Alterations of Myocardial Dynamic Stiffness Implicating Abnormal Crossbridge Function in Human Mitral Regurgitation Heart Failure


Abstract—Mitral regurgitation (MR) causes ventricular dilation, a blunted myocardial force-frequency relation, and increased crossbridge force-time integral (FTI). The mechanism of FTI increase was investigated using sinusoidal length perturbation analysis to compare crossbridge function in skinned left ventricular (LV) epicardial muscle strips from 5 MR and 5 nonfailing (NF) control hearts. Myocardial dynamic stiffness was modeled as 3 parallel viscoelastic processes. Two processes characterize intermediate crossbridge cycle transitions, B (work producing) and C (work absorbing) with Q_{10} of 4 to 5. No significant differences in moduli or kinetic constants of these processes were observed between MR and NF. The third process, A, characterizes a nonenzymatic (Q_{10}=0.9) work-absorbing viscoelasticity, whose modulus increases sigmoidally with [Ca^{++}]. Effects of temperature, crossbridge inhibition, or variation in [MgATP] support associating the calcium-dependent portion of A with the structural “backbone” of the myosin crossbridge. Extension of the conventional sinusoidal length perturbation analysis allowed using the A modulus to index the lifetime of the prerigor, AMADP crossbridge. This index was 75% greater in MR than in NF (P=0.02), suggesting a mechanism for the previously observed increase in crossbridge FTI. Notably, the A-process modulus was inversely correlated (r^{2}=0.84, P=0.03) with in vivo LV ejection fraction in MR patients. The longer prerigor dwell time in MR may be clinically relevant not only for its potential role as a compensatory mechanism (increased economy of tension maintenance and increased resistance to ventricular dilation) but also for a potentially deleterious effect (reduced elastance and ejection fraction). (Circ Res. 2002;90:66-72.)

Key Words: mitral regurgitation ■ heart failure ■ myocardial stiffness ■ crossbridge function ■ prerigor dwell time

Heart failure in mitral regurgitation (MR) is accompanied by impaired ventricular function and altered myocardial function involving defects in excitation-contraction coupling\(^2\) and in crossbridge function. The latter is evidenced by a 50% lower myofibrillar Ca\(^{++}\)-activated myofibrillar ATPase\(^1,4\) and an 85% increased crossbridge force-time integral (FTI).\(^5\) Reduced ATPase suggests slowing of the rate-limiting prerigor step of the crossbridge cycle (ie, ADP release from AMADP\(^6\)) or slowing of an earlier step (eg, the intermediate phosphate-release step from AMADPP\(^6\)). Consequently, we first assessed crossbridge intermediate reaction kinetics using sinusoidal length perturbation analysis in skinned MR and NF myocardium. Because preliminary experiments showed no alterations in these kinetics, the present study repeated the sinusoidal analyses in preparations that were conditioned by regular twitch activity immediately before skinning. This better complies with conditions present in our previous FTI measurements\(^8\) because phosphorylation levels within previously stimulated skinned strips better comply with intracellular phosphorylation levels in our previous study on excitable strips. These results also did not reveal obvious shifts in intermediate crossbridge reaction rates. However, by extending our methodology to include detection of the putative AMADP prerigor state of the crossbridge cycle, we provide evidence that increased dwell time in this state may contribute to increased crossbridge FTI in MR.

Materials and Methods

Patient Selection and Biopsy Procedure
Subepicardial myocardium was obtained from 4 male and 1 female patients with mitral regurgitation and New York Heart Association Class II-III heart failure symptoms, aged 66.8±7.6 years. Control subepicardial myocardium (NF) was obtained from 2 male and 3 female nonfailing coronary artery bypass patients, aged 61.6±7 years, with normal left ventricular contraction patterns. The mean ejection fraction in MR (0.67±0.03) was not significantly different than in the control group (0.69±0.03, P=0.8). Medications are summarized in the online data supplement, available at http://www.circresaha.org. The committee on Human Research of the University of Vermont approved the study. Patients gave informed, written consent before participating. The anterior segment of the left

Original received April 26, 2001; revision received November 15, 2001; accepted November 26, 2001.
From the Departments of Molecular Physiology & Biophysics (L.A.M., W.B., N.R.A., D.W.M.) and Surgery (B.J.L., F.P.I.) and the Cardiology Unit (M.M.L.W.), University of Vermont, Burlington Vt.
Correspondence to Louis A. Mulieri, PhD, Dept of Molecular Physiology and Biophysics, Given Building, University of Vermont, Burlington, VT 05405-0068. E-mail mulieri@physiology.med.uvm.edu
© 2002 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org
ventricular free wall was biopsied shortly after cardioplegic arrest. No complications resulted from the biopsy procedure.

**Muscle Strip Preparation**

Biopsies were submerged in butanedione monoxide (BDM) Krebs-Ringer solution and subsequently dissected into two to four thin strips from each heart. Half of the strips from each heart were skinned before skinning (nonconditioned strips). For details, see the Muscle Strip Preparation section in the online data supplement, available at http://www.circresaha.org.

### Apparatus

Skinned strips were suspended between a force gauge and piezoelectric motor in a muscle bath maintained at 27 ± 0.1°C or 35 ± 0.1°C. Length (eddy current transducer) and force (semiconductor strain gauge) signals were monitored by a strip chart recorder and oscilloscope and acquired by an A/D converter using custom software for data processing and presentation. Strips were activated by incremental increase in [Ca²⁺] by exchanging equal volumes of relaxing solution for pCa 4.5 activating solution to attain pCa values of 8, 7, 6, 5.75, 5.5, and 5. (See the online data supplement, available at http://www.circresaha.org, for solution details, and see Force Measurements and Sinusoidal Analysis section).

### Sinusoidal Analysis

Small-amplitude (0.25% strip length, peak-to-peak) sinusoidal length perturbation (42 frequencies between 0.125 to 100 Hz) was used to probe strip viscoelastic properties related to crossbridge kinetics and passive viscoelasticity. Force and length changes were divided by cross-sectional area and reference length of strips, respectively, to obtain stress and strain values. We modeled the frequency-dependent dynamic modulus Y(f) (“dynamic stiffness” in normalized form) as 3 viscoelastic components in parallel (Equation 1). The first component with modulus A represents a nonenzymatic process; the second and third components with moduli B and C represent enzymatic process-B and process-C, respectively. Where

\[ Y(f) = A(i2\pi f/\alpha) + B(i/f(1+i/\gamma)) + C(i/f(1+i/\gamma)) \]

where \( i = \sqrt{-1} \) and \( \alpha = 1 \) Hz. A, B, and C are coefficients (in Nm⁻²) that characterize the modulus of each component, and \( b \) and \( c \) are characteristic frequencies (Hz) of the enzymatic processes B and C, respectively. The dip frequency \( f_d \) is a universal exponent proportional to the phase shift, \( \phi \), between length change and the component of force generated by the A-process (\( \phi = k(2\pi f) \)). Components with moduli B and C are representative of enzymatic processes because their characteristic frequencies \( b \) and \( c \) have high Q₁₀ values (Table). They are conventionally attributed to dynamic processes of the crossbridges.

We previously considered the term \( A(i2\pi f/\alpha) \) to represent a fixed, passive viscoelastic property in cardiac strips. This term is now expanded to include both fixed (\( A_{\infty} \)) and activation-dependent (\( A_{\infty} \)) viscoelastic portions. The A-process is present in identical form in the relaxed, active, and rigor states, with A differing only in magnitude according to changes in \( A_{\infty} \). As shown later, \( A_{\infty} \) bears a constant relation to crossbridge activity as gauged by constancy of the ratio of \( A/B \) moduli as degree of activation is varied.

### Results

#### Myocardial Dynamic Stiffness Characteristics

The frequency dependence of total dynamic stiffness and the phase angle between force change and length change in NF are shown for an individual myocardial strip preparation in Figure 1. The average for all strips is shown in Figure 2 (left). The solid curves are fits of Equation 1 to the data points. Individual contributions to the total dynamic stiffness from the 3 viscoelastic components in Equation 1 are shown in Figure 2 (right). The negative sign of the B modulus accounts for the prominent “dip” in the total dynamic stiffness curves of Figure 1. The magnitude of the dip and the position of its nadir (“dip frequency”) are controlled by the relative magnitudes of the oppositely directed B and C moduli and by the difference in their characteristic frequencies (\( b \) and \( c \)). The A-process component of Equation 1, \( A(i2\pi f/\alpha) \), adds a linearly rising viscosity and constant phase angle to the total

---

*Parentheses enclose \( Q_{10} \) values obtained between 27°C and 35°C.*

# Tension and Stiffness Parameters*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonconditioned Myocardium</th>
<th>Conditioned Myocardium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension, mN/mm²</td>
<td>NF (9 strips)</td>
<td>MR (10 strips)</td>
</tr>
<tr>
<td>18.9±4.0</td>
<td>11.5±5.0</td>
<td>0.18</td>
</tr>
<tr>
<td>[Ca⁺³]o, μmol/L</td>
<td>1.7±0.15</td>
<td>1.4±0.14</td>
</tr>
<tr>
<td>Hill n₀</td>
<td>4.5±1.2</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td>1-Hz stiffness</td>
<td>242±25</td>
<td>198±12</td>
</tr>
<tr>
<td>100-Hz stiffness</td>
<td>608±64</td>
<td>534±63</td>
</tr>
<tr>
<td>Dip frequency, Hz</td>
<td>4.3±0.17</td>
<td>4.4±0.17</td>
</tr>
<tr>
<td>A, kN/m²</td>
<td>220±32 (0.9)</td>
<td>250±64 (0.9)</td>
</tr>
<tr>
<td>k</td>
<td>0.09±0.01 (1.0)</td>
<td>0.10±0.14 (0.9)</td>
</tr>
<tr>
<td>B, kN/m²</td>
<td>−376±76 (1.3)</td>
<td>−573±140 (1.2)</td>
</tr>
<tr>
<td>h, Hz</td>
<td>3.97±0.13 (4.4)</td>
<td>4.31±0.13 (4.7)</td>
</tr>
<tr>
<td>C, kN/m²</td>
<td>525±117 (1.5)</td>
<td>811±221 (1.3)</td>
</tr>
<tr>
<td>c, Hz</td>
<td>11.2±0.36 (3.4)</td>
<td>11.5±0.63 (3.9)</td>
</tr>
</tbody>
</table>
dynamic stiffness, accommodating its upward tilt as frequency increases (Figure 1).

The A-process term was required to adequately fit the total dynamic stiffness data independent of whether resting, activated, or permanent rigor conditions prevailed (Figure 3). This is a key observation on which our new analysis was based. The omnipresence of the A-process supports our previous choice to associate it with parallel passive viscoelastic components, such as the extracellular collagen/elastin complex and intracellular titin. However, as shown qualitatively in Figure 3 and quantitatively in Figure 4, coefficient A increases with increased calcium concentration or rigor and this requires including other myofilibrillar structures as contributors to process A.

To determine whether increases in A involve enzymatic or passive mechanisms, we measured the $Q_{10}$ of $k$ and $A$ coefficients during crossbridge cycling and during rigor. The $Q_{10}$ (27°C to 35°C) of $k$ is 0.95±0.01 (n=35) during calcium activation and 0.93±0.001 (n=28; $P=NS$) during rigor. The corresponding $Q_{10}$ values for A are 0.83±0.07 and 1.02±0.001 ($P=NS$). The similarity of $Q_{10}$ values for calcium activation and rigor supports a common origin, while their near-unity values suggest a nonenzymatic process unlike the enzymatic crossbridge cycling processes B and C ($Q_{10}$ of b and c=3.4 to 5.2, Table). $Q_{10}$ near unity are consistent with associating the A-process with a passive structural phenomenon such as polymer viscoelasticity.

Including our finding that A and B moduli increase proportionately as calcium concentration is raised (Figures 3 and 4 and Equation 2), we hypothesize that the calcium-dependent portion of process-A ($A_{Ca}$) reflects the viscoelastic properties of the passive portions of the crossbridge “backbone” (passive portions of S1 and S2). We neglected any possible viscoelastic compliance of the thin filaments because decreased I-band length does not increase the characteristic frequency of the B-process. The large increase in A mod-

**Figure 1.** Total dynamic stiffness and phase angle of a nonconditioned NF strip preparation during steady-state maximal contracture. Values (○) were obtained at sinusoidal length perturbation frequencies of 0.125 to 100 Hz (amplitude L=0.00125 L2.1). Solid lines drawn according to least-squares fit of Equation 1.

**Figure 2.** Left, Average total dynamic stiffness and phase angle for all nonconditioned NF strips (mean±SEM, 9 strips, 5 hearts). Solid lines drawn according to Equation 1 using the mean-of-fit parameter values from all strips as given in the Table. Fit discrepancies arise from between-strip differences in ratios of parameter values rather than inadequacy of fitting regimen. Right, Calculated average dynamic stiffness of individual processes A, B, and C. Equation 1 was used to calculate average stiffness moduli and phase angles of the 3 viscoelastic model components. Stiffness and phase at each frequency represent the mean±SEM of the least-squares fit to each individual nonconditioned NF strip. The B curve was assigned a negative sign to facilitate plotting.

**Figure 3.** Effects of calcium concentration or rigor on A- and B-process moduli. A-process stiffness (thick solid lines) retains the same frequency dependence as $[Ca^{2+}]$ increases from pCa 8 to pCa 5 while its A modulus doubles. The A modulus at pCa 8 is the noncalcium-dependent portion of the A-process ($A_{nonCa}$). The rise in A with $[Ca^{2+}]$ is the calcium-dependent portion of the A-process ($A_{Ca}$). B-process modulus is essentially zero at pCa 8 and rises to its maximal value at pCa 5. During rigor, A rises 6-fold above the pCa 8 value while retaining a similar frequency dependence. The noncontractile epicardium strip (dots) exhibits a typical $A_{nonCa}$-like stiffness but is totally insensitive to pCa or rigor conditions. Solid lines indicate same NF strip and experimental conditions as in Figure 1.
ulus accompanying development of rigor (ATP withdrawal, Figure 3) is expected since all crossbridges entering the rigor state cease cycling and remain connected to actin (ie, the B modulus approaches zero).

As a test of this hypothesis, we selectively suppressed crossbridge cycling during full activation with the crossbridge inhibitor BDM15 or by actin filament depolymerization with gelsolin16 (Figure 5). These treatments leave only a calcium-independent portion of the A-process \((A_{\text{Ca}})\) that is equal to the pretreatment, pCa 8, value of A. These observations support the proposed crossbridge origin of the calcium dependent \((A_{\text{Ca}})\) portion of the A modulus.

To explore the basis for the proportional increases in \(A_{\text{Ca}}\) and B with rising activation, a subset of NF myocardial strip preparations were used to evaluate the effect of [MgATP] on the slope \((dA/db)\) of the A versus B relation at a constant pCa 5. The slope of the A versus B relation increases as [MgATP] declines (Figure 6). The least-squares regression line for these data are given by Equation 2:

\[
dA/db = (0.83 \pm 0.08) + (0.90 \pm 0.07)/[\text{MgATP}],
\]

\(r^2 = 0.98, P = 0.001.\)

A rise in the slope of the A versus B relation as [MgATP] declines is consistent with transfer of crossbridges from a population undergoing the B-process to a population characterized by the \(A_{\text{Ca}}\)-process, since the prerigor AMADP and rigor AM states are favored at low [MgATP]. The increase in \(dA/db\) slope can also be understood as a progressive increase in time spent by each crossbridge in the AMADP state \((A_{\text{Ca}}\)-process\) relative to the time spent in the AMADPPi state \((B\)-process\) (For more details, see Conceptual Scheme in the online data supplement, available at http://www.circresaha.org).

To gain further insight into the structural basis of \(A_{\text{Ca}}\), we prepared a connective tissue strip, devoid of contractile tissue, from the epicardial surface of a biopsy (See Experimental Results in the online data supplement available at http://www.circresaha.org). The epicardium strip was subjected to the same skinning, storage, and sinusoidal length perturbation as for myocardial strips. The epicardium exhibited the typical frequency dependence of the myocardial \(A_{\text{Ca}}\)-process (Figure 3, filled circles). These results support associating \(A_{\text{Ca}}\) with the viscoelastic properties of extracellular connective tissue.

**Figure 5.** Selective abolition of \(A_{\text{Ca}}\) by BDM or gelsolin treatment. Nonconditioned NF strips were activated at decreasing levels of pCa in normal activating solution (curve 1), pCa 8 or pCa 5 activating solution with 30 mmol/L BDM (curve 2), and finally at pCa 8 or pCa 5 activating solution after depolymerization of actin filaments by gelsolin treatment (curve 3). \(A_{\text{Ca}}\) values were obtained by subtraction of A at pCa 8 from A at each pCa \((\text{i.e., } A_{\text{Ca}} = A_{\text{Ca}} - A_{\text{Ca}})\) followed by normalization to A at pCa 5.

**Figure 6.** Dependence of A versus B slope on MgATP concentration. A and B moduli were evaluated in nonconditioned NF muscle strips \((n=5)\) at pCa 8 and pCa 4.5 with [MgATP] varied between 10 and 0.5 mmol/L. Slope of the A versus B relation \((dA/db)\) was evaluated by subtraction of pCa 8 values of A and B from respective values at pCa 4.5. Slopes were normalized by their values at normal MgATP (5 mmol/L) to reduce effects of between-stripe variance. Regression line fit to data:

\[
dA/db=(0.83 \pm 0.08) + (0.90 \pm 0.07)/[\text{MgATP}],\]

\(r^2 = 0.98, P = 0.001.\)
Dynamic Stiffness Properties of MR Versus NF Myocardium

In maximally activated myocardium, although there were slight differences in the total dynamic stiffness and dip frequencies between MR and NF, none were significant in either nonconditioned or conditioned states (Table). However, unlike between-group comparisons, there were significant within-group differences in effects of conditioning in both MR and NF. The Hill coefficient of the tension-pCa relation was lowered by 1.7 (P=0.05) in NF and by 1.3 (P=0.01) in MR. Conditioning increased total dynamic stiffness in both groups at low frequency (NF: 33%, P=0.03; MR: 86%, P=0.03) but not at high frequencies. Conditioning raised dip frequency in MR (by 9%, P=0.03) but not in NF (Table). In resting myocardium, dynamic stiffness, as assessed by A-process modulus was 81±16 kN/m² versus 100±16 kN/m² in nonconditioned MR versus NF myocardium (P=0.4). The MR and NF resting moduli increased by ~2-fold but remained not significantly different in conditioned myocardium. Phase parameter k also was not significantly different in resting MR versus NF myocardium.

The lack of significant differences between the total dynamic stiffness curves of MR and NF in both nonconditioned and conditioned states, although conditioning produced significant within-group effects, suggests there are no major effects of MR on the conventional kinetic parameters of crossbridge cycling. Comparison of the relevant model parameters (next section) confirms this.

Comparison of Viscoelastic Parameters of MR and NF Myocardium

The Table summarizes the parameters of the model fitted to the dynamic stiffness data, including the Q₁₀s of their temperature dependence. None of the 6 parameters of the fits of Equation 1 to the dynamic stiffness data from MR were significantly different than those in NF, regardless of conditioning state. Characteristic frequency b of the B-process rose 30% as pCa was lowered from 8 to 5 in both MR and NF (pCa_{0}=5.77 in NF and 5.72 in MR; n=2.3 in NF and 2.5 in MR). There were no significant differences in pCa dependencies of these parameters between MR and NF.

There were within-group differences in effect of conditioning on the 6 stiffness parameters corresponding to the significant effects on total dynamic stiffness of MR and NF described above. Conditioning increased the A modulus in MR (by 52%; paired, P=0.05) more than in NF (by 31%; paired P=NS) while k decreased in MR (by 10%; paired P=0.02) but not in NF (by 10%; paired P=NS). There were only insignificant effects (paired t tests) of conditioning on B and C moduli. Conditioning caused characteristic frequency b to increase slightly in both NF (by 7%) and MR (by 14%), but only the latter increase was significant (paired, P=0.03). Characteristic frequency c did not change significantly in NF or MR.

Discussion

Our results show that the kinetic parameters of our 6 parameter crossbridge model are not significantly altered in MR regardless of conditioning state (Table). This implies that changes in the force-generating step of the crossbridge cycle do not account for increased crossbridge FTI in MR. Therefore, we turned our attention to the possibility that a later step in the crossbridge cycle might be altered—specifically, that a prolongation of the prerigor AMADP state might account for increased FTI.

Comparison of Relative AMADP Dwell Times in MR and NF Myocardium

We used the slope of the A versus B relation (eg, Figure 4) generated by varying pCa to estimate the relative time spent by a crossbridge in the prerigor AMADP state compared with time spent in the force-generating state. The inverse relation between [MgATP] and the slope of the A versus B relation at a constant pCa (Figure 6) provides the main basis for interpreting this slope as an index of the relative dwell time of the AMADP state (see Conceptual Scheme in the online data supplement available at http://www.circresaha.org). Comparison of the A versus B slopes in MR and NF is shown for averaged data in Figure 7. These data were obtained by varying pCa. The range from pCa 8 to 5.75 was chosen to cover physiological values and to avoid departures from linearity. Increased slope consistently occurred below pCa 5.75 (see Figure 4), possibly indicating ATP depletion as this increase in dwell time likely explains the increase in AMADP dwell time observed (Figure 6).

The linear regressions of A on B in Figure 7 were significant (r²=0.97, P=0.01 for MR; r²=0.95, P=0.05 for NF). In MR, the slope of the regression line was 75% greater (P=0.02) than in NF myocardium (0.35±0.03 versus 0.20±0.04, respectively). Thus, there is an apparent 75% increase in dwell time of the prerigor AMADP state relative to the dwell time of the force-generating state. Since there were no significant changes in B-process kinetics, most of this 75% increase in dA/db in MR is attributable to increased AMADP dwell time alone (see last paragraph in Conceptual Scheme in the online data supplement available at http://www.circresaha.org). By prolonging the crossbridge “on-time,” an increase in AMADP dwell time likely explains the increase in FTI in MR (85% at 21°C) since our observed
increase in AMADP dwell time rises from 75% to 88% after correcting for the 16°C temperature difference between the two studies (Q_{10} of \text{dA/dB}=0.75 in MR and 0.69 in NF, calculated from data used for the Table). Similarly, the previously observed 50% decrease in myofibrillar actomyosin ATPase activity in MR^{4,17} can be explained by the 75% increase in AMADP dwell time (61% after temperature correction) assuming the entire ATPase turnover time is equal to the AMADP dwell time.

There is also good agreement between our estimate of the Q_{10} of the AMADP dwell time and the Q_{10} of FTI. In NF, the latter is 0.48 (calculated from values at 21°C and 37°C^{18}), but unlike AMADP dwell time, this Q_{10} is determined by the temperature sensitivities of both force (ie, crossbridge recruitment) and on-time. Removing the effect of temperature on the force component of FTI (Q_{10} of isometric tension=1.41^{19}) yields a Q_{10} of 0.67 for on-time. This compares well with our Q_{10} of 0.61±0.04 (n=9) for AMADP dwell time in NF.

Myosin changes are not likely involved in prolongation of the rate-limiting step, because our previous studies using purified myosin show that its actin-activated ATPase activity is not altered in MR.20 Troponin T (TnT) isoform shifts are not likely involved either, since in contrast to the adult-to-fetal TnT isoform shift reported in dilated cardiomyopathy,21 none have been observed in MR.22 MR-related differences in phosphorylation of myofibrillar proteins could account for the depressed ATPase or increased FTI, but this possibility is contraindicated since appropriate conditioning effects on the crossbridge kinetics in MR did not occur (Table).

Because of the strong dependency of AMADP dwell time on [MgATP], it is tempting to speculate that changes in the energy supply system, rather than changes in contractile or regulatory proteins account for increased FTI in MR. One possible candidate is altered myofibrillar-bound creatine kinase (MBCK) since MBCK is an important component of the phosphocreatine-CK MgATP-regenerating system.23 For example, a reduced activity or concentration of MBCK that reduces myofibrillar [MgATP] from 5 to 1 mmol/L would cause a 75% rise in \text{dA/dB} (Figure 6). Since we observed an inverse relation between A modulus and ejection fraction (see below), and others observed that declining total CK activity is inversely correlated with LV ejection fraction in MR patients,24 CK depression may be the common link. If depression of MBCK activity contributes to these correlations, it is likely that it also contributes to depressed intramyofibrillar [MgATP] and elevated A moduli in our skinned preparations, because normal MBCK activity is probably necessary to maintain saturating myofibrillar ATP levels even in the presence of exogenous CK in the skinned-strip muscle bath.23

**Clinical Relevance of Findings**

Although average ejection fraction (EF) was normal in our MR patients, decreased EF and stroke work (independent of afterload) and depressed end-systolic elastance have been observed in other studies.26,27 Since increased passive myocardial stiffness is correlated with decreased EF in MR patients,28 we examined the possible role of the A modulus in contributing to variations in EF within our MR group. We found a significant inverse correlation (r^2=0.84, P=0.03, n=5) between EF and A modulus, indicating a 10% decrease in EF for each 12% increase in A. In view of the quantitatively similar inverse relation between in vivo myocardial stiffness and EF reported by Corin et al,28 we suggest the possibility that increases in A modulus may contribute to reduced EF. However, this speculation must be confirmed using a larger population of MR patients. Increased time spent in the force-bearing portion of the crossbridge cycle (ie, increased \text{dA/dB}, Figure 7) relative to its power stroke could increase internal resistance to sarcomere shortening and lengthening and cause internal dissipation of myocardial work output. Interestingly, our previous measurements of myosin motility in MR showed a trend toward reduced average filament sliding velocity (0.9 versus 1.2 μm/s) including a clear 90% to 100% reduction in filament velocities in the 1.7 to 2.5 μm/s range.29 Thus, increased AMADP dwell time could contribute to increased internal loading, decreasing shortening velocity, and increasing myocardial stiffness in MR during ventricular ejection and filling.

**Limitations of Study**

The major technical limitation of this study is the uncontrolled effect of series compliance at the ends of the preparations. All stiffness moduli were obtained without compensation for differences between applied length changes and actual sarcomere length changes. Although this problem can be avoided by using an online sarcomere gauge to record actual sarcomere length change simultaneously with resulting force change (see, eg, Wannenburg et al^{29}), the apparatus used for our experiments did not have this capability. However, using a computational approach, we were able to estimate the extent to which end compliance affected our stiffness measurements.

We simulated the effects of a wide range of hidden end compliance to examine resulting errors in evaluating model parameters (see the online data supplement available at http://www.circresaha.org). The results demonstrate that the presence of a large series compliance (as much as 4 times greater than the maximum possibly present) has virtually no effect on our ability to faithfully recognize the presence of 3 parallel viscoelastic elements. Further, by assuming reasonable values of hidden end compliance determined by independent tests, we established that parameter values (A, B, C, k, b, and c) of the apparent parallel elements differed from those of the real elements by no more than 26% (range: 7% to 26%). In cases where errors are as large as 26%, it is possible that our failure to detect changes in conventional intermediate crossbridge reactions in MR myocardium are partly attributable to increased variance associated with differences in end compliance between preparations. However, it is notable that estimates of prerigor dwell time are barely affected by even large variations in hidden end compliance. Dwell-time indices are obtained from the A/B ratio, which is underestimated by only 5% in the presence of reasonable end compliance (versus up to 26% errors in absolute values). The underestimation increases to 8% if an unreasonably large end compliance is assumed.

Another potential limitation arises because of the closeness of characteristic frequencies b and c in cardiac muscle. This
taxes the resolving power of the fitting algorithm. We also addressed this potential problem by computational methods. The results for simulated data in which $c/b$ ranged from 2.8 to 5 show that possible errors in evaluating $A$, $B$, and $C$ are less than 4%, 8%, and 8%, respectively (see the online data supplement available at http://www.circresaha.org). Since there was little difference in characteristic frequencies $b$ or $c$ between NF and MR myocardium, these small errors will cause even smaller errors in estimating differences between $A/B$ ratios and dwell times in the two groups.

Acknowledgments
This work was supported by National Institutes of Health Grants R01 HL54821 (D.W.M.) and R01 HL55641 (N.R.A.).

References
Alterations of Myocardial Dynamic Stiffness Implicating Abnormal Crossbridge Function in Human Mitral Regurgitation Heart Failure

_Circ Res._ 2002;90:66-72; originally published online December 6, 2001; doi: 10.1161/hh0102.103221

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/90/1/66

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/12/19/hh0102.103221.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT TO:

ALTERATIONS OF MYOCARDIAL DYNAMIC STIFFNESS IMPLICATING
ABNORMAL CROSS-BRIDGE FUNCTION IN HUMAN MITRAL REGURGITATION
HEART FAILURE

L. A. Mulieri, W. Barnes, B. J. Leavitt¹, F. P. Ittleman¹, M. M. LeWinter², N. R. Alpert,
D. W. Maughan

Department of Molecular Physiology & Biophysics, U. Vermont, Burlington VT 05405
¹Department of Surgery and ²Cardiology Unit, U. Vermont, Burlington VT 05405

Supported by National Institutes of Health R01 HL54821 (D.W.M) & R01 HL55641 (N.R.A.)

Address for reprints: Dr. Louis A. Mulieri, Ph.D.
Dept. Molecular Physiology and Biophysics
Given Building, University of Vermont
Burlington, VT 05405-0068
Telephone:(802)656-2540,
E-mail: mulieri@physiology.med.uvm.edu
Materials and Methods

Patient medications.

NF patients’ medications included: beta blockers (number of patients, drug) (4, atenolol; 1, propanolol; 2, metoprolol); long-acting nitrates (1, isosorbide dinitrate); diuretics (1, dyazide); calcium channel blockers (1, amlodipine; 2, diltiazem); and anti-arrhythmics (1, disopyramide).

MR patients’ medications included: beta blockers (1, atenolol); angiotensin converting enzyme inhibitors (1, lisinopril; 2, ramipril); other vasodilators (1, losartan; 1, terazocin); diuretics (1, furosemide; 1, hydrochlorthiazide).

Muscle strip preparation.

After a 60 min recovery from surgical trauma in oxygenated BDM-Krebs solution, the subepicardial biopsies were dissected into thin strips (mean diameter and length of 112±5 μm and 382±14 μm, respectively in NF and 118±5 μm and 392±21 μm, respectively in MR). Half of the strips were ligated with single silk filaments (15 μm diameter), transferred to a skinning vessel, and stretched to 2.1 – 2.3 μm sarcomere length and remained quiescent before skinning (Non-conditioned strips). Half of the strips from each heart were electrically stimulated at 37° C and 1 Hz for 1 h immediately prior to skinning (Conditioned strips). Comparison of conditioned and non-conditioned myocardium was made because the original observation of increased crossbridge FTI10 included continuous twitching at 1 Hz. Our conditioning protocol, followed by rapid cooling and skinning in the presence of calyculin was intended to establish and maintain similar myofibrillar phosphorylation levels as in the prior FTI experiments. Skinning was carried
out at 21°C for 1 hr in 1.6 ml of skinning solution (see Solutions). Strips were subsequently washed with relaxing solution and stored at 4 °C overnight. One strip was used the next day and the remaining 2 or 3 strips were kept in storage solution at -20 °C for subsequent experiments.

Final preparation involved washing a tethered strip in relaxing solution containing 30 mmol/L BDM and clamping a small aluminum "T"-clip on each end to delimit a uniform segment (~0.4 mm length) for study. The preparation with attached clips was cut free and transferred to a 30 μL drop of relaxing solution on the glass bottom of a mineral oil-filled aluminum chamber. The strip was mounted on the force and length control hooks, and stretched incrementally to a sarcomere spacing of 2.1 μm (estimated with an inverted microscope and filar micrometer). Strip cross-sectional area was obtained by calculating the equivalent circular cross-sectional area from strip diameters measured from the top and side views (mirror). The mean diameter of NF strips was 112±5 μm which was not significantly different than 118 ± 5 μm (p=0.34) in MR.

**Solutions.**

*Relaxing solution* contained 5 mmol/L MgATP, 15 mmol/L phosphocreatine (PCr), 240 units/mL creatine kinase (CK), 0.25 mEq/L free phosphate, 1 mEq/L free Mg²⁺; 5 mmol/L EGTA, 0.1% w/v calyculin, 1 μmole/L DTT, and 20 mmol/L BES buffer (pH 7.0), pCa 8 (0.13 mmol/L CaCl₂). Ionic strength was adjusted to 175 mEq/L with added sodium methane sulfonate. All solutions were formulated by solving equations describing the ionic equilibria².

*Activating solution* (pCa 4.5) was the same as relaxing solution, except the total concentration of
CaCl₂ was 5.01 mmol/L.

Skinning solution was the same as relaxing solution except PCr and CK were omitted and 0.5% v/v Triton X100 was added, followed by a 50% v/v dilution with glycerol.

Storage solution was the same as skinning solution except Triton X100 was omitted and 10 μg/mL leupeptin was added, followed by a 50% v/v dilution with glycerol.

**Force measurements and sinusoidal analysis.**

Force and length change data were acquired via A/D (16-bit board DT2838, Data Translation Inc., Marlboro, MA). The length and force signals from the piezomotor displacement transducer and strain gauge were digitized and the elastic and viscous components of dynamic stiffness were calculated from the change in tension and length at each frequency³.

Bandwidth and linearity of the force gauge (AE 801, SensoNor) and displacement transducer (KD2810-1U, Kaman Instruments) were adequate to provide accurate force and length change signals over the perturbation frequency bandwidth of 100 Hz without compensation. Apparatus stiffness, estimated by substitution of an aluminum strip in place of a muscle strip, was much greater (10⁴) than muscle strip values and was therefore neglected.

No correction for damaged-end compliance was applied because, as described below, estimates of end-compliance effects on apparent stiffness moduli of the A- and B-processes are moderate and proportional, thereby maintaining the A/B ratio within a 5% error band. Effects on high-
frequency stiffness (1 kHz) were shown to be small because separate quick-release experiments demonstrated that the chord stiffness of maximally activated preparations (pCa5) was 14% to 33% of that in rigor. If significant end-compliance were present, contracture stiffness would be lowered more than rigor stiffness because excess compliance from irresponsive or partially responsive sarcomeres during contracture becomes stiff during rigor. Since 15 - 40% of cross-bridges connect to thin filaments during full activation and 100% connect during rigor, we expect ratios of activation to rigor stiffness to be 15-40% if no damaged-end compliance is present. Agreement with our observed 14%-33% range confirms our preparations are not unusual. Since quick-release measurements only give information about purely elastic end-compliance effects we also assessed errors in the experimental frequency range of 0.125-100 Hz using a simulation method. These results confirm minimal effects, especially on the A/B ratios (See “Quantitative effects of series compliance on viscoelastic model parameters” below.). Our use of 30 mM BDM in the skinning solution during application of the T-clips reduces damaged-end effects because paralysis of cross-bridge function prevents the spread of crush-injury within the strips. Retention of a circular cross-section in the clamped regions of the muscle strip also helps preservation of sarcomere function.

No correction for preparation deterioration was required since pilot experiments indicated rundown of maximal activation averaged 3.1 ± 1% from beginning to end of experiments providing
rigor was not induced before return to full activation. These data were not available in the main study because routine experiments terminated in rigor runs. In preparations re-activated after reversal of rigor the run-down was observed to be high as 35 - 40%.

Strip stress was calculated as the quotient of the amplitude of the force change over the strip cross-sectional area. Strip strain was calculated as the change in strip length divided by $L_{2.1}$, where $L_{2.1}$ is the strip length at a sarcomere spacing of 2.1 $\mu$m. Dynamic stiffness, $Y(f)$, was calculated at each frequency ($f$) as stress/strain. Nonlinear muscle response was avoided by use of appropriately small amplitudes of length oscillations (0.125 % $L_{2.1}$). Linearity was confirmed by cross-correlation analysis showing the total harmonic distortion in the 2$^{nd}$ and higher orders averaged 1.4 % ± 0.2 % of the total force response.

Three component viscoelastic model and its fitting.

It is important to note that the representation of the $A$-, $B$-, and $C$-processes as 3 parallel mechanical elements in our model (Equation 1) is not meant to imply a physical correspondence with 3 separate structural elements that are permanently in parallel within the muscle. The model connotes 3 independent physical processes (states or state transitions) inherent in the muscle and cross-bridge cycle. The cross-bridge related portions of these processes occur sequentially in each cycling cross-bridge; however, asynchronous cycling of these cross-bridges causes all processes to be present simultaneously in activated muscle. At any given moment the total population of randomly cycling cross-bridges distributes itself among all 3 processes. Thus, on a population
basis all 3 independent processes continually interact with the measuring apparatus and, therefore, can be represented as a parallel mechanical arrangement.

A fundamental difference between our and Kawai's approach is that our $Y(f)$ represents the total dynamic stiffness of the myocardial preparations, whereas Kawai's $Y(f)$ represents the difference between active and resting dynamic stiffness\(^4\). Furthermore, Kawai uses gluteraldehyde-fixed and rigor-state preparations as reference materials to correct for limited bandwidth and stray series compliance in the apparatus\(^4\). Because of adequate bandwidth and relatively stiff connections, such system corrections with the present apparatus were deemed unnecessary over the range of frequencies used (0.125 - 100 Hz).

Differences between our process-$A$ and Kawai's should also be noted. Unlike Kawai\(^5\), our $A$-process is not an exponential process with a single characteristic frequency. Furthermore, as shown in the Print Results, our $A$-process includes both a cross-bridge related component and a non-cross-bridge related component, whereas Kawai's $A$-process contains no such distinction.

The reiteration algorithm of Kawai & Brandt\(^3\), modified to include our non-exponential $A$-process, was used to fit Equation 1 to the data by a non-linear routine that evaluated the parameters of all components simultaneously to minimize the overall root-mean-square deviation of $Y(f)$ from the data. The modifications to accommodate the $A$ term of Eq. 1 add iterations with $k$ starting at 0.05 and incrementing in 0.002 step up to $k = 1.5$. At each step, the remaining 5
parameters of Eq. 1 are minimum r.m.s. fitted. This is followed by selection of the k value giving overall lowest r.m.s error.

**Test of resolving power of fit algorithm.** The ratio of c/b characteristic frequencies measured in this study averages about 4 (3.96 ± 0.6 in NF myocardium and 4.36 ± 0.6 in MR). Consequently, we were concerned whether the fitting algorithm had sufficient power to correctly resolve the B and C processes when c and b differ by less than 5-fold\(^1\). The effect of diminishing c/b ratios on fitting errors was measured using the following procedure:

a. The six-parameter fit of Eq. 1 to the experimental data ("original fit") of the muscle strip in Print Figure 1 was used to generate 4 sets of simulated data. Each set was simulated using Eq. 1 with the same 6 original fit parameters from the muscle strip, except that c frequency was altered to give c/b ratios of 5, 3.3, 2.8, and 2.

b. Realistic noise was added to the simulated data at each frequency according to the difference between the original fit of Eq. 1 and the real data in Print Figure 1. This "noise" contains both apparatus and biological components that are present during all experiments, as well as the unknown fitting errors specific to the shortcomings of the fit algorithm. A characteristic of Eq. 1 is that the frequencies at which maxima and minima occur in the viscous and elastic moduli shift as the ratio of c/b is changed. Consequently, before adding the noise values derived from Print Figure 1 to the simulated data at different c/b ratios, we altered its frequency distribution. This maintained the original alignment of the noise profile with the frequencies of characteristic maxima and minima of the simulated curves. E.g., before adding
the noise values to the simulated stiffness curve having a c/b ratio of 5, the frequency scales of the elastic and viscous noise distributions were multiplied by 1.30 and 1.35, respectively. For a c/b ratio of 3.3, the multipliers were 1.0 and 1.0; for a c/b = 2.8, the multipliers were 0.83 and 0.86; etc.

c. Our fitting program was then used to perform least-squares re-evaluation of the 6 parameters of Eq. 1 for each noisy simulated data set. Figures 1 and 2 show the effect of c/b ratio on the differences between the six parameters fit to the noisy simulated data and the six parameters that were used to generate each simulated data set.

Figure 1 (black curve) shows the average deviation (as r.m.s. error) between the fitted total dynamic stiffness curve and the simulated total dynamic stiffness data over the 2- to 5-fold range of c/b frequencies. This average deviation varies between 10 and 11.9 kN/m², with little indication of a systematic increase as c/b decreases. These deviations arise mainly from the original noise that was added to the simulated data because they are about equal to the average deviation of the original fit to the actual data from the muscle strip (11.1 mN/mm², shown as X symbol in Figure 1). Errors in evaluating B and C moduli remain less than 8% as c/b falls to 2.8 but they increase steeply for lower values. Errors in A are less than 2% over the whole range of c/b between 5 and 2.
Figure 1. Effect of c/b Frequency Ratio on Modulus Errors in Fitting Equation 1 to Simulated Data. Percent errors between Eq. 1 fit moduli and simulated data in which the ratio of characteristic frequencies was varied between 2 and 5. Simulated data was generated from Equation 1 with added “noise” derived from fitting errors in Print Figure 1 (see text). The Deviation curve is the average of the r.m.s. errors between fitted and actual viscous or elastic moduli.

Figure 2 shows the fitting errors in c, b, and k also are not larger than 8% for c/b ratios between 2.8 and 5. These findings suggest there is little interference with fitting resolution for c/b ratios above 2.8. Since only 2-strips in the present report had c/b values less than 2.8 (NF, 2.39; MR, 2.32), we conclude the resolving power of our fitting algorithm is adequate to detect and accurately quantitate the A-, B-, and C-processes even when c frequency is as low as 2.8 times b
frequency.

**Figure 2.** Effect of $c/b$ Frequency Ratio on Characteristic Frequency Errors in Fitting Equation 1 to Simulated Data. Percent errors between fit characteristic frequencies $b$ and $c$ or phase constant $k$ and simulated data. Same data sets as in Figure 1.

We also tested whether the fitting algorithm would erroneously extract 2-exponential processes from noisy data generated from a single exponential process. We generated a single exponential stiffness curve from Eq. 1 using the same values for the 6 parameters as in Print Figure 1 except that we set the $C$ modulus to zero. We added the same noise to this simulation as was present in
the original real data of Print Figure 1 (i.e., differences between fitted, 3-process curve and actual data). This noise biased our result toward finding a second exponential process in the simulated data because some of its frequency profile is centered around the frequencies that were associated with the original C process. The fitting algorithm did return a “rogue” C-process, but its modulus and characteristic frequency were well outside the range of those commonly extracted from our real experimental data sets. (Rogue characteristic frequency c was at least 20-fold higher (271 Hz) than myocardial values. Rogue C modulus (50.5 kN/mm²) was only 10% of companion B modulus while for myocardial data, the C modulus averages 132% of the companion B modulus.) When forced to fit an extra process to a data set, our fitting program accommodated by making the “rogue” modulus very small with its characteristic frequency so large that it was well above the experimental bandwidth investigated. Remarkably, the forced fitting of a rogue C process to the data did not interfere with the ability of the fitting algorithm to correctly quantitate the existing B-process. There was excellent agreement between fitted and actual values of B moduli and b frequencies (−489 vs. −504 kN/mm² and 4.3 vs. 4.3 Hz, respectively). Fitted vs actual values of A and k were equally good (205 vs. 201 kN/mm² and 0.11 vs. 0.12).

We also considered that increases in A and B might be a co-variance effect, representing an increased in error in fitting increasing B and C moduli as activation rises. We reject this explanation on the following experimental grounds. The $Q_{10}$s of the A and B (or C) moduli would have to be similar if A were derived from errors in fitting B and C. This is not the case since A actually decreases
with increased temperature ($Q_{10} = 0.83 \pm 0.07$) while B and C increase substantially. $Q_{10}$ of B = $1.27 \pm 0.07$ and $Q_{10}$ of C = $1.32 \pm 0.07$; both are significantly different than the $Q_{10}$ of A ($p \leq 0.05$). The increase in B and C moduli with temperature is most likely due to cross-bridge recruitment. Thus, the possibility that A increases with increased activation due to larger fitting errors at higher levels of activation is not supported by the data, because the experimental data are opposite to that based on this premise. Furthermore, the phase-frequency relation of the $A_{Ca}$ process (state) as described by Eq. 1 is independent of frequency. The phase angle of both B and C processes strongly dependent on frequency. This makes it unlikely that errors in fitting B and C are absorbed into the frequency-independent A term. Finally, as described in the previous paragraph, when we fitted an A+B+C relation to simulated data that was generated by an A+B model, we observed less than an 8% difference between evaluated and actual A parameters. This result shows that, even when the fitting errors are as extreme as those produced by forcing a 3-process fit to 2-process data, the fitting algorithm does not make use of alteration of the A process parameters to compensate for B+C process errors.

In conclusion, although it is generally true that multi-component fitting can be problematic when characteristic frequencies or time constants are closely spaced, our analysis of resolving power demonstrates such problems are small and can be neglected for the particular conditions of our experiments.

**Effects of series compliance on evaluation of parallel viscoelastic model parameters.**

Although the quick-release measurements described above (p. 4) suggest that damaged-end
compliance can be neglected at high frequencies, we examined whether end-compliance might have larger effects in the 0.125 – 100 Hz, experimental range. Of additional concern was the possibility that end-compliance does not influence each of the A, B, and C processes similarly. This was of particular concern because the ratio of apparent A and B moduli is important for our experimental interpretations. We addressed these concerns using simulated stiffness data from a 4-component, series-parallel model consisting of our standard 3-component (parallel $A + B + C$ processes) model connected mechanically in series with an end-compliance or "S-process". These data were analyzed with our standard fitting algorithm that assumed only the standard 3-component, parallel $A + B + C$ process model. Comparison of the apparent parameters of the assumed 3-component model with the actual 4-component parameters allows quantitative estimates of end-compliance effects.

The dynamic stiffness modulus of the 4-component model is given by Equation 3.

$$Y_{4\text{-Comp.}} = \frac{S(f)Y(f)}{[S(f)+Y(f)]}$$  \hspace{1cm} \text{Eq 3.}$$

$Y(f)$ is the standard 3-component, parallel element model represented by:

$$Y(f) = A (i2\pi f/\alpha)^k - B if/(b+if) + C if/(c+if),$$  \hspace{1cm} \text{i.e., print Eq. 1},

and $S(f)$ is the single-component, series element represented by:

$$S(f) = S(i2\pi f/\alpha)^q$$  \hspace{1cm} \text{Eq. 4.}$$

$S$ is the stiffness modulus of the end compliance (kN m$^{-2}$) and $q$ is a unitless exponent proportional to the phase shift, $\phi$, defined similarly as in print Eq. 1.
We used Equation 4 to model the dynamics of the damaged-end process because it accurately represents passive dynamic properties of resting, activated, and rigor myocardium (see Print Paper). It is likely that the clamped ends of muscle strips are inhomogeneous, consisting of intact, partially damaged, and irresponsive sarcomeres. However, during activation we expect more uniform properties to prevail. The 150 μm-long, crimped collars of the aluminum T-clips that encase the ends of the muscle strip severely restrict diffusion of substrates/metabolites from and to the bath. Therefore, responsive regions within the clamps will be depleted of ATP during activation, resulting in rigor. It is also likely that during activation, irresponsive and partially active sarcomeres within the clamped region would also be in rigor or, at least, protected from stretch by the stiffness of parallel, rigor sarcomeres thus creating mechanical homogeneity.

Figure 3 shows the effect of progressively decreasing the stiffness of the series component on the Nyquist curves calculated from Eq. 3. For these curves the 6 parameters of the standard, parallel element portion of the series-parallel model were held constant at the mean values from 5-NF preparations (see legend). Series compliance clearly reduces the overall size of the Nyquist curve, severely lowering both elastic and viscous moduli. Of particular interest is that the apparent ability of the B-process to deliver net work output (negative viscosity region of the Nyquist curve) can be completely obliterated by the positive viscosity of a hidden series compliance (S=200 curve).
Figure 3. Effect of adding a series compliance on the apparent Nyquist plots of a standard 3-element, parallel model. Data points generated from Equation 3 with stiffness modulus, $S$, of the series compliance progressively decreasing from infinity. Parallel element moduli and kinetic parameters were held constant at the following mean values from NF myocardium. A, B, and C moduli = 221.8, 313, and 463 kN/m$^2$, respectively. Characteristic frequencies $b$, and $c = 3.54$ and 12.2 Hz and $q = k = 0.088$.

We fitted a standard 3-element, parallel model to the 4-element, series/parallel model data shown in Figure 3. There was virtually no degradation in ability of our fitting algorithm to detect the presence of the 3 original parallel elements even though the simulated data were corrupted by the effects of the added series element. This was true even for a severely altered Nyquist curve
generated with $S = 150 \text{ kN/m}^2$. The correlation coefficients and average r.m.s errors between fitted curves and simulated data ranged between 0.978 - 0.999 and 2.97 - 3.86 kN/m$^2$, respectively as $S$ ranged between 150 and $\infty$. The effects of increasing end-compliance on the magnitudes of the apparent parameters evaluated with our standard fitting algorithm under the assumption of a 3-element, parallel model are plotted in Figures 4 and 5.

**Figure 4.** Effects of hidden series compliance on the apparent stiffness moduli of the 3-element parallel elastic portion of a series/parallel model. Apparent stiffness moduli, A, B, and C of our standard 3-element parallel model were evaluated by applying our fitting algorithm to the simulated data (Fig. 3) from a 4-element series/parallel model. Actual values of stiffness moduli of the 3-element parallel elastic portion of the model are plotted at the right side of the graph at $S = \infty$. The dimensionless ratio of A/B moduli (-100A/B curve) is plotted to emphasize the insensitivity of this parameter to the presence of a hidden series compliance.
**Figure 5.** Effects of hidden series compliance on the apparent kinetic parameters of the 3-element parallel elastic portion of a series/parallel model. Apparent kinetic parameters, $k$, $b$, and $c$ of our standard 3-element, parallel model were evaluated by applying our fitting algorithm to the simulated data (Fig. 3) from the 4-element series/parallel model. Actual values of the kinetic parameters of the 3-element parallel elastic portion of the model are plotted at the right side of the graph at $S = \infty$. Parameters $b$ and $c$ in Hz, parameter $k$ is dimensionless.

**Estimate of actual stiffness errors in present study due to damaged-end compliance.**

Although we were not able to measure the actual end-compliance present during activation in the preparations studied, we were able to estimate reasonable values using the stiffness measurements obtained from these preparations in rigor solution combined with literature
estimates of cross-bridge stiffness. In sarcomere length-controlled experiments during maximal activation, a quick-release of 0.01 \text{l}_o carried out within 0.11 ms (equivalent to a quarter-wave of a 2.27 kHz sinewave) is sufficient to discharge full activation tension\(^7\). In the absence of end-compliance our maximal developed tension (18.9 kN/m\(^2\)) would also be discharged by a 0.01 \text{l}_o release. Thus, the stiffness of the participating fraction of cross-bridges is estimated to be 1890 kN/m\(^2\) per \text{l}_o (i.e., 18.9 kN/m\(^2\) / 0.01 \text{l}_o). This value underestimates total cross-bridge stiffness because generally, only 15-40\% of all cross-bridges are recruited during maximal activation. In the present case we can estimate the degree of cross-bridge recruitment during maximal activation from the ratio of \(A\)-process stiffness developed during maximal activation (\(A_{\text{Ca}}\)) to the \(A\)-process stiffness recruited by rigor buildup (\(A_{\text{Ca, Rigor}}\)), i.e., the combined structural stiffness moduli of all cross-bridges participating in a contracture divided by the combined structural stiffness moduli of all cross-bridges. Because \(A_{\text{Ca}} = 120 \pm 23\) kN/m\(^2\), and \(A_{\text{Ca, Rigor}} = 641 \pm 78\) kN/m\(^2\), we estimate 19\% of all cross-bridges are recruited during maximal contractures hence, stiffness during 100\% recruitment is 1890/0.19 = 9947 kN/m\(^2\). When a muscle strip is in rigor, there is 100\% recruitment of cross-bridges to the \(A_{\text{Ca, Rigor}}\)-process but the overall strip stiffness is lower than cross-bridge stiffness because of series compliance in the ends of the preparation. Our measured \(A_{\text{Ca, Rigor}}\) stiffness modulus of 641 kN/m\(^2\) generates a strip stiffness of 1487 kN/m\(^2\) at 2.27 kHz, the sinewave frequency equivalent in the published quick-release study (i.e., \(Y_{\text{Ca, Rigor}}\)).

\[
2.27 \text{kHz} = A_{\text{Ca, Rigor}} (2\pi f)^k = 641(2\pi \cdot 2270)^{0.088}.
\]

Hence, the stiffness of the end regions is 1748 kN/m\(^2\) per \text{l}_o (i.e., 1/(1487\(^{-1}\) - 9947\(^{-1}\)) = 1748). This corresponds to an end stiffness modulus, \(S\), of 754 kN/m\(^2\) (i.e., \(S = 1748/[2\pi \cdot 2270]^{0.088}\)). Using this value of \(S\) in Equation 3 to generate
simulated series-parallel data and fitting Print Eq. 1 to this data yields apparent A, B, and C moduli of 174. -266 and 339 kN/m², respectively. These values are 21, 15, and 26% below the actual A, B, and C values (i.e., when S = ∞). Similarly, the apparent kinetic parameters are k = 0.094, b = 3.83 Hz, and c = 8.62 Hz. These values are 7, 8, and -21 % above the actual values, respectively. Although these % errors in stiffness moduli might be considered large, one should note that A and B are reduced proportionately as series compliance increases (A and B curves are parallel between S values of 700 and 1000, Fig. 4). Because of this proportional effect on A and B, the apparent ratio of A/B moduli are underestimated by no more than 5 - 8% for S values ranging from 400 to 1369 kN/m² (-100A/B curve in Fig. 4). The closeness of our estimate to the true value of A/B further justifies our use of apparent A/B moduli to accurately characterize changes in the AMADP dwell time, even in the presence of hidden end-compliance.

A worst-case scenario, independent of cross-bridge stiffness assumptions, was also assessed by assuming that the measured rigor stiffness moduli originate entirely in the clamped ends. Under this assumption we substituted the experimentally determined average rigor values of A and k (641 and 0.088, respectively) for S and q in Eq. 4. The apparent A, B, and C moduli are 167, -256 and 322 kN/m², respectively. These values are 24, 18, and 30 % below the actual A, B, and C values. The apparent kinetic parameters, k, b, and c for this worst case scenario are 7, 8, and -23 % above the actual values.
Experimental Results.

The epicardial strip data in Print Figure 3 were obtained at pCa5 and 5 mmol/L ATP at 35° C, with the only arbitrary condition being to pre-stretch the connective tissue strip sufficiently (43% above slack length; giving a total tension of 6.4 kN/mm²) so that A was similar to the mean $A_{\text{nonCa}}$ value of myocardial strip preparations. The stiffness-frequency data were well fit by $A(i2\pi f/\alpha)^k$, with $A = 89$ kN/m² and $k = 0.058$. Lowering temperature to 27° C revealed the $Q_{10}$ s of both A and k to be 0.89, similar to the 0.83 and 0.95 values for A and k, respectively, found in muscle strip preparations at pCa5 (see Print Results). Lowering the calcium concentration to pCa 8 caused a slight increase (n.s.) in dynamic stiffness modulus ($A = 90$ kN/m² and $k = 0.059$) with little change in their $Q_{10}$ s (0.95). There was also insignificant change in dynamic stiffness of the connective tissue strip upon removal of ATP from the bathing solution ($A = 83$ kN/m², $k = 0.054$). However, gluteraldehyde fixation of the connective tissue strip raised the A modulus 11-fold and reduced k 18-fold, suggesting conversion of viscosity-linked collagen fibrils to a heavily cross-linked, elastic network.

Discussion.

Conceptual Scheme. The following provides a basis for interpretation of our results in the context of a cross-bridge model. During normal cross-bridge cycling, cross-bridges progress sequentially through weak-binding and strong-binding states. The strong-binding state includes a force-maintaining state before ADP is released from AMADP$^7$. As illustrated in Fig. 6, the measured B modulus of the dynamic stiffness is associated with a transition between a pre-force
(weak-binding) state and a post-force (strong-binding) state prior to the AMADP (force-maintaining) state. The measured C modulus of the dynamic stiffness is associated with a transition between a strong-binding state and a detached state$^{9,10}$.

**Figure 6.** Summary of a conceptual model that associates the 3 components of Print Eq. 1 with their corresponding physical-chemical processes (curved arrows) or state and with their structures of origin (zigzag lines). The non-enzymatic, $A$-process, (first term in Eq. 1), arises from two myocardial properties. One is the polymer-like, passive viscoelastic behavior of the S1 and S2 portions of the cross-bridge that determines the $A_{Ca}$ modulus. Additional passive viscoelastic contributions to the $A$-process as $A_{nonCa}$ arise from connective tissue paralleling the sarcomeres in the extracellular space and possibly from titin filaments within the myofibrils$^{11}$. Note that the $A$- process represents a state in the cross-bridge cycle, unlike the $B$ - and $C$ -processes that represent transitions between cross-bridge states. The two enzymatic processes, $B$ and $C$, arise from transitions between intermediate states in the cross-bridge cycle and are represented by the second and third terms in Print Eq. 1. The B modulus represents transition between pre-force (weakly binding) and force-maintaining (strongly binding) cross-bridge states occurring at a
characteristic frequency, b. This gives rise to cross-bridge force and work production. The C modulus represents transition between force-maintaining (strongly binding) and detached cross-bridge states occurring at a characteristic frequency, c. The C- process includes work absorption by exertion of a braking force that opposes filament sliding. The absorbed work is dissipated if sliding is sufficient to break the actomyosin bonds.⁸,⁹

To extend this cross-bridge model, we hypothesize that the pre-rigor, AMADP state gives rise to the $A_{Ca}$ portion of the A modulus. This hypothesis is based on our observations of constancy of the slope of the A vs. B relation with rising activation (see Print Figs 4 and 7) and because the frequency dependence and $Q_{10}$ s of the $A$-process dynamic stiffness are the same as for the rigor state. We propose that $A_{Ca}$ represents primarily the visco-elastic modulus of the passive structural backbone of the actin-myosin link (i.e., the S1 and S2 components of the cross-bridge, including the actin-S1 bond, but excluding all dynamic mechanical properties associated with conformational changes in the myosin motor domain and excluding the dynamics of formation and abolition of the actin-S1 bond). Further, we propose $A_{Ca}$ is externally manifest only when actin-myosin links are in the pre-rigor AMADP or rigor AM states. $A_{Ca}$ is not detected during other portions of the cross-bridge cycle because the dynamic state of the bond between S1 and the thin filament (during intervening B-processes) introduces a large, dominating series compliance (B and C moduli are 3 to 4 times more compliant than the rigor $A_{Ca}$ modulus at b and c frequencies). This large series compliance dominates the mechanical behavior in the form of the B- and C-processes except when the cross-bridges are in the pre-rigor, non-cycling $A_{Ca}$-state. Given this interpretation and assuming that every cycling cross-bridge participates in each intermediate step of the cross-bridge cycle, the A, B, and C moduli provide an index of the number and lifetime of
cross-bridge participation in each of the 3 processes. Since each cross-bridge time-shares a mechanical connection to the measuring apparatus as it progresses through each of the 3 processes, the fraction of all cycling cross-bridges involved in any one of the 3 processes corresponds to the fraction of cycle time spent by a given cross-bridge in that process/state. Thus the A, B, and C moduli can provide an index of cross-bridge dwell time in each of the 3 states. Similarly, the slope of the A vs. B relation during rising activation levels can indicate the ratio of dwell-times of the two states.

It must be noted however, that numerical values of the A vs. B slope, dA/dB, may not directly measure the ratio of dwell times of the two states. The unitary contribution to measured \( A_{Ca} \) by a single cross-bridge during the \( A \)-state may be different than its unitary contribution to the measured B during its \( B \)-state. This is possible not only because of effects of differences in dwell time on measured moduli but also because MR could alter the unitary stiffness associated with each of the 3 processes differently. In the present study however, we found no evidence for MR-related changes in B modulus, characteristic frequency \( b \), or maximal isometric tension. Consequently we can interpret MR-related changes in dA/dB to indicate changes in dA alone. Further, since values of \( A_{Ca} \) in the rigor state (i.e., \( A_{Ca} \)-state dwell time = infinity) are not significantly different in MR compared with NF myocardium (557 ± 87 kN/m² vs 641 ± 78 kN/m², \( p = 0.5 \)) we assume that the unitary stiffness of each cross-bridge during the \( A_{Ca} \)-state is not altered in MR. Thus, we interpret percent changes in dA/dB in MR myocardium as direct indications of percent changes in the average dwell time of the \( A_{Ca} \)-state (i.e., the AMADP state). We note that additional advantages of using dA/dB as an index of relative dwell time are
its independence on degree of activation between pCa8 and pCa5.75 and its insensitivity to end-compliance.

**Comparison of Experimental Results with Previous Publications.** Our values of isometric contracture tension and its calcium dependence (pCa50 = 5.77, nH1 = 4.5, Table 1 of Print publication) are comparable to reported values (temperature corrected using Q10 = 1.0612) for human NF right ventricular trabeculae (pCa50 = 6.2, nH1 = 4.513) and for human NF left ventricular myocytes (pCa50 = 6.1, nH1 = 3.814; pCa50 = 5.73, nH1 = 3.7215). Our finding of insignificant changes in the tension-pCa relation in MR accords with two previous studies in more severe heart failure due to dilated cardiomyopathy13,15. A third study reported increased calcium sensitivity (pCa50 = 6.28) that was reversed (pCa50 = 6.0) by PKA phosphorylation14.

We are aware of only two studies of myocardial stiffness in human NF myocardium using sinusoidal length perturbation analysis16,17. Our total dynamic stiffness modulus at 100 Hz (608 kN/m², Table 1) compares well with one study16 (597 kN/m², Table 1, length = 5 mm, cross-sectional area = 0.5 mm²). However, the previously reported dip frequencies of 0.78±0.06 Hz at 22°C16 and 1.1 ± 0.05 Hz at 27°C17 are 28 and 40% lower than our value of 4.3 ± 0.17 at 35°C (after correction for the temperature differences using a Q10 = 2.9). Our finding of no significant difference in dip frequency between failing MR and NF myocardium, contrasts with the 46%16 and 30%17 lower dip frequencies found in the more severely failing, end-stage dilated cardiomyopathy in the previous studies.
Our value of dip frequency in NF (4.3±0.17 Hz) accords with values (corrected for temperature differences) reported in V₃-myosin dominant myocardium from both rabbit (3.9 Hz¹⁸) and hypothyroid rat (4.0 Hz¹⁹). However, it is 22% - 25% lower than in euthyroid rat¹⁷,²⁰ (after correction for the 2:1 ratio of dip frequencies in V1 vs. V3 myosin muscles¹⁹ and temperature). Our values of b and c frequencies in human NF myocardium are also approximately 50% lower than in ferret myocardium (Figure 2, with [Pᵢ] = 0.25 mmol⁴) and 55 to 70% lower than in rat¹⁷,²⁰ myocardium (Using Q₁₀=2.9 and 2:1 V1:V3 corrections). Our finding that cross-bridge kinetics are increased 30% by raising [Ca⁺⁺] from pCa8 to pCa5 contrasts with the much larger, 140% increase observed in rat myocardial preparations by Wannenburg et.al.²⁰. This difference may be related to the absence of significant V1 myosin in human myocardium and to our use of a higher [Ca²⁺] at maximal activation.

The hyperbolic curve we obtained between dA/dB and [MgATP] (Print Figure 7) resembles that relating rigor dwell time to [MgATP] in single molecule, laser-trap measurements²¹. We did not separate rigor dwell time from pre-rigor dwell time in our Aₖₐ-state dwell time estimates because the laser-trap estimates indicate it is much shorter than that of the AMADP state in our study.

References


19. Rossmanith GH, Hoh JF, Kirman A, Kwan L.J. Influence of V1 and V3 isomyosins on the mechanical behaviour of rat papillary muscle as studied by pseudo-random binary noise
