Cycling Cross-Bridges Increase Myocardial Stiffness at Submaximal Levels of Ca^{2+} Activation

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ABSTRACT Permeabilized multicellular preparations of canine myocardium were subjected to controlled length changes to investigate the extent to which cross-bridges augment passive stiffness components in myocardium at low levels of Ca^{2+} activation. When the preparations were immersed in pCa 9.0 solution (negligible free [Ca^{2+}]) they behaved as simple elastic systems (i.e., tension increased proportionately with length). In contrast, when the muscles were stretched in Ca^{2+} activating solutions, tension rose much more rapidly during the initial phase of the movement than thereafter. Several lines of evidence suggest that the nonlinear response represents the displacement of populations of cycling cross-bridges that are perturbed by interfilamentary movement and take some time to recover. 1), The stiffness of the initial phase increased proportionately with the level of Ca^{2+} activation. 2), The magnitude of the short-range response increased with stretch velocity. 3), The initial response was reversibly reduced by 5-mM 2,3-butanedione monoxime, a known cross-bridge inhibitor. The initial stiffness of the passive elastic (pCa 9.0) response was equivalent to the Ca^{2+} dependent component at 2% (pCa $\sim$ 6.2) of the maximal (pCa 4.5) level. These results suggest that cross-bridges may significantly affect diastolic chamber stiffness.

INTRODUCTION

Normal cardiac function is critically dependent on the resistance to flow that the ventricular chambers present to returning venous blood. If the chambers are compliant, blood can enter through the atrio-ventricular valves without hindrance. Conversely, if the walls are stiff, ventricular pressure rises sharply when blood enters and the pressure differential across the mitral and triscuspid valves is reduced. Less blood flows through the valves and ventricular filling is diminished.

The stiffness of the ventricular walls depends not only on their thickness but also on the proteins and structural elements of which they are composed (Price, 1991). Titin molecules, microtubules, and intermediate filaments contribute to the properties of individual myocytes whereas the collagen matrix forms an additional extracellular component. Together these structures form a passive source of wall stiffness in that they resist further extension when stretched but do not themselves actively generate force. Although different elements will contribute different portions of the passive stiffness as muscle length is varied (Granzier and Irving, 1995), the term ''passive elastic component'' will be used throughout this work to describe their combined effect.

The passive elastic component forms the heart's minimal stiffness. Consider a section of ventricular wall subjected to a length change Δx that is sufficiently small that the passive elastic stiffness can be considered as a constant k_P. Under most circumstances, the measured change in wall tension ΔF cannot be less than the corresponding change in passive tension, $\Delta F_P = k_P \times \Delta x$. There is however an obvious situation where ΔF will be greater than ΔF_P. This occurs when the contractile apparatus is activated in which case the measured change in force will reflect both the extension of the passive elastic component and that of any cross-bridges that were attached between the thick and thin filaments at the beginning of the movement. The exact profile of the tension response during the stretch will depend on the cross-bridge cycling kinetics, but the measured change in tension (at least during the initial stages of the movement before cross-bridges have been forcibly detached) must be greater than that due to the passive component alone.

Although it is obvious that the stiffness of a muscle stretched under these conditions will scale with the number of attached cross-bridges, it is less clear whether the cross-bridge contribution to ventricular stiffness is significant under conditions mimicking early diastole. Cross-bridges certainly remain attached between the filaments throughout the cardiac cycle (Lu et al., 2001, and references therein), but whether there are sufficient numbers of them to substantially augment the passive elastic component during diastole is uncertain. If this were to be the case, attached cross-bridges could have a profound impact on ventricular function and might even form a potential target for therapeutic intervention. The experiments described here address this important issue by assessing the impact of submaximal cross-bridge activity on the stiffness of canine myocardial preparations.

METHODS

Preparations

The myocardial preparations used in these experiments were isolated from a control group of mongrel dogs subjected to a sham operation similar to that described by Wolff et al. (1995) as part of an ongoing study of tachycardia-induced heart failure. Left ventricular sections (20–40 g) were excised from the canine hearts at the end of terminal studies, immediately frozen between

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paired metal plates precooled in liquid nitrogen and stored at −80°C for periods of up to three months. Canine preparations were used in preference to those isolated from rat or mouse hearts because the mechanical properties of the canine samples at low levels of Ca^{2+} activation are more likely to approximate those of human myocardium during early diastole. Diastolic function almost certainly depends on the duration of the cardiac cycle and dogs and humans have similar resting heart rates (~60 beats per minute, bpm) whereas those of small rodents are typically in excess of 350 bpm. The expression ratio of the N2BA and N2B titin isoforms in humans is also much closer to that of dogs than of rodents (Cazorla et al., 2000).

Permeabilized multicellular preparations were prepared from the frozen sections as described previously (Patel et al., 2001; Fitzsimons et al., 2001). Briefly, small pieces (~0.5 g) of tissue were thawed in 10 ml of ice-cold relaxing solution (in mM: 100 KCl, 20 imidazole, 4 MgATP, 2 EGTA, and 1 free Mg^{2+}) and mechanically disrupted (3 s, 12,500 rpm) using a tissue homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). Multicellular preparations (600–1200 × 100–150 μm) were separated from the homogenate by centrifugation and chemically permeabilized in fresh relaxing solution containing 1% (v/v) Triton X-100 (30 min at room temperature). Once skinning was complete, the preparations were washed three times in relaxing solution and stored on ice for up to 12 h.

The experiments described in this paper were performed using 16 preparations from 5 hearts. Two additional control experiments were performed using multicellular preparations isolated in an identical manner from fresh (i.e., nonfrozen) canine tissue. No systematic experimental differences were apparent between the preparations obtained from the fresh and frozen tissue samples.

SDS vertical agarose gel electrophoresis analysis (Warren et al., 2003) was performed on samples of canine myocardium subjected to a sham experimental procedure (including mechanical disruption, chemical permeabilization with Triton X-100, exposure to solutions with 30-μM free Ca^{2+}, and prolonged storage (>8 h) on ice) similar to that used with the experimental preparations. Densitometry traces showed that ~80% of the protein mass attributed to titin consisted of the intact N2BA and N2B isoforms.

Solutions

pCa (=$-\log_{10}[Ca^{2+}]$) solutions (pH 7.0) contained (in mM) 20 imidazole, 14.5 creatine phosphate, 7 EGTA, 4 MgATP, 1 free Mg^{2+}, free Ca^{2+} ranging from 1 nM (pCa 9.0) to 32 μM (pCa 4.5), and sufficient KCl to adjust the ionic strength to 180 mM. Muscles were immersed for 30 s in a solution with a reduced Ca^{2+} buffering capacity (Campbell and Moss, 2002) immediately before activation to minimize diffusion delays. The final concentrations of each metal, ligand, and metal-ligand complex were calculated using the computer program developed by Fabiato (1988) and the stability constants listed by Godt and Lindley (1982). All experiments were performed at 15°C.

Mechanical measurements

Individual preparations (length 913 ± 53 μm, width 130 ± 12 μm) were connected between a motor (Model 312B, time for 100-μm step ~1 ms, Aurora Scientific, Ontario, Canada) and a force transducer (Model 403, resonant frequency ~600 Hz, Aurora) using a technique described in detail by Campbell and Moss (2002). At the beginning of each experiment, sarcomere length was adjusted to 2.31 ± 0.02 μm in pCa 9.0 solution and the cross-sectional area of the preparation was determined (assuming a circular profile).

In many of the experiments, a laser beam (HeNe, 10 mW, Melles Griot, Irvine, CA) was then projected through the muscle so that the sarcomere length of the preparation could be controlled in real time using a laser diffraction technique (Campbell and Moss, 2002). One of the first-order diffraction lines was imaged onto a lateral effects photodiode detector (Model 1239 diode, Model 301-DIV differential amplifier, bandwidth 5 kHz, UDT Instruments, Baltimore, MD) that produced an output signal proportional to the position of the centroid of the incident light. This arrangement was ideal for the relaxed and submaximally activated preparations used in these experiments where end-compliance effects were minimal and the diffraction line was sharply defined. (The validity of the technique under these conditions is confirmed in Fig. 2 that shows that the muscle was subjected to comparable length changes and exhibited similar mechanical properties when stretched with and without sarcomere length control.) Unfortunately, sarcomere length control could not be successfully implemented at higher levels of Ca^{2+} activation. When the muscles were activated in solutions with pCa values ≤ 5.8 (isometric tension ≥ 30% maximal Ca^{2+} activated tension) stable striation patterns were still evident from video microscopy (Fig. 1 *C*), but regional heterogeneity in the striation pattern resulted in a broader, more diffuse, diffracted beam. Under these circumstances, the width of the diffracted beam was comparable to the expected movement of the beam centroid due to changes in overall muscle length. Consequently, sarcomere length could not be reliably determined from the photodiode detector and sarcomere length control proved unfeasible.

Signals representing force, motor position (equivalent to muscle length), and (where appropriate) sarcomere length were sampled (16-bit resolution, DAP5216a, Microstar Laboratories, Bellevue, WA) at rates ≥ 1.5 kHz and saved to computer files for later analysis. Individual experiments were controlled by software (SLControl) developed in our laboratory and could be performed under fiber length, tension, or (at lower levels of Ca^{2+} activation) sarcomere length control. Experiments utilizing real-time control were implemented using the processing power of the DAP5216a board. In these situations, the motor command voltage was continually adjusted by a feedback algorithm in such a way as to minimize the difference between the controlled value (tension or sarcomere length) and a predefined target waveform stored in on-board RAM. The update rate was determined by the specified sampling frequency but could be set to as high as 5 kHz at which time the overall system response was limited by the resonant frequency of

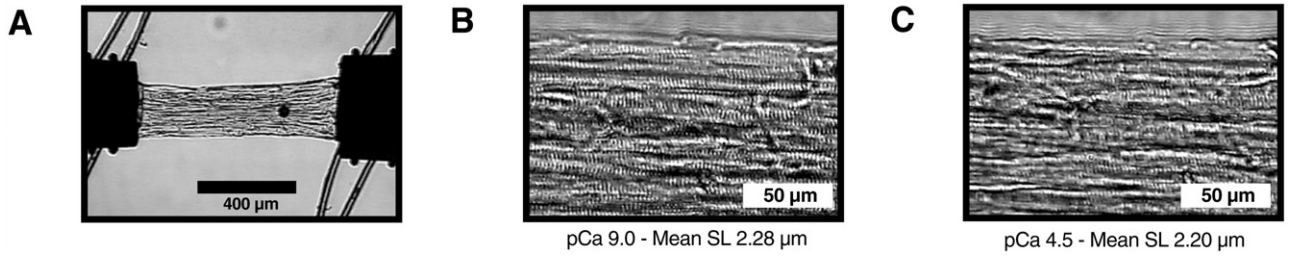

FIGURE 1 (*A*) Myocardial preparations were attached between stainless steel troughs connected to a force transducer (*left*) and a motor (*right*). Translucent filaments running diagonally across the image are single strands of dental floss that help to secure the muscle attachments. See Fig. 1., Campbell and Moss (2002). (*B* and *C*) Stable striation patterns were maintained at all levels of Ca^{2+} activation. SL is an abbreviation of sarcomere length.

the force transducer and the step time of the motor. Further details are available at http://www.slcontrol.com.

Analysis

Short-range stiffness values were calculated from the gradient of regression lines fitted to *x-y* plots of force against length and expressed as Young's Modulus values (Campbell and Moss, 2002). The stiffness values shown in Fig. 5 were calculated from plots of force against muscle length since sarcomere length records were not normally available at the highest levels of Ca^{2+} activation. All other stiffness measurements were performed using sarcomere length data. Experimental results are reported as mean ± SE.

RESULTS

Fig. 2 compares the mechanical properties of a relaxed and a submaximally activated multicellular myocardial preparation. When the muscle was immersed in pCa 9.0 solution (negligible free Ca^{2+} concentration) it behaved like a simple elastic system, i.e., tension changed proportionately with length. In contrast, the contracting muscle exhibited a clear short-range stiffness (Rack and Westbury, 1974), i.e., tension rose more quickly during the first ~15-nm half-sarcomere^{-1} of the movement (an increase in sarcomere length from 2.31 to 2.34 μm) than thereafter. Note that the short-range response was evident whether or not the contracting muscle was stretched under sarcomere length control. It is thus an inherent feature of activated striated muscle and cannot be attributed to end-compliance effects.

The simplest way of explaining these records is to assume that the tension response measured in pCa 9.0 solution represents the passive elastic component (titin and collagen filaments, etc.). The response of the muscle in pCa 6.0 solution (submaximal activation) is then the sum of the passive response and an additional contribution from cycling cross-bridges. According to this theory the short-range response represents the increased strain in cross-bridge links that are stretched when the muscle is extended. If the movement persists, highly strained cross-bridges detach rapidly from the thin filament and force drops transiently before settling at a maintained plateau once the cross-bridges have adopted new steady-state distributions characteristic of the interfilamentary velocity (Campbell and Moss, 2002).

An obvious prediction of this theory is that the muscle's initial stiffness should increase with the level of Ca^{2+} activation. In Fig. 3, the short-range response became more prominent as the free intracellular Ca^{2+} concentration was progressively raised and was so large in saturating pCa 4.5 solution that it completely dominated the muscle's passive properties.

The idea that the measured tension response represents the sum of contributions from the passive elastic component and from a variable number of cycling cross-bridges gains further support from the experiment illustrated in Fig. 4. When the muscle was immersed in pCa 6.0 solution the tension response to an imposed stretch could be reduced to very near

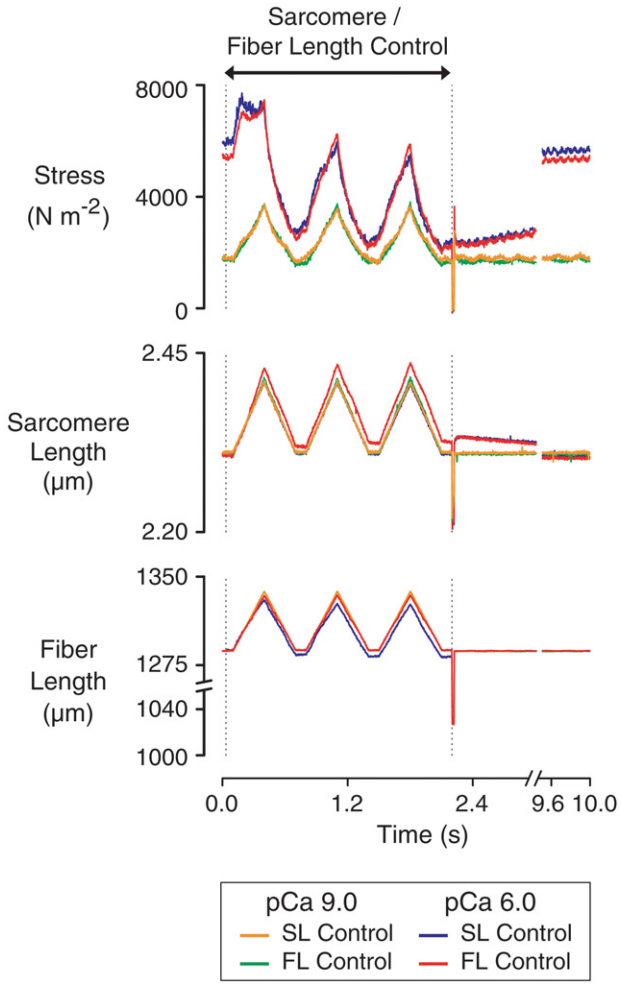

FIGURE 2 Length change 0.04 l_0, velocity ± 0.12 l_0 s^{-1}, intertriangle interval 0.1 s. Isometric tension in pCa 6.0 solution is ~0.06 of the maximal Ca^{2+} activated tension and recovers to steady state within ~7 s of the end of the perturbation. Note the break in each time axis between 3.0 and 9.5 s. The sarcomere length signal is invalid during the k_{tr} step (0.2 l_0, 20-ms duration) because the imposed length change is sufficiently large to move the diffracted laser beam off the position sensitive detector. In all subsequent figures showing sarcomere length traces, sarcomere and fiber length records are clipped to the graph axes during the k_{tr} step so that the quality of the length control can be more readily assessed. Records indicated as "SL Control" were imposed under sarcomere length control during the specified period; those labeled as "FL Control" under fiber length control. Sarcomere length control emphasizes the initial phase of the tension response but does not change the basic features.

that measured in pCa 9.0 solution by the addition of 5-mM 2,3-butanedione monoxime (BDM). BDM is a known cross-bridge inhibitor (Bagni et al., 1992), and the present results can be explained if a 5-mM concentration is sufficient to prevent cross-bridges from binding between the myofilaments. Under these conditions, no cross-bridges would be able to generate force or contribute to the stretch response and the muscle's mechanical properties would reflect solely the underlying passive elastic component.

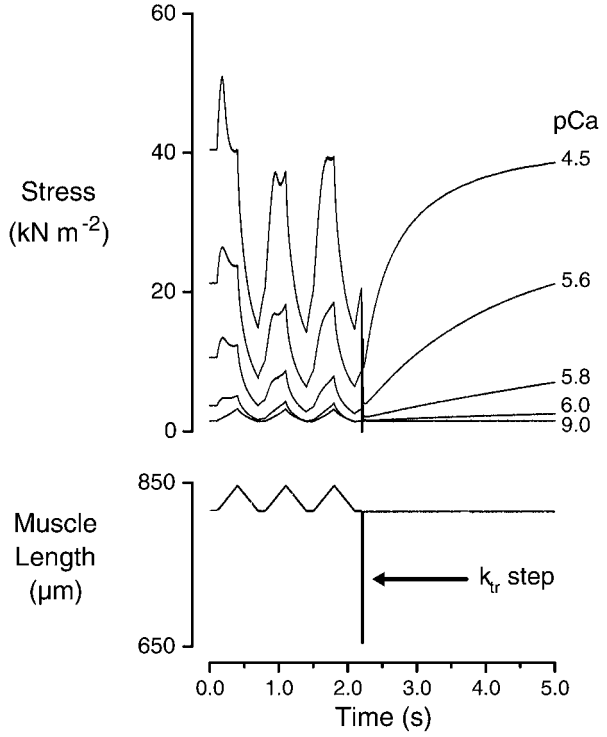

FIGURE 3 Length change 0.04 l_0, velocity ± 0.12 l_0 s^{-1}, intertriangle interval 0.1 s.

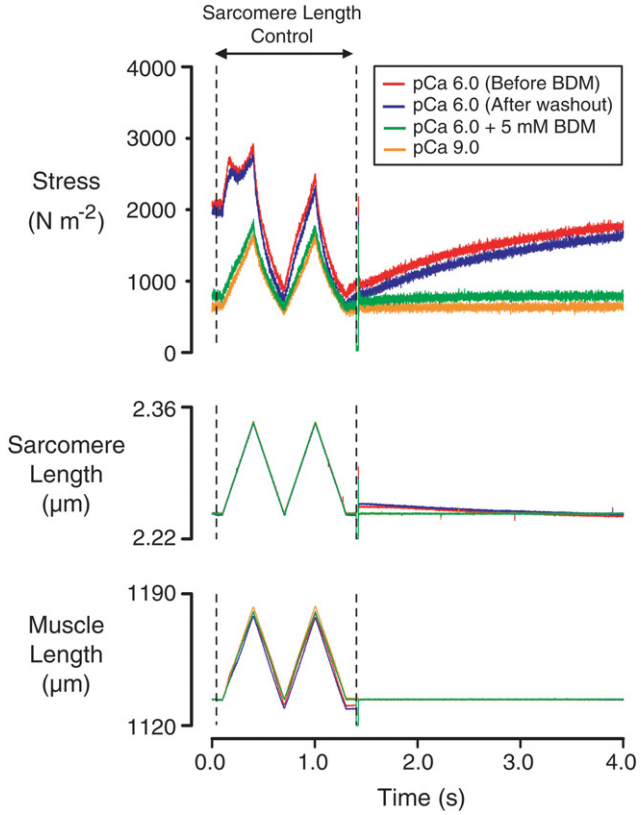

FIGURE 4 Length change 0.04 l_0, velocity ± 0.12 l_0 s^{-1}, intertriangle interval 0.001 s. Isometric tension in pCa 6.0 solution (without BDM) is ~0.08 of maximal Ca^{2+} activated tension.

Magnitude of the parallel elastic component

Fig. 5 illustrates a method that can be used to partition the initial stiffness of a contracting muscle into passive elastic and actively generated cross-bridge components. Muscle stiffness is plotted against Ca^{2+} activated tension and a regression line is fitted to the experimental data. The *y* intercept of this line corresponds to the initial stiffness of a muscle generating zero active force (i.e., with no cycling cross-bridges) and is therefore equal to the stiffness of the passive component. The resulting value (30 ± 14 kN m^{-2}, Young's Modulus) is small compared with the stiffness of contracting muscles in the present experiments. When the canine myocardial preparations were maximally activated in pCa 4.5 solution they developed an isometric tension of 40 ± 3 kN m^{-2}. Their initial stiffness under these conditions (1.58 ± 0.14 MN m^{-2}) was ~50 times greater than that attributed to the passive component.

History-dependence

The stiffness of the short-range response in contracting preparations depended on the muscle's history of movement. When two or more identical length changes were imposed the initial stiffness of the second and subsequent responses was always reduced when the movements were separated by intervals of less than a few seconds (Figs. 2–4). Fig. 6 presents an analysis of this thixotropic behavior (Campbell and Lakie, 1998; Campbell and Moss, 2000, 2002) at low levels of Ca^{2+} activation where sarcomere length control could be used to minimize any confounding effects from series compliance in the muscle attachments.

Myocardial preparations were subjected to paired triangular movements separated by intervals that ranged from 1 ms to 10 s in different trials. Relative stiffness was reduced by ~50% immediately after the first length change and recovered toward unity (i.e., equal first and second stretch stiffness values) at a rate of 0.21 s^{-1} thereafter. As in the case of submaximally activated skeletal muscle fibers (Campbell and Moss, 2002), this rate was similar to the rate of tension recovery after a quick length step (0.28 ± 0.02 s^{-1} measured in the same activations) in agreement with the suggestion that the history-dependence of the short-range response represents a temporary reduction in the number of attached cross-bridges.

Velocity-dependence

Fig. 7 *A* shows experimental records for a submaximally activated preparation subjected to a series of stretches with an ~20-fold range of velocities. Both the maximal force produced during the short-range response and the force at the extreme stretch length increased with the velocity of interfilamentary movement.

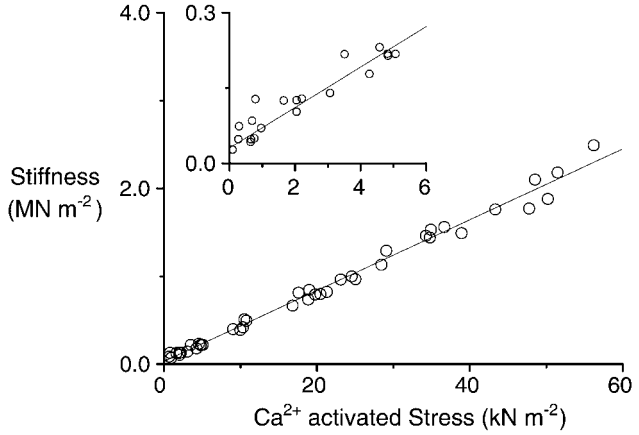

FIGURE 5 Short-range stiffness (expressed as Young's Modulus) plotted against Ca^{2+} activated stress (defined as the difference between the isometric tension of the contracting fiber and the corresponding tension measured in pCa 9.0 solution). Collated data from 13 preparations. The inset is an enlargement of the *y* axis intercept. The mean value of muscle stiffness measured directly in pCa 9.0 solution (46 ± 6 MN m^{-2}) was not significantly different (*t*-test, $p > 0.05$) from the *y* axis intercept determined by linear regression (30 ± 14 MN m^{-2}). Additional experiments (not shown) using six different preparations measured the tension response of myocardial preparations immersed in solutions with pCa values of 6.4, 6.6, 6.8, and 7.0. None of the resulting stiffness values were statistically different (*t*-test, $p > 0.05$) from the corresponding values measured in pCa 9.0 solution.

Many previous reports (Noble, 1977; Chiu et al., 1982; de Tombe and ter Keurs, 1992; Stuyvers et al., 1997, 1998) have suggested that relaxed myocardium presents a viscous resistance to movement and at first thought the records presented in Fig. 7 are reminiscent of this type of behavior. However, closer examination shows that it is unlikely that the short-range responses measured in the present work can be attributed to a simple viscosity arranged in parallel with the passive elastic component.

Since viscous elements produce a force directly proportional to their lengthening velocity, the tension responses shown in Fig. 7 can only be explained if the viscous element accelerates continuously throughout the initial stage of the response and then lengthens at a constant speed during the latter stages of the movement (Bagni et al., 1995). This does not seem to occur in the present experiments. Not only were the preparations stretched under sarcomere length control but also there was no discernible change in the velocity of sarcomere lengthening at the end of the initial phase. A second argument against a pure viscosity concerns the relationship between muscle force and sarcomere length during the movement. If the muscles exhibited significant viscous properties, the apparent stiffness of the muscle (i.e., the gradient of the initial phase of each response in Fig. 7 *B*) would increase with stretch velocity. In fact, the initial phases superposed.

Tension control

Ventricular walls in the intact heart are obviously not subjected to controlled length changes. Instead the chambers enlarge during diastole to accommodate blood entering through the atrio-ventricular valves. The analogy is not exact, but for an isolated myocardial preparation this is

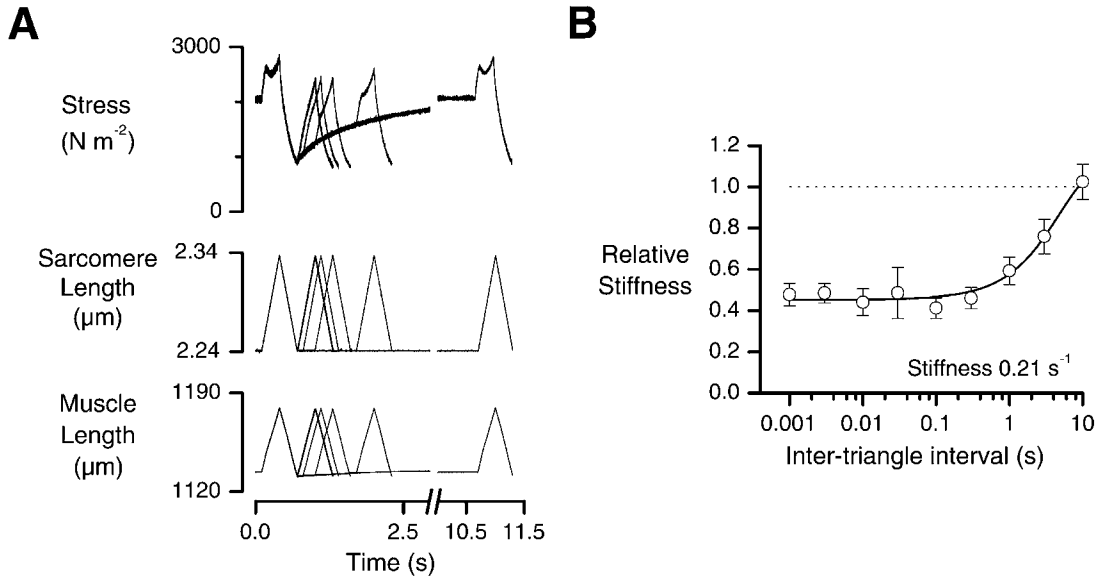

FIGURE 6 (*A*) Length change 0.04 l_0, velocity ± 0.12 l_0 s^{-1}. pCa 6.0. Isometric tension is ~0.08 of maximal Ca^{2+} activated tension. Records show superposed tension, sarcomere, and muscle length records for trials with intertriangle intervals ranging from 0.001 to 10 s. Responses separated by 10 s are indistinguishable. (*B*) Symbols show the mean (± SE) relative stiffness for each intertriangle interval from 5 preparations in pCa 6.0 or pCa 6.1 solution. Isometric tension was between 3% and 10% of the maximal Ca^{2+} activated force under these conditions. Muscle stiffness was calculated from the gradient of a straight line fitted to the first 50 ms of an *x*-*y* plot of tension against sarcomere length. Relative stiffness was defined as the second stretch stiffness divided by the corresponding first stretch value. Solid line is a best fit of the form $y = [a - b \times \exp(-c \times \Delta t)]$ with $c = 0.21$ s^{-1}.

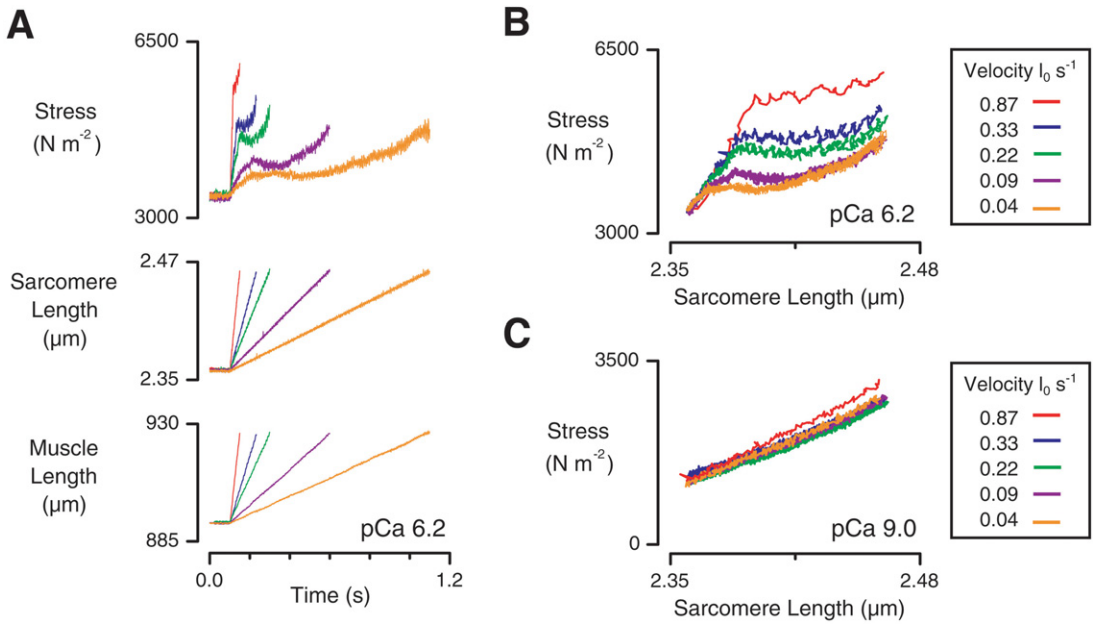

FIGURE 7 (*A*) Length change 0.04 l_0. pCa 6.2. Isometric tension is ~0.03 of maximal Ca^{2+} activated tension. (*B*) *x-y* plots of tension against sarcomere length for the experimental records shown in panel *A*. (*C*) *x-y* plots for the same preparation stretched at the same velocities in pCa 9.0 solution.

probably more akin to lengthening in response to a steadily increasing load. Fig. 8 shows that contracting myocardial preparations also exhibit a short-range stiffness in this type of protocol.

An *x-y* plot of force against sarcomere length (Fig. 8 *B*) shows two distinct regions where the muscle's apparent stiffness is high. The first region, at the very beginning of the movement, corresponds to the short-range response measured under length control where cross-bridges stiffen the sarcomere. The second region of high stiffness occurs near the end of the movement and probably reflects nonlinearities in the passive elastic elements. These structures were stretched considerably further in this experiment (0.09 l_0) than in most of the length control experiments (0.04 l_0). Since they behave as stiffening elastic elements (providing ever-increasing resistance the further they are stretched (Granzier and Irving, 1995)), they would curtail further extension at long sarcomere lengths. A similar region of high stiffness was observed at long sarcomere lengths in muscles stretched under tension control in pCa 9.0 solution (Fig. 8 *C*) though under these conditions the short-range response was absent.

DISCUSSION

The experimental results presented in this paper lend strong support to the view that the short-range tension response of contracting myocardium represents the displacement of populations of cycling cross-bridges. Not only is the response reversibly reduced in the presence of BDM (Fig. 4) but also the muscle's initial stiffness scales linearly with isometric force (Fig. 5). In itself, this is not particularly surprising; the stiffness of contracting muscle is well known to scale (at least to a first approximation) with the number of bound cross-bridges (Ford et al., 1981). What is so intriguing about the present results is that the cross-bridge component of myocardial stiffness was comparable to the passive elastic stiffness at very low levels of Ca^{2+} activation.

This finding is important because it implies that ventricular filling might be influenced by cross-bridge activity during the diastolic phase of the cardiac cycle. The myocardium's basal level of stiffness (i.e., the stiffness of the parallel elastic component) would be doubled if just 2% of the cross-bridges bound during a maximal contraction remained attached during diastole (Fig. 5). Smaller increments to passive tension would require correspondingly fewer cross-bridges.

It should also be noted that relatively small changes in chamber stiffness due to diastolic cross-bridge activity probably have a disproportionately large effect on ventricular function. This is because cross-bridges provide their greatest resistance to movement during the initial stages of an imposed stretch and will therefore exert their greatest influence when the tricuspid and mitral valves first open and the ventricles start to distend. Since this phase of the cardiac cycle normally accounts for ~85% of ventricular filling (Brutsaert and Sys, 1997), it seems likely that attached cross-bridges could have a particularly significant effect.

This hypothesis could be tested directly in experiments using intact hearts rather than the permeabilized preparations used in the present work. Such measurements could also investigate the precise Ca^{2+} threshold of contractile activity in a rhythmically beating heart. We acknowledge that the intracellular Ca^{2+} concentration required for significant contractile force in the present experiments (~2% maximal

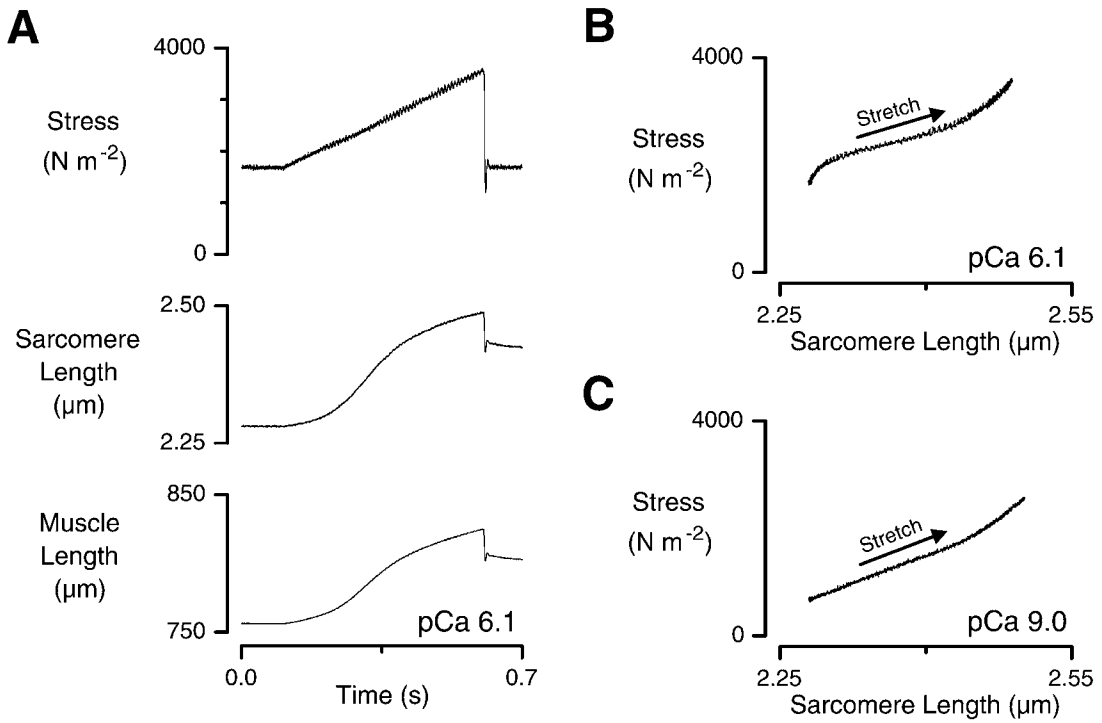

FIGURE 8 (*A*) Stress change 2000 N m^{-2}, velocity 4000 N m^{-2} s^{-1}. pCa 6.1. Isometric tension is ~0.04 of the maximal Ca^{2+} activated tension. (*B*) *x-y* plot of tension against sarcomere length for the lengthening phase of the movement. (*C*) *x-y* plot for the same preparation stretched under tension control in pCa 9.0 solution.

activation, pCa 6.2, 630 nM) was higher than the basal concentration of 75–200 nM normally assumed in working hearts (see p. 35 in Bers, 1991). However, we would contend that this does not detract from the significance of our observations. The most important finding of this work is not the precise intracellular Ca^{2+} concentration at which myocardial stiffness was significantly increased but rather the relative level of activation at which this occurred.

The precise threshold for Ca^{2+} activation in a working heart may be very different from that of the isolated permeabilized preparations used in the present experiments. The tension-pCa relationship for instance is significantly left-shifted in intact muscle compared to that measured in permeabilized preparations (Gao et al., 1994). It probably also depends on the prevailing temperature (37°C in a working heart vs. 15°C in the present experiments) and the phosphorylation status of regulatory proteins. Such effects might well underlie quite large differences in the precise Ca^{2+} concentration at which cross-bridges started to influence ventricular stiffness. However, these effects would not alter the finding that ventricular stiffness is significantly increased at very low levels of Ca^{2+} activation unless they simultaneously changed the relationship between stiffness and developed force for single cross-bridge links.

Although these arguments have potentially far-reaching implications, it is important to recognize some of the limitations of the present work. Perhaps one of the most important concerns the choice of experimental tissue. The multicellular preparations used in these experiments were chosen because they could be isolated directly from samples of left ventricular myocardium. The main advantage of this is that their mechanical properties may be more representative of ventricular wall dynamics than those of atrial preparations or trabeculae. One of the disadvantages is that the multicellular preparations may be more susceptible to mechanical damage during prolonged experiments at high levels of Ca^{2+} activation than some other types of tissue.

Evidence for this view comes from Fig. 3. At low levels of Ca^{2+} activation (pCa $\geq$ 5.8), the tension responses to the second and third stretches were almost identical whereas the second and third responses measured in solutions with pCa values of 5.6 and 4.5 were significantly different. Similar experiments performed in skeletal muscle fibers and the underlying theory (Campbell and Moss, 2000) suggest that the second and third stretch responses at a given level of Ca^{2+} activation should be identical irrespective of the pCa value of the bathing solution. The most obvious explanation for this discrepancy is that regional heterogeneity developed in the cardiac preparations at the higher levels of Ca^{2+} activation.

Significant sarcomere inhomogenities were not apparent at the low levels of Ca^{2+} activation at which the vast majority of experiments were performed. Under these conditions, the preparations produced repeatable, consistent results over multiple trials. The experimental data shown in Fig. 6 *B* for example was collated from a minimum of 27 trials conducted under sarcomere length control in each of 5 different

preparations. No systematic trends in the experimental data were evident during the course of these prolonged experiments.

Another point worthy of discussion is the sarcomere length (2.31 ± 0.02 μm) at which these experiments were performed. The mean value, although shorter than that used in many other experiments using permeabilized cardiac preparations, e.g., Fitzsimons et al. (2001), is slightly longer than that likely to be encountered in the intact heart under normal physiological conditions. This is certainly not ideal but proved necessary in the present experiments for reliable sarcomere length control. If the resting sarcomere length was reduced considerably below this level, the laser diffraction pattern was not sufficiently distinct for adequate control. The advantage of minimizing the effects of potential series compliance in the measurements at low levels of Ca^{2+} activation was judged to outweigh the disadvantage of performing the experiments at a slightly extended length.

It also seems unlikely that experiments performed at shorter sarcomere lengths would have led to significantly different conclusions. The most important finding of this work is that the Ca^{2+} dependent portion of myocardial stiffness was equal to the passive elastic stiffness at just 2% of maximal Ca^{2+} activation, a value that is much lower than commonly assumed. If the experiments were performed at shorter sarcomere lengths (where the passive elastic component is less stiff (Granzier and Irving, 1995)), the Ca^{2+} dependent component would probably have been equal to the passive component at an even lower level of Ca^{2+} activation.

It should be noted that other workers have concluded that cross-bridges do not influence diastolic mechanical properties. De Tombe and ter Keurs (1992) and Stuyvers et al. (1997, 1998) used rapid length changes and sinusoidal oscillations to probe the visco-elastic properties of rat trabeculae. They concluded that the muscles exhibited a significant viscosity during diastole and attributed the effect to an interaction between the thin filament and titin. More recent studies suggest that this interaction may involve the PEVK region of the titin molecule (Kulke et al., 2001; Yamasaki et al., 2001).

This interpretation is very different from that proposed here. Fig. 7 *C* shows that in the present experiments, myocardial preparations immersed in pCa 9.0 solution did not exhibit significant velocity-dependent mechanical properties. Moreover the velocity dependent effects measured in Ca^{2+} activating solutions do not seem to be due to a simple viscosity. Instead, the larger short-range forces produced by fast stretches seem to reflect velocity dependence of the elastic limit, i.e., fast stretches produce a larger short-range force than slow stretches by stretching a constant stiffness spring further. A similar theory has already been proposed to explain the limited velocity dependence of the Short Range Elastic Component (SREC) of relaxed intact frog skeletal muscles (Hill, 1968; Campbell and Lakie, 1998).

Supporting evidence comes from the *x-y* plots of force against sarcomere length shown in Fig. 7 *B*. As already mentioned, the initial phases of the *x-y* plots for different stretch velocities superpose, indicating that the muscles have the same initial stiffness. The muscle's elastic limit is indicated by the magnitude of the length change at the transition between the first and second phases of the response and there seems little doubt that it is increased at the fastest stretch velocities. This behavior is consistent with a cross-bridge mechanism if fast stretches displace cross-bridges further before they detach than slow stretches (as would be the case if the cross-bridge detachment rate was only affected to a minor degree by cross-bridge strain).

Perhaps the apparent discrepancy between these measurements and those of de Tombe and ter Keurs (1992) and Stuyvers et al. (1997, 1998) reflects important differences between the experimental conditions. De Tombe and ter Keurs and Stuyvers et al. performed their experiments at higher temperatures (25°C vs. 15°C in the present experiments) and used much faster length changes ($\sim$1 l_0 s^{-1} or greater). Since the magnitude of a viscous response increases proportionately with stretch velocity, it is possible that the viscous effects measured in their work would have been vanishingly small in the present experiments that generally used stretch velocities of order 0.1 l_0 s^{-1}.

More importantly, de Tombe and ter Keurs and Stuyvers et al. performed their experiments using rat trabeculae whereas the present experiments used myocardial preparations obtained from canine left ventricles. When we performed pilot experiments using rat trabeculae, the relative contribution of the passive elastic component was much greater than that observed in the present experiments. The rat trabeculae still exhibited a short-range response that increased with stretch velocity and was temporarily reduced by movement but it was much less prominent at low levels of Ca^{2+} activation than in the canine preparations.

It is difficult to judge whether this represents a difference between trabeculae and ventricular wall preparations or a difference between rat and dog hearts. The disparity could certainly be explained if trabeculae have a greater extracellular collagen component than normal myocytes. Alternatively, the difference could reflect different levels of titin isoform expression in the two species. Dogs seem to express roughly similar amounts of the N2BA and the N2B titin isoforms whereas rats express almost exclusively the shorter (stiffer) N2B isoform (Cazorla et al., 2000).

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