Comparison of Crossbridge Dynamics Between Intact and Skinned Myocardium From Ferret Right Ventricles

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This study compares the crossbridge kinetics of intact and skinned preparations from ferret cardiac muscles at 20°C to determine whether skinning causes any alteration in the crossbridge response to an imposed length change. A papillary or trabecular muscle was isolated from the right ventricle, the muscle length adjusted to give the maximum twitch tension (T\text{max}), and the preparation was subjected to Ba\textsuperscript{2+} contracture. When steady tension developed, the length of the preparation was perturbed sinusoidally in 19 discrete frequencies, ranging from 0.13 to 135 Hz, and at a small peak-to-peak amplitude (0.25% T\text{max}). We identified three exponential processes in the sinusoidal force–response to the imposed length oscillation, and these were labeled processes B, C, and D in order of increasing speed. A slow process, A, normally present in fast-twitch skeletal muscles, is very small or absent in cardiac muscles. Process B is an exponential delay, and the muscle produces oscillatory work on the forcing apparatus; processes C and D are exponential advances in which the muscle absorbs work. The preparation was chemically skinned and activated in the presence of (mM) Ca\textsubscript{EGTA} 6 (pCa 4.55), MgATP 5, magnesium propionate 1, and phosphate 1, pH 7.0, with ionic strength adjusted to 200 mM with potassium propionate. We found that the crossbridge kinetics were not altered by the skinning procedure. The apparent rate constants extracted from the sinusoidal analysis were nearly identical in Ba\textsuperscript{2+} contracture (intact preparation) and in Ca\textsuperscript{2+} activation (skinned preparation), and the Nyquist plots were similar. Because the rate constants changed sensitively with the substrate (MgATP) concentrations, we concluded that the substrate is adequately supplied during Ba\textsuperscript{2+} contracture in the intact preparation. Our study demonstrates the compatibility of results obtained from an intact and from a skinned preparation. (Circulation Research 1991;68:772–781)

It now is known that macromolecular projections from the thick filament, called myosin crossbridges, are responsible for force generation\textsuperscript{1} in all muscle types. These crossbridges cyclically interact with the actin that makes up the thin filaments and transduce chemical energy stored in ATP into useful work.\textsuperscript{2−4} It also is known that the myosin and ATP complex goes through a series of state changes as the complex interacts with actin.\textsuperscript{5−8} Thus, to have a thorough understanding of the transduction process, it is necessary to establish the elementary steps\textsuperscript{9} of contraction and to determine the kinetic constants that identify these steps. These studies must be performed on a structured muscle system so that steric constraints exist; otherwise, the kinetic constants may not have a realistic meaning. It is preferable to conduct experiments during maximal activation so that complications from partial activation can be avoided.

We approached this problem by applying small length perturbations to an isometrically contracting muscle and by following concomitant tension changes. With this method, it is possible to deduce the rate constants that specify crossbridge kinetics\textsuperscript{10−13} and to examine how these rate constants might be modified by environmental changes. We developed this technique with fast-twitch skeletal muscles and found it useful for assessing crossbridge kinetics. We now apply the technique to cardiac muscles to elucidate the crossbridge cycle scheme in this preparation.

The first step is to study the crossbridge kinetics of an intact myocardium and obtain the basic response

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of myocytes when contractile proteins are bathed in the natural cytosol. In a cardiac preparation, however, it is difficult to produce a steady contraction similar to the tetanus condition in skeletal muscles. This difficulty can be overcome by the use of Ba\(^{2+}\) contracture, during which tension can be maintained for many minutes. It is known that Ba\(^{2+}\) enters myocytes\(^{14}\) and binds to troponin C to induce a contraction.\(^{15}\) Several groups have successfully used this method of activation to characterize isometric tension and related crossbridge properties of myocardium.\(^{16-20}\) These studies indicated that the crossbridge kinetics can be followed by the perturbation analysis method.

The next step is to use a skinned preparation. In this system, one can perfuse ions of experimental interest into the myofilament lattice space normally occupied by the cytosol and follow their effects on contractile proteins. By studying the effects of MgATP, MgADP, and phosphate ions, one can create a crossbridge scheme and deduce necessary kinetic constants.\(^{21,22}\) Furthermore, with the skinned preparation, it may be possible to find solution conditions that give rise to the same crossbridge kinetics as in the intact preparation. Since we reported that the muscle in Ba\(^{2+}\) contracture preserved cellular integrity,\(^{23}\) it is possible to determine whether the crossbridge kinetics are the same in Ba\(^{2+}\) contracture in intact preparations and in Ca\(^{2+}\) activation in skinned preparations. We further examine whether solution parameters are correctly chosen to produce comparable results.

### Materials and Methods

#### Chemicals and Solutions

H\(_4\)EGTA, Na\(_2\)H\(_2\)ATP, Na\(_3\)CP (creatine phosphate), KH\(_2\)PO\(_4\), KHPO\(_4\), H\(_2\)O, glucose, 3-(N-morpholino)propanesulfonic acid (MOPS), and glutaraldehyde were purchased from Sigma Chemical Co., St. Louis; H\(_2\)EDTA, CaCO\(_3\), KOH, CaCl\(_2\) \(\cdot\) 2H\(_2\)O, BaCl\(_2\) \(\cdot\) 2H\(_2\)O, KCl, MgCl\(_2\) \(\cdot\) 6H\(_2\)O, NaH\(_2\)PO\(_4\), H\(_2\)O, MgO, NaCl, NaOH, propionic (Prop) acid, and Triton X-100 were purchased from Fisher Scientific Co., Itasca, Ill. Creatine kinase (CK) was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and NaHCO\(_3\) from EM Science, Cherry Hill, N.J. All chemicals were of analytical grade.

Tyrode’s solution and its variations were used for intact muscle experiments. The control Tyrode’s solution contained (mM) NaCl 130, CaCl\(_2\) 2.5, KCl 4, MgCl\(_2\) 1, NaH\(_2\)PO\(_4\) 0.435, NaHCO\(_3\) 10, and glucose 5.6. CaCl\(_2\) was deleted in calcium-free Tyrode’s solution and was replaced with 2 mM BaCl\(_2\) for a solution to induce Ba\(^{2+}\) contracture. These were stored in separate baths (100 ml each), which were constantly oxygenated with a gas mixture (98% O\(_2\)-2% CO\(_2\)) and transfused. When equilibrated with this gas mixture, these solutions attained a Po\(_2\) of 540–570 mm Hg and a pH of approximately 7.4,\(^{25}\)

For skinned preparations, the skinning solution contained\(^{22}\) (mM) EGTA 5, MgATP 2, free ATP 5, NaProp 132, imidazole 6, and 1% (vol/vol) Triton X-100. The relaxing solution contained EGTA 6, MgATP 2, free ATP 5, inorganic phosphate (P) 8, KProp 48, NaProp 62, and MOPS 10. The wash solution contained MgATP 0.3, P, 8, KProp 103, NaProp 75, and MOPS 10. The activating solution contained CaEGTA 6, MgATP 6.1, MgProp 0.54, KP 1, Na\(_3\)CP 15, NaProp 26, KProp 71, NaN\(_3\) 10, MOPS 10, and 160 units/ml CK. (CK unit is as specified by Boehringer Mannheim Biochemicals.) CaEGTA was not initially included in the activating saline and was added as needed. NaN\(_3\) was used to inhibit mitochondrial ATPase. Rigor solution contained (mM) P 8, NaProp 76, KProp 103, and MOPS 10. After induction of rigor, 3.6 mM EDTA was added to the rigor solution.

In solutions used for skinned muscle experiments, total sodium was maintained at 78 mM, the ionic strength was adjusted to 200 mM, and pH was adjusted to 7.00±0.01. EGTA, CaEGTA, EDTA, and P, were added as neutral potassium salts, MgATP and CP as neutral sodium salts, and free ATP as Na\(_3\)K\(_2\)ATP (a neutral salt). Individual concentrations of multivalent ionic species were calculated using our computer program, which assumed multiple equilibria with the following apparent association constants (log values at pH 7.00): CaEGTA, 6.28; MgEGTA, 1.61; CaATP, 3.70; MgATP, 4.00; CaCP, 1.15; and MgCP, 1.30. According to this calculation, pCa of the activating solution was 4.55; Mg\(^{2+}\), 1 mM; MgATP\(^{2-}\), 5 mM; and free ATP, 0.5 mM.

#### Intact Muscle Preparations

Male ferrets (approximately 800 g body wt) were anesthetized with sodium pentobarbital (80 mg/kg i.p.), hearts were removed quickly, and right ventricular papillary or trabecular muscles were excised and placed in a Petri dish that contained oxygenated Tyrode’s solution. Each end of the excised muscle was tied by a silk thread to a small tungsten wire (125-μm diameter) hook in the dissecting dish.

The preparation was transferred to an experimental chamber and mounted horizontally between a length driver and a tension transducer. The experimental chamber likewise contained oxygenated and transfused Tyrode’s solution (1 ml), and its temperature was controlled to 20.2±0.2°C. The muscle was electrically stimulated with a pulse of 5-msec duration and a voltage of 20–50% above the threshold level via a pair of platinum plate electrodes that were 7 mm apart and mounted alongside the preparation. The preparation was stabilized by repetitive stimuli at the rate of 12 per minute for more than 60 minutes before it was used for experiments.

The muscle length was adjusted to give the maximum twitch tension; this length was termed L\(_{\text{max}}\). At this point, the diameter of the preparation was determined with an ocular micrometer, and the cross-sectional area was estimated. The muscle...
length ranged from 1.8 to 3.3 mm, and the diameter ranged from 140 to 500 μm. These values were used to calculate tension and elastic and viscous moduli of intact preparations shown in Figure 1 and Table 1. The sarcomere length, determined by optical diffraction using a He-Ne laser, ranged from 2.2 to 2.3 μm at Lmax, in agreement with earlier work.24–26

Skinned Muscle Preparations

An intact preparation was placed in the calcium-free Tyrode’s solution for approximately 20 minutes, until the twitch contraction disappeared. The preparation was subsequently treated with the chemical skinning solution (1 ml) that included 1% Triton X-100 for 60 minutes.27 The skinning solution was agitated constantly, and its temperature was maintained at 20°C. The skinned muscle then was bathed in a relaxing solution (1 ml). At this point, the muscle length, the sarcomere length, and the diameter were measured. Although the muscle length and the sarcomere length did not change, the diameter increased by 11.0±0.7% (n=8) with skinning. The diameter thus obtained was used for calculating the tension and elastic and viscous moduli of the skinned preparations shown in Figure 2 and Table 1. To compare the intact and skinned preparations, the tension and modulus values of the skinned preparations were adjusted by the average cross-sectional area increase (indicated by an asterisk in Table 1), because the diameter value before skinning was not always measured.

Sinusoidal Analysis

A preparation was subjected to small-amplitude sinusoidal length oscillations (peak-to-peak amplitude, 0.25% Lmax) at 19 discrete frequencies ranging from 0.13 to 135 Hz. Tension and length signals were digitized simultaneously and processed on-line to yield a complex modulus [Y(f), a frequency response function] that is defined as the ratio of stress to strain in the frequency domain (f). Stress is defined as the force (newton) per unit cross-sectional area (square meter), and strain is the fractional length change (dimensionless). The time required to collect one complex modulus spectrum was approximately 24 seconds. Data were corrected for the system response by using the frequency response function of a fixed preparation with 2.5% glutaraldehyde for 10 minutes. The frequency profile of the complex modulus data was displayed on a CRT screen for visual inspection of the results. The details of the sinusoidal analysis technique were published previously.10,12

Results

Experiments With Intact Myocardium

Of seven preparations used for experiments with intact myocardium, five were papillary muscles and two were trabecular muscles. A preparation was dissected and mounted in the control Tyrode’s solution, and then it was incubated 9–21 minutes in a Ca2+-free Tyrode’s solution until the twitch tension disappeared. The purpose of this step was to obtain steady Ba2+ contracture without spontaneous twitch contractions.16,18 The sinusoidal analysis was performed at this time to obtain the complex modulus of the relaxed muscle (Figure 1, triangles with dotted lines). Ba2+ contracture then was induced. As shown earlier,18,19,28 2 mM BaCl2 was sufficient to induce Ba2+ contracture. This condition produced a stable tension that could last in excess of 30 minutes. We performed the sinusoidal analysis in the initial 5–11 minutes because a nuclear magnetic resonance study has demonstrated previously that the ATP and phosphocreatine levels drop after 25 minutes in Ba2+ contracture in rabbit papillary muscles.29 In our experience, however, there was no change in the rate constants of exponential processes for the initial 34 minutes, the longest period for which we followed the time course. We infer from this observation that the rigor state did not develop and that the ATP concentration remained at the physiological level during this period of time under our experimental conditions.

The complex modulus data Y(f) are plotted in Figure 1. The open circles represent data obtained from muscles in Ba2+ contracture. The dynamic modulus (=|Y(f)|) and phase shift [=argY(f), the tension change relative to the length change] are plotted against frequency in Figures 1A and 1B, respectively. Figure 1A shows that the dynamic modulus assumes the minimum value at approximately 1 Hz and increases appreciably toward the higher frequency. Figure 1B shows that phase shift is negative at frequencies of 0.5 Hz or less and becomes positive at frequencies of 0.7 Hz or more, exhibiting a biphasic profile. Figure 1C is a Nyquist plot, which is a plot of the real part of Y(f) in the abscissa and the imaginary part of Y(f) in the ordinate. This plot is convenient for the identification of exponential processes, because one semicircle represents one exponential process.30 The data shown in Figure 1 are the averaged results over seven experiments (the averaging was performed at each frequency); the individual data were similar to those shown in Figure 1.

Since three semicircles (plotted with open circles) are identified in Figure 1C, we assume that Y(f) can be resolved into three exponential processes labeled (B), (C), and (D):

\[ Y(f) = H - B/(1+b/\omega) + C/(1+c/\omega) + D/(1+d/\omega) \]  

(1)

where \( i = \sqrt{-1} \). The three processes are marked in Figure 1C. We found that in activated myocardium, a slow process A was either very small or absent; hence, we did not include this term in Equation 1. Process A normally is present in fast-twitch skeletal muscles10,12,23 and corresponds to phase 4 of step analysis.32,33 In Equation 1, we denote the characteristic frequency of an exponential process by a lowercase letter (b, c, d) and each magnitude by an uppercase letter (B, C, D). H
is the elastic modulus extrapolated to zero frequency. We also define the elastic modulus extrapolated to the infinite frequency as $Y_\infty = H - B + C + D$. This parameter represents the number of attached crossbridges.

Process B has a negative polarity in Equation 1 because the corresponding arc goes through the fourth quadrant in the Nyquist plot (Figure 1C) and the phase shift is negative (Figure 1B). This fact shows that, at frequencies around $b$, the muscle generates "oscillatory work" on the forcing apparatus. In a cardiac preparation, magnitude $B$ is smaller than magnitude $C$, and $b$ and $c$ are close together. Consequently, an arc going through the fourth quadrant is not as evident as in fast-twitch skeletal muscles or insect muscles. Instead, process C appears to have an extra curvature at the lower frequency end (first quadrant). As a result, the frequency at which the maximum oscillatory work is produced is approximately 0.35 Hz; this is lower than the characteristic frequency $b$ (approximately 1.2 Hz). Process B corresponds to "phase 3" or "delayed tension" in step analysis, and $2\pi b$ corresponds to the apparent rate constant of this phase.

In contrast, the muscle absorbs work from the forcing apparatus at frequencies around $c$ and $d$. These high-frequency components correspond to phase 2 of step analysis. Our results (Figure 1C) indicate that there are two exponential processes identifiable in the high-frequency range, and their corresponding rate constants are $2\pi c$ and $2\pi d$. Positive polarity of processes C and D in Equation 1 indicates that they are exponential advances. The correlation between processes of sinusoidal analysis and phases of step analysis were published previously.

Solid lines in Figure 1 represent the result of fitting the data to Equation 1. In the Nyquist plot, calculated values by Equation 1 are also entered as filled circles (Figure 1C). As is seen in these plots, fitting is satisfactory, and Equation 1 adequately describes the complex modulus data of a cardiac preparation in Ba$^{2+}$ contracture in the frequency range we observed. The rate constants $2\pi b$, $2\pi c$, and $2\pi d$ are found to be, on the average ($n=7$), $7.6\pm0.7$, $26\pm2$, and $682\pm62$ sec$^{-1}$, respectively ($\pm1$ SEM throughout the text). These results are summarized in Table 1.

The isometric tension ($P_0$) developed in Ba$^{2+}$ contracture was on the average 49±4 kN/m$^2$. This value is slightly smaller than that reported in maximally tetanized ferret papillary muscle (63±19 kN/m$^2$); in separate studies it was reported that Ba$^{2+}$ tension was less than Ca$^{2+}$ tension in rabbit papillary muscles. After Ba$^{2+}$ contracture, the bathing solution was replaced with the control Tyrode’s solution. In 11–15 minutes, the regular twitch response resumed when stimulated electrically. We infer from this observation that the Ba$^{2+}$ contracture does not cause a permanent change in the preparation and that the muscle recovers from the Ba$^{2+}$ contracture.

Experiments With Skinned Myocardium

Skinned myocardium was prepared as described above. Of seven preparations used for this part of the
TABLE 1.  Tension and Parameters of Exponential Processes of Activated Cardiac Muscles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Intact</th>
<th>Skinned</th>
<th>Skinned*</th>
<th>Units</th>
<th>$p$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension</td>
<td>$P_0$</td>
<td>49±4</td>
<td>26±2</td>
<td>32±3</td>
<td>kN/m$^2$</td>
<td>0.005</td>
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<tr>
<td>Rate constant</td>
<td>2$\tau_b$</td>
<td>7.6±0.7</td>
<td>8.6±0.7</td>
<td>8.6±0.7</td>
<td>sec$^{-1}$</td>
<td>0.33†</td>
</tr>
<tr>
<td>Rate constant</td>
<td>2$\tau_c$</td>
<td>26±2</td>
<td>30±2</td>
<td>30±2</td>
<td>sec$^{-1}$</td>
<td>0.18‡</td>
</tr>
<tr>
<td>Rate constant</td>
<td>2$\pi d$</td>
<td>682±62</td>
<td>703±52</td>
<td>703±52</td>
<td>sec$^{-1}$</td>
<td>0.80‡</td>
</tr>
<tr>
<td>Constant</td>
<td>$H$</td>
<td>0.33±0.05</td>
<td>0.27±0.04</td>
<td>0.34±0.05</td>
<td>MN/m$^2$</td>
<td>0.89§</td>
</tr>
<tr>
<td>Magnitude</td>
<td>$B$</td>
<td>0.82±0.09</td>
<td>0.58±0.05</td>
<td>0.72±0.06</td>
<td>MN/m$^2$</td>
<td>0.37§</td>
</tr>
<tr>
<td>Magnitude</td>
<td>$C$</td>
<td>2.09±0.14</td>
<td>1.28±0.12</td>
<td>1.57±0.14</td>
<td>MN/m$^2$</td>
<td>0.02</td>
</tr>
<tr>
<td>Magnitude</td>
<td>$D$</td>
<td>0.90±0.10</td>
<td>0.48±0.07</td>
<td>0.59±0.08</td>
<td>MN/m$^2$</td>
<td>0.03</td>
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<tr>
<td>Elastic modulus</td>
<td>$Y_s$</td>
<td>2.50±0.18</td>
<td>1.44±0.13</td>
<td>1.78±0.16</td>
<td>MN/m$^2$</td>
<td>0.01</td>
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<tr>
<td>Ratio</td>
<td>$P_0/Y_s$</td>
<td>2.07±0.25</td>
<td>1.85±0.13</td>
<td>1.85±0.13</td>
<td>%$L_{max}$</td>
<td>0.45‡</td>
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<tr>
<td>Experiments</td>
<td>$n$</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SEM. $L_{max}$, muscle length to give maximum twitch tension.

†Adjusted for cross-sectional area increase ($=1.11^2$). Student’s $t$ test was performed between the intact and adjusted skinned values, and the $p$ value is shown.

§Statistically insignificant ($p>0.05$).

experiments, four were papillary muscles, and three were trabecular muscles; two of the papillary muscles were the same preparation used for experiments with intact myocardium. Before activation, the preparation was washed to remove EGTA; then the solution was replaced twice with a "base solution" that contained all ingredients necessary for activation except for CaEGTA. The muscle was equilibrated for approximately 2 minutes in this base solution and subjected to the sinusoidal analysis to obtain the complex modulus of relaxed muscle. The results are shown in Figure 2 (triangles and dotted lines). This was followed by an injection of 66 mM CaEGTA in 10 mM MOPS (pH 7.00) into the bath (1:10 in volume) for the final concentration of 6 mM Ca$^{2+}$. Changes in pH due to mixing were minimal ($\leq0.02$), and the resulting pCa was 4.55.

As reported previously, we found that pCa 4.55 is adequate to generate maximum calcium tension. The Mg$^{2+}$ concentration of the activating solution was 1 mM; P$_i$, 1 mM; total ATP, 6.1 mM; and pH 7.00. We chose these particular ionic conditions because it was reported in other muscle types that the intracellular Mg$^{2+}$ concentration is approximately 1 mM,38,39 P$_i$ is 1.4 mM,40 total ATP is 6.2 mM,41 and pH is approximately 7.0.42 Intracellular ionic compositions in frog skeletal muscles were summarized by Godt and Maughan.43

When a steady tension developed, the sinusoidal analysis was performed. Complex modulus results averaged over seven experiments are plotted in Figure 2 (circles). As seen in Figure 2, the frequency profile of the Ca$^{2+}$ activation is similar to that of Ba$^{2+}$ contracture, and Equation 1 adequately describes the complex modulus data from Ca$^{2+}$ activation. The rate constants 2$\pi b$, 2$\pi c$, and 2$\pi d$ are found to be, on the average ($n=7$), 8.6±0.7, 30±2, and 703±52 sec$^{-1}$, respectively. These values are slightly higher than those from the intact preparation, but their difference is not statistically significant ($p>0.05$, Table 1). $P_0$ measured with Ca$^{2+}$ activation was 26±2 kN/m$^2$ (Table 1), and it was about half of the tension measured in Ba$^{2+}$ contracture. Our $P_0$ value compares to 20.7 kN/m$^2$ on skinned trabeculae of guinea pig.44 Magnitude parameters in a skinned preparation also were about half of those in an intact preparation. However, it can be deduced from Table 1 that the relative significance ($P_0:B:C:D$) is approximately the same between intact and skinned preparations.

The tension to stiffness ratio, $P_0/Y_s$, represents the degree of instantaneous length release required to abolish full active tension. The ratio was found to be 2.07% and 1.85% (Table 1) for intact and skinned preparations, respectively, and their difference is not statistically significant (Table 1). In comparison, Polack and Krueger estimated the ratio to be 1.6% by measuring sarcomere length changes in the midportion of rat myocardium; Shibata and his colleagues observed 1.8% by a segment-clamp experiment in rabbit papillary muscles. These values are not very different from our measurements, indicating that the contribution of the damaged end (if any existed) should be very small in our preparations. These values of myocardium compare to 1.2% in skinned rabbit sapho fibers and 0.4% in intact frog muscle fibers. The reason for the difference between different muscle preparations is not immediately apparent.

After the experiments were finished, the preparation was activated again and subsequently brought into the "high rigor" state by three washes with the rigor solution. To remove magnesium, concentrated K$_2$EDTA (40 mM, pH 7.00) was added for the final concentration of 3.6 mM as soon as steady rigor tension developed (approximately 3 minutes). The complex modulus then was measured and plotted in Figure 2 (squares and broken lines). The complex modulus of the rigor preparation is characterized by a small phase advance (2–4°) and the large dynamic modulus that increases slightly with an increase in frequency. This
FIGURE 2. Complex moduli of skinned preparations averaged over seven experiments. Triangles: Data obtained while preparations were in relaxing solution. Circles: Data obtained during Ca\textsuperscript{2+} activation. Open circles represent actual data, closed circles and solid lines represent theoretical projections based on Equation 1 (see text). Squares: Data obtained after rigor induction. Units of all moduli are meganewtons per square meter. Frequencies used were 0.13, 0.25, 0.35, 0.5, 0.7, 1.0, 1.4, 2, 3.2, 5, 7.5, 11, 17, 25, 35, 50, 70, 100, and 135 Hz.

Panel A: Dynamic modulus plotted against frequency; panel B: phase shift plotted against frequency; panel C (Nyquist plot): viscous modulus (ordinate) plotted against elastic modulus (abscissa). For activated preparations, three frequency points (1, 11, 100 Hz) are indicated; for relaxed preparations, only two frequency points (0.13, 100 Hz) are shown, and these are almost superimposed with the 1 Hz point of Ca\textsuperscript{2+} activation; for preparations in rigor, only five frequency points (0.13, 0.25, 1, 11, 100 Hz) are shown.

FIGURE 3. Mechanical equivalence of activated cardiac muscles. H, C, and D are elasticity; component (B) is the power generator; components (C) and (D) are devices to dissipate work and to generate heat. Viscosity of dashpots are $Q_c$ and $Q_d$ for (C) and (D), respectively. Maximum energy exchange occurs at frequencies $b$, $c$, and $d$ for devices (B), (C), and (D), respectively.

Frequency profile is consistent with the complex modulus of the rigor preparation of rabbit psoas.\textsuperscript{10}

Discussion

In the present study, we find that the myocardial complex modulus can be interpreted in terms of three exponential processes (B, C, and D) whether the method of activation is Ba\textsuperscript{2+} contracture (intact preparation) or Ca\textsuperscript{2+} activation (skinned preparation) (Figures 1 and 2, circles). These exponential processes are absent in the relaxed myocardium or when it is brought into the rigor state (Figures 1 and 2, triangles and squares). From these observations, we conclude that these three exponential processes reflect the kinetics of actively cycling crossbridges. An equivalent mechanical diagram to demonstrate these processes is shown in Figure 3, which correlates directly with Equation 1. The significance of this diagram is to demonstrate that three processes and the elasticity $H$ are arranged in parallel within a half sarcomere. In Figure 3, the component (B) is a force generator, and the components (C) and (D) are devices that dissipate mechanical energy. Our eventual goal is to determine the molecular correlates of these components.

Because the polarity of process B is negative and that of C is positive (Equation 1), the dynamic modulus decreases to the minimum at approximately 1 Hz (Figures 1A and 2A, circles). This is known as the minimum frequency ($f_{\text{min}}$) and is used among cardiac physiologists\textsuperscript{17,20} as an indicator of the crossbridge kinetics. In a cardiac preparation, $f_{\text{min}}$ is close to the characteristic frequency $b$. As mentioned, muscle produces oscillatory work when the phase shift is negative. This happens at the low frequency end of activated preparations (Figures 1B and 2B, circles). Interestingly, phase shift is slightly but consistently negative in intact myocardium during relaxation at frequencies between 0.35 and 0.7 Hz (Figure 1B, triangles). This observation indicates that the
muscle is slightly active and implies that a threshold level of Ca\(^{2+}\) may be present in the myoplasm when the muscle is placed in the Ca\(^{2+}\)-free Tyrode’s solution and oxygenated. This weak activity may relate to the mass transfer of the myosin head toward the thin filament observed in rat papillary muscles at the low Ca\(^{2+}\) concentration.\(^{47}\) The negative phase shift is much diminished in skinned and relaxed myocardium (Figure 2B, triangles).

In activated myocardium, we do not find process A, which normally is present in skeletal muscle\(^{10,31}\); instead, \(H\) assumes a significant value that is larger than the elastic modulus of relaxed myocardium (Figures 1 and 2). Previously, we attributed process A to a filament sliding that results in sarcomere rearrangements, because this component disappeared when a fraction of crossbridges was covalently cross-linked to actin in rabbit psoas fibers.\(^{48}\) It is possible that a rigid sarcomere structure of myocardium, represented by a steep rise in the length–tension diagram during diastole,\(^{24,26}\) is responsible for the absence of process A. Because of this absence, the appearance of process B is enhanced to cause a delayed rise in tension when the muscle length is increased. Thus, stretch activation, delayed tension rise, an absence of process A, and a large \(H\) seem to be the results of the same underlying mechanisms. Because of the presence of a large \(H\), the steady-state tension level increases as the muscle is stretched and after the transients subsided: if the stretch is \(\Delta L\), then the steady-state tension increase is \(H\Delta L\). This phenomenon is an addition to the Frank-Starling law\(^{49–51}\) at the crossbridge level, because it contributes to an increase in \(P_o\) in response to a stretch. It generally is agreed that an increased calcium availability in myoplasm and an increased calcium sensitivity of troponin C are primary causes of the Frank-Starling law.\(^{49}\)

The Nyquist plots of activated cardiac preparations (Figures 1C and 2C, circles) are reminiscent of those from rabbit soleus and semitendinosus fibers.\(^{33,52}\) The complex moduli of these slow-twitch fibers are represented by a large process C and smaller processes B and D; process A is almost nonexistent. It is reasonable that the Nyquist plot of cardiac preparations is similar to that of slow-twitch fibers, when we realize that slow myosin light chains are common in both muscle types\(^{53}\) and that the \(\beta\)-isoform of myosin heavy chain is either identical in both muscle types\(^{54,55}\) or homologous.\(^{56}\) It also is known that the myosin in ferret myocardium is primarily the \(V_\beta\) (\(\beta \)) isoform.\(^{57}\)

Our Nyquist plots from activated preparations are similar to that of Steiger\(^{58}\) on freeze-dried rabbit papillary muscles for the frequency range studied (\(\leq 5\) Hz). The ratio \(B:C\), which indicates the relative significance of process B with respect to process C, appears to be larger in Steiger’s report\(^{58}\) than in the current report. It is possible that extra cross-links may have formed between thick and thin filaments in a freeze-dried preparation, and these cross-links would have contributed to the enhanced appearance of process B, as happens in partially cross-linked psoas fibers.\(^{38}\) Our observation that processes B and C are present and process A is absent in myocardium is consistent with the prediction based on the time course of tension transients in response to step length changes in myocardium.\(^{16,23,34}\) Our results also are consistent with the dynamic stiffness of myocardium.\(^{18,19}\)

Our results show that the rate constants of intact and skinned preparations are almost identical (Table 1) and Nyquist plots from these preparations are similar (Figures 1C and 2C). We infer from these observations that the composition of our activating solution is a close approximation to the in situ myoplasmic constituents. This inference is based on our earlier observation in rabbit skeletal fibers that processes B and C are sensitive to MgATP concentration\(^{12,13,21}\) and that process B also is sensitive to P, and Ca\(^{2+}\) concentrations.\(^{11,12,22}\) Our preliminary observations on cardiac preparations (unpublished data) indicate similar MgATP effects. A slight shift in frequency plots between intact and skinned preparations (Figures 1A and 1B, and 2A and 2B) or a slight difference in the rate constants (Table 1) could be compensated for if the MgATP concentration in skinned preparations is lowered slightly. The solution parameters we chose are close to cytosolic compositions known for other muscles. These are 1 mM for Mg\(^{2+}\), \(0.1,0.2\) 1.4 mM for P,\(^{40}\) and 6.2 mM for total ATP,\(^{41}\) pH 7.0,\(^{42}\) and are reviewed by Godt and Maughan.\(^{43}\)

Our results differ from those of Rossmannith et al\(^{17}\) (see their Figures 3 and 4), which showed that skinned myocardium exhibited approximately 2 times faster \(f_{\text{min}}\) (approximately our characteristic frequency \(b\)) than intact myocardium in 3–5-week-old rats that had exclusively \(V_\beta\) myosin isoform. This difference may relate to the fact that they used 10 mM MgATP for skinned fiber experiments; it also is possible that MgATP and P, sensitivities are different depending on the type of myosin isoforms and the animals. Our results, which show that the rate constants are approximately the same in intact and skinned myocardium (Table 1), imply that skinned myocardium can be made to behave similarly to intact myocardium under our experimental conditions. This validates skinned myocardium as a preparation to test crossbridge dynamics. Our results further demonstrate that the myocardium in Ba\(^{2+}\) contracture maintains a steady and adequate cytosolic MgATP supply and that Ba\(^{2+}\) contracture is a useful method of activating an intact myocardium to study contractile mechanisms.

One may ask whether the crossbridge kinetics with a Ba\(^{2+}\) activation are the same as with a Ca\(^{2+}\) activation. In earlier studies, it was shown that Ba\(^{2+}\) enters the myocyte via Ca\(^{2+}\) channels\(^{14}\) and binds to troponin C to activate thin filaments.\(^{15}\) It also was shown, in skinned myocardium, that tension transients in response to step length changes\(^{37}\) and frequency-dependent stiffness spectra\(^{17}\) were similar in Ba\(^{2+}\) activation to those in Ca\(^{2+}\) activation, whereas
the sensitivity of the myofilaments to Ba2+ was lower than that to Ca2+. Thus, it seems safe to assume that the crossbridge kinetics are not different between the maximum Ca2+ and Ba2+ activations. The phase that corresponds to process D has not been reported in myocardium previously17-19,58 because of the low frequency range used (<60 Hz) in earlier studies. In glycerinated skeletal and insect fibrillar muscles, there is an initial fast recovery phase followed by a slower recovery phase after stretch.59 The initial phase corresponds to process D found in the present study. The slower phase corresponds to process C and reflects detachment of cross-bridges.12,13,21,59 In comparison, Ford et al58 fitted phase 2 of electrically stimulated intact frog fibers into four exponentials; Shimizu and Tanaka60 fitted the same phase of skinned rabbit psoas fibers up to two to three exponentials. The significance of process D or faster elements of phase 2 in a crossbridge cycle remains to be established.

In Table 1, the P0 value measured in a skinned preparation with Ca2+ activation (26 kN/mm2) was about half that of an intact preparation in Ba2+ contracture (49 kN/mm2). The magnitude parameters were likewise diminished in the skinned preparation. There are two possible reasons for diminished tension and magnitude parameters: 1) a swelling of the lattice spacing, or 2) a mismatch in ionic strength. We observed, on the average, 11% expansion of the muscle width after skinnning. This value compares with 28% on mechanically skinned frog semitendinosus fibers61 or to 8% on saponin skinned murine toe muscles.62 We used a cross-sectional area of the skinned preparation for tension and modulus normalization in the skinned preparation; this alone accounts for 23% of the lower tension (1.11^2=1.23). For this reason, the tension and modulus values were recalculated after the factor of the area increase was considered (Table 1, column indicated by an asterisk). With this correction, the magnitude parameters H and B are not statistically different between the intact and skinned preparations (Table 1), and tension is increased to 32 kN/mm2. In addition, compressing the skinned fibers to the intact level (e.g., with 3–4% dextran T500) is known to increase P0 in skeletal muscles63; this increase accounts for another 20–25% of the tension.

The ionic strength (200 mM) we used for activating skinned preparations may be higher than that of cytosol: Godt and Maughan63 estimated intracellular ionic strength of frog fibers to be 178 mM when the effect of the fixed charges along contractile filaments is ignored. The P0 value increases when the ionic strength is lowered without significantly changing the rate constants in skeletal fibers6; a decrease of 22 mM would increase P0 by approximately 10%. A combination of these factors may explain the apparently lower tension in skinned fibers. Likewise, the combination explains the diminished magnitude parameters of exponential processes in skinned fibers. We infer from these facts that the number of attached crossbridges may be approximately 10% less in skinned preparations than in intact preparations under our experimental conditions.

Although the P0 of skinned myocardium during Ca2+ activation (26 kN/m2) is similar to that (20.7 kN/m2) reported by Kentish,44 it is less than that on skinned myocytes (114 kN/m2) reported by Fabiato.64 This is because the presence of collagen and other extracellular elements in myocardium account for 40% of myocardial volume65 and because Fabiato carried out his experiments at lower ionic strength (160 mM), as discussed by Kentish44 and Yue and his colleagues.36

In conclusion, we showed that the crossbridge kinetics of a skinned myocardium can be made to behave similarly to those of an intact myocardium by choosing solution parameters. Although experiments with the intact myocardium offer basic kinetic data when the contractile proteins are bathed in the natural cytosol, those with skinned myocardium offer insights into chemical modifications of crossbridge kinetics. These data are essential to the characterization of molecular mechanisms of contraction both in normal and pathological conditions of myocardium.

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**KEY WORDS**

- crossbridge kinetics
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Comparison of crossbridge dynamics between intact and skinned myocardium from ferret right ventricles.

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