of k_{tr} , the rate constant of force redevelopment after mechanical release and restretch (Fitzsimons et al., 2001b) observed previously

in mouse skinned myocardium, suggesting that at least some of the processes underlying the two rate constants are similar (Campbell et al., 2004). When the time course of delayed force development at submaximal activations was fit with a double exponential equation, both the fast and slow recovery processes were found to exhibit activation dependence (Table I). At each level of submaximal activation, the faster recovery process was predominant, as judged by the relative amplitudes of the two rate processes; however, as the level of activation was reduced, the relative amplitude of the slower process increased, reaching ~40% of the total force response at the lowest level of activation studied here. Similarly, as the amount of stretch was increased, the values of k_{df} , k_1 , and k_2 at every level of activation decreased and the amplitude of the slow component increased as a fraction of the total delayed force response. This suggests that greater stretch induced greater cooperative inactivation of the thin filament presumably as a consequence of progressively greater disruption of cross-bridges strongly bound to the thin filament. In turn, delayed force redevelopment would be slowed due to the time required for cooperative recruitment of cross-bridges to force-generating states, as suggested by the model of Campbell, et al. (2004).

Effect of NEM-S1 on Stretch Activation

In this study, a strong binding derivative of myosin subfragment 1, NEM-S1, was used to explore the possibility that the stretch activation response is mediated by increased cooperative binding of cross-bridges to the thin filament as a consequence of stretch. The use of

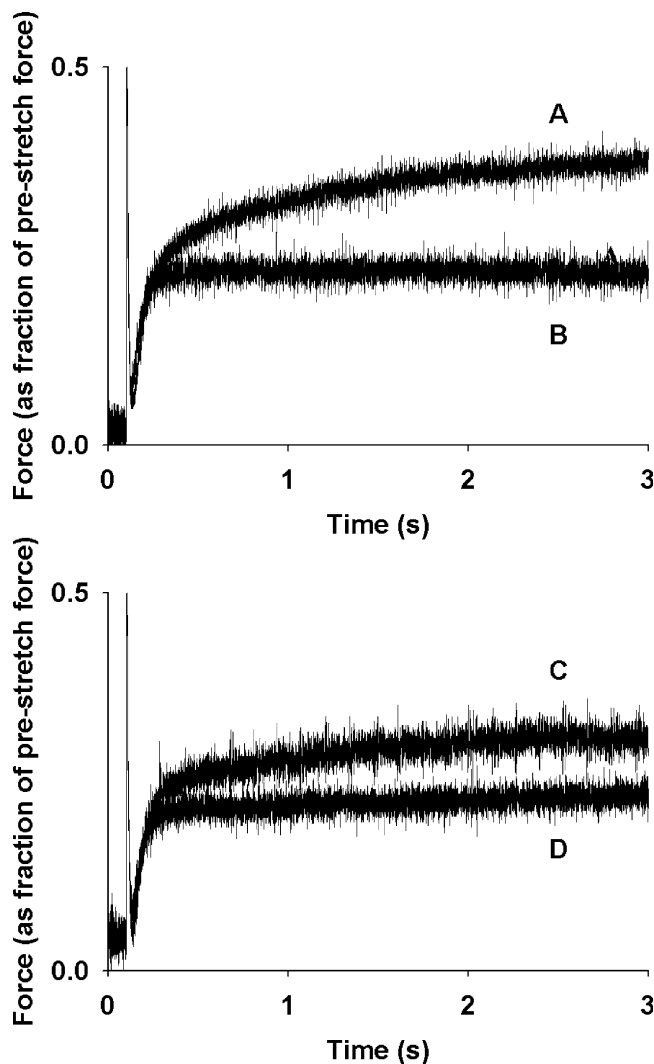


Figure 10. Effect of NEM-S1 on stretch activation at low and intermediate levels of activation. Stretch activation responses after a 1% of muscle length stretch in an untreated fiber (A and C) and the same fiber treated with 1.0 μM NEM-S1 (B and D), at a low level of activation (top), $P/P_0 = 0.18$ (A) and $P/P_0 = 0.19$ (B), and at an intermediate level of activation (bottom), $P/P_0 = 0.42$ (C) and $P/P_0 = 0.40$ (D). In this fiber, the phase 3 rate constants of force development were (A) 5.5 s^{-1} (low activation level) and (C) 10.7 s^{-1} (intermediate activation level) in the absence of NEM-S1 and (B) 22.1 s^{-1} (low activation level) and (D) 19.4 s^{-1} (intermediate activation level) in the presence of 1.0 μM NEM-S1.

NEM-S1 to probe cross-bridge roles in the regulation of force development offers the advantage that while it binds strongly to actin NEM-S1 does not itself generate force (Swartz and Moss, 1992). In the present study, the myocardial preparations were bathed in very low concentrations of NEM-S1 so that there were no appreciable effects on the Ca^{2+} sensitivity of force or on resting force at pCa 9.0. This feature of experimental design simplified the interpretation of stretch activation responses because prestretch forces and free Ca^{2+} concentrations were similar in the presence and absence of

NEM-S1. Of course, we cannot exclude the possibility that by keeping NEM-S1 concentrations low we have underestimated the activating effects of strong binding cross-bridges in stretch activation.

NEM-S1 treatment resulted in dramatic increases in the overall rate of rise of force (k_{diff}) in phase 3 while decreasing the amplitude (P_3) of the force transient (Fig. 10; Table II). Furthermore, at submaximal activations, where delayed force development was best fit by a double exponential, NEM-S1 increased the rate constants for both the fast (k_1) and slow (k_2) rate processes and significantly reduced the overall contribution of the slower process to delayed force development. Regardless of the approach used to assess the rate of delayed force development, the effects on rate and amplitude were greater when NEM-S1 concentration was increased. Earlier studies suggested that the rise in force in phase 3 is due to rapid recruitment of nonforce-generating cross-bridges into force-generating states (Getz et al., 1998; Linari et al., 2004a), at least in insect flight muscle. Linari et al. (2004a) suggested that stretch-induced distortion of cross-bridges bound to the thin filament displaces tropomyosin (Tm), which relieves steric blocking of binding sites on actin and allows additional cross-bridges to bind. Furthermore, as was seen in the present study, the phase 3 transient was multi-exponential at low levels of activation but a single exponential at high levels of activation, suggesting that cooperative recruitment of cross-bridges slowed the rate of force development at low levels of activation. From this model, it would also be predicted that larger stretches might cause greater displacement of tropomyosin and allow more cross-bridge binding and more delayed force, as observed here (Fig. 7).

The amplitude and rate of the force transient (phase 3) due to stretch are both sensitive to the activation level of the fiber, similar to the activation dependence of force redevelopment after a mechanical release/restretch maneuver designed to reduce or eliminate strongly bound cross-bridges (Fitzsimons et al., 2001b). At the level of the myofilaments, regulation of contraction is initiated by Ca^{2+} binding to troponin C (TnC), which alters the interactions of thin filament proteins, resulting in the movement of Tm to allow the binding of cross-bridges on actin and generation of force. Recent studies have shown that strongly bound cross-bridges cooperatively activate the thin filament through allosteric effects within a regulatory strand (for reviews see Gordon et al., 2000; Moss et al., 2004). Thus, the activation dependence of the rate of force development depends both on Ca^{2+} binding to TnC and on cross-bridges binding to actin, such that at saturating Ca^{2+} the rate of force development is fast and cross-bridge recruitment is minimal while at low Ca^{2+} the rate of force development is slow and cross-bridge recruitment is maximal.

TABLE III
Effect of NEM-S1 on Phases 1 and 2 in Response to a 1% Stretch

Group	Activation Level (P/P _o)	P ₁ (P ₁ /P _o)	P ₂ (P ₂ /P _o)	k _{rel} s ⁻¹
Control (13)	0.19 ± 0.02	0.350 ± 0.020	0.039 ± 0.009	224 ± 12
+ 0.5 μM NEM-S1 (13)	0.18 ± 0.02	0.343 ± 0.022	0.036 ± 0.008	215 ± 11
+ 1.0 μM NEM-S1 (8)	0.20 ± 0.03	0.328 ± 0.018	0.020 ± 0.009	208 ± 11
Control (13)	0.41 ± 0.02	0.347 ± 0.016	0.029 ± 0.006	208 ± 13
+ 0.5 μM NEM-S1 (13)	0.44 ± 0.03	0.340 ± 0.019	0.020 ± 0.004	202 ± 12
+ 1.0 μM NEM-S1 (8)	0.36 ± 0.04	0.318 ± 0.022	0.017 ± 0.007	199 ± 9

Data are means ± SEM, with number of experiments performed in each case in parentheses. The amplitudes are normalized to prestretch isometric force, and the apparent rate constant of force decay (k_{rel}) was obtained by fitting a single exponential to the time course of decay, i.e., $y = a(1 - \exp(-k_1 \times x))$, where "a" is the amplitude of the single exponential phase and k_1 is the rate constant of decay, as explained in the text.

Thin filament regulation of cross-bridge binding is widely believed to involve changes in the position of Tm, which is the basis of the model of activation proposed by McKillop and Geeves (1993). In the absence of Ca²⁺, Tm blocks myosin binding sites on actin but when Ca²⁺ binds to TnC, alterations in the interactions of thin filament proteins results in movement of Tm such that cross-bridges can bind to actin and generate force. In this regard, it has been shown that Tm assumes different positions on the thin filament possibly corresponding to different regulatory states (Vibert et al., 1997, Maytum et al., 1999) and opening the possibility that interactions between adjacent Tm molecules participate in the spread of activation along the thin filament due to cross-bridge binding (Palmiter et al., 1996; Lehman et al., 2000).

In the present experiments, binding of NEM-S1 to the thin filament appears to accelerate the recruitment of endogenous cross-bridges to strongly bound, force-generating states. NEM-S1 has previously been shown to increase myosin subfragment-1 binding to thin filaments and to increase force and the rate of force development in skinned preparations from both skeletal muscle and myocardium (Fitzsimons et al., 2001a,b). This can be understood as an increase in the probability of cross-bridge binding due to NEM-S1, which cooperatively accelerates the transition to a force-generating state by displacing Tm, thereby allowing endogenous cross-bridges to bind to actin or to make the transition from weakly bound to force-generating states.

The Mechanism of Activation Dependence of the Delayed Force Response to Stretch

The finding in the present study that NEM-S1 accelerated the kinetics and reduced the amplitude of stretch activation (Fig. 10; Table II), particularly during submaximal Ca²⁺ activations, strongly suggests that stretch induces cooperative recruitment of cross-bridges to strong-binding states. NEM-S1 presumably mimics the activating effects of strong binding cross-bridges, which accelerates the rate of rise of delayed force. The finding

that NEM-S1 actually reduced the amplitude of stretch activation can be explained if the thin filament-activating effects of NEM-S1 supercede the activating effects of stretch to cooperatively recruit additional cross-bridges. These results are consistent with the idea that the kinetics and amplitude of phase 3 in myocardium are dependent on the number of strongly bound cross-bridges, similar to the mechanism proposed previously for stretch activation in insect flight muscle (Linari et al., 2004a).

The cooperative recruitment of cross-bridges is a dynamic process (Moss et al., 2004) that is slower than any single kinetic step in the activation of force development (Campbell et al., 2004). Thus, the cooperative recruitment of cross-bridges as part of the stretch activation response would be expected to slow the rate of delayed force development and at least partly explains the activation dependence of k_{df} and also the large discrepancy in the rates of phase 2 and phase 3 in the force response to stretch. At high levels of activation, a large fraction of available cross-bridges are already strongly bound to actin, which leaves few myosin heads for recruitment in response to stretch. In such cases, the delayed increase in force would occur at maximal or near maximal rates. At low levels of activation, a much smaller fraction of available cross-bridges are strongly bound to actin, which leaves a larger number of myosin heads for recruitment. In these cases, the stretch-induced increase in force would be slowed due to the time taken to cooperatively recruit cross-bridges to force-generating states. Consistent with these ideas, progressively increasing the level of activation not only increased the rate of delayed force development, k_{df} (Fig. 9), but also reduced the amplitude of delayed force development when normalized to prestretch force (Fig. 6). Increasing the amount of stretch (from 0.5% to 2.5% of initial muscle length) at any level of activation also reduced the apparent rate constant of delayed force development, k_{df} (Fig. 9), and increased P₃ (Fig. 6). Greater amounts of stretch presumably disrupted larger numbers of cross-bridges, which would leave more sites available for cross-bridge

binding to actin and thereby slow the overall rate of force development due to the longer time required to fully reestablish cooperative activation of the thin filament. Consistent with this idea, the fractional contribution of the slow phase of force recovery (described by the rate constant k_2) increased as the amount of applied stretch was increased (Table I).

The greater effect of NEM-S1 to accelerate the rate of delayed force development (Table II) at lower levels of activation (e.g., $P/P_o \sim 0.2$) compared with higher levels of activation (e.g., $P/P_o \sim 0.4$) is consistent with the greater availability of unbound cross-bridges at low levels of activation, which in turn increases the effects of cooperative recruitment on the kinetics of force development (Linari et al., 2004a). Since cooperative recruitment of cross-bridges slows the overall rate of the stretch activation response, the net effect of NEM-S1 is to accelerate force development by accelerating the transition of cross-bridges to force-generating states.

Our finding that the shape of the force transient at low levels of activations after NEM-S1 treatment tends to become more nearly single exponential, rather than biexponential (Fig. 10; Table II), further supports the idea that cooperativity in cross-bridge binding contributes significantly to the rate and amplitude of the delayed increase in force after stretch. Examination of Table II reveals that both the fast and slow rate processes observed at low levels of activation were accelerated by NEM-S1, indicating that cooperative recruitment of cross-bridges contributes to both processes either directly or indirectly. However, the finding that NEM-S1 significantly reduces the contribution of the slow process to the overall response suggests that the slow process is primarily a consequence of cooperativity in cross-bridge binding. Based on this suggestion, it is possible that the slow phase would be abolished at much higher concentrations of NEM-S1, which we were unable to achieve in these experiments due to substantial increases in Ca^{2+} -independent force at NEM-S1 concentrations much $>1 \mu M$.

The amplitude of phase 3, P_3 , is proportional to the number of additional cross-bridges recruited to force generating states as a consequence of stretch. Linari et al. (2004a) proposed that the number of cross-bridges recruited by stretch of insect flight muscle is controlled by a positive feedback process related to the number of cross-bridges bound to actin before stretch. In our experiments, a small amount of NEM-S1 reduced P_3 and therefore the number of cross-bridges recruited by stretch, even though this amount of NEM-S1 had little effect on the isometric force before stretch. This can be explained if we consider that NEM-S1 binds strongly to actin but does not produce force, thereby inducing the greater activation that is characteristic of increases in strong binding cross-bridges but reducing the number of new cross-bridges recruited after stretch, i.e., NEM-S1

binding to actin before stretch increases the probability of cross-bridge binding to the thin filament even before stretch. Overall, our data indicate that NEM-S1 reduced the number of cross-bridges recruited by stretch but increased the rate of delayed force development presumably as a result of prestretch recruitment of cross-bridges.

Even with the application of NEM-S1, there is still a stretch activation response (Fig. 10). The most likely explanation for the residual stretch activation is that in addition to the cooperative recruitment of cross-bridges, stretch also increases force either by changes in the thin filament regulatory strand, as suggested by Linari et al. (2004a), or changes in registration of unbound myosin with binding sites on actin (Wray, 1979), or both. Of these two possibilities, it is difficult to tell which is more important, since there is stretch activation even at maximal activation, where it is unlikely that stretch could significantly further activate the thin filament regulatory strand, so that the delayed activation of force would be due mainly to changes in thick/thin filament registration. In any case, such considerations suggest that the stretch-induced delayed force development is due to a combination of both cooperative and noncooperative recruitment of cross-bridges to force-generating states. Our finding that NEM-S1 substantially and preferentially reduced the amplitude of the slower phase of force redevelopment (Table II) is consistent with this suggestion, since NEM-S1 would be expected to preferentially reduce the amplitude of a process that is principally mediated by recruitment of cross-bridges to strongly bound states.

Because stretch activation at saturating Ca^{2+} concentrations is comprised of just the fast phase (Table I), the contribution of cooperative cross-bridge binding is likely to be relatively small. However, as the level of Ca^{2+} activation is reduced, the contribution of the slow phase to the overall response increases to a maximum of $\sim 40\%$ (assessed by relative amplitudes of the fast and slow phases). Thus, the cooperative recruitment of cross-bridges becomes an increasingly important component of the stretch activation response as the level of activation is lowered, and therefore, the rate of delayed force development (k_{dt}) is slowed. Conversely, at higher levels of activation where cooperativity in cross-bridge binding is less prominent in the response to stretch, the rate of delayed force development is faster.

Physiological Consequences of Stretch Activation in Myocardium

Earlier work has suggested that stretch activation plays a role in cardiac function by modulating power output during systolic ejection, such that strength of contraction increases with the amount of stretch of ventricular myocytes (Davis et al., 2001). Results of the present study indicate that both the rate and amplitude of the

stretch activation response in murine myocardium vary with the level of prestretch activation. Furthermore, the delayed development of force that is characteristic of stretch activation is mediated by a combination of improved registration of unbound myosin heads with thin filament binding sites and enhanced activation of the thin filament. This increased activation may be a consequence of thin filament strain, as suggested previously by others (Linari, et al., 2004a), but evidence presented here indicates that cooperative recruitment of cross-bridges (Campbell, et al., 2004) also contributes significantly to the stretch activation response. Regardless of the underlying mechanism, stretch-induced recruitment of cross-bridges could contribute to cardiac output by increasing the force and speed of ventricular contraction during ejection.

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