Impact of osmotic compression on sarcomere structure and myofilament calcium sensitivity of isolated rat myocardium

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MYOFILAMENT LENGTH-DEPENDENT activation (LDA) is a phenomenon found in all striated muscle, but to varying extents, being a prominent feature of cardiac muscle (2, 4, 7, 15, 16) and the indirect flight muscle of insects (33) but is less prominent in skeletal muscle (17, 26, 32). LDA is defined as an increase in the amount of active force developed at longer sarcomere length for a given concentration of activating calcium ions. LDA is the underlying cellular mechanism that underlies the Frank-Starling law of the heart (16). This increase in myofilament calcium sensitivity is commonly measured by the EC50 parameter, that is, the calcium concentration at which the active developed force is half that observed at a saturating calcium concentration. An understanding of the molecular mechanism(s) underlying LDA has remained elusive. A popular hypothetical mechanism for LDA is that changes in interfilament lattice spacing, usually estimated as changes in fiber width, are directly linked to changes in myofilament calcium sensitivity and active tension development. Much of the evidence for this view has come from experiments that show that when fiber width (and presumably, lattice spacing) is reduced by an externally applied osmotic pressure, myofilament calcium sensitivity increases (1, 3, 8, 9, 11, 22, 25, 34–36). The consensus interpretation of these data (the lattice spacing hypothesis) is that the decrease in lattice spacing induced by sarcomere lengthening is the important factor in myofilament length-dependent activation and that this effect can be mimicked at a shorter sarcomere length by artificially compressing the myofilament lattice with large polymeric compounds, such as Dextran T500.

However, Konhilas and colleagues (16, 18), using X-ray diffraction to directly measure myofilament lattice spacing as a function of sarcomere length and osmotic pressure, demonstrated that there are many circumstances where changes in lattice spacing are not well correlated with changes in myofilament calcium sensitivity. For example, when a small amount of dextran (1%) was used to shrink the myofilament lattice so as to mimic the lattice spacing change induced by a change in sarcomere length, myofilament calcium sensitivity was found to be unaffected. These findings are not consistent with the lattice spacing hypothesis, as are other recent observations from our laboratory, as reviewed by Konhilas et al. (16); hence, the role of interfilament spacing in modifying myofilament calcium sensitivity is at present unclear. Instead, there may be an osmotically sensitive mechanism within the sarcomere that plays a role in myofilament calcium sensitivity which is not directly related to lattice spacing. This led us to propose that the structural changes responsible for the increase in myofilament calcium sensitivity upon osmotic compression by dextran include changes in the structure of the thick filament. If true, this would have important implications for the interpretation of all experiments on striated muscle that use osmotic compressive agents, such as dextran.

Here we tested our hypothesis in skinned myocardium by correlating sarcomere structure with myofilament calcium sensitivity over a wide range of externally applied osmotic pressure at sarcomere length of 2.20 μm. We found that whereas lattice spacing decreased monotonically with increasing osmotic pressure, myofilament calcium sensitivity increased discontinuously over a narrow range of osmotic pressure between 0.24 and 0.38 kPa. This abrupt transition in myofilament calcium sensitivity was well correlated with changes in the external osmotic pressure.

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relative position of the myosin heads as estimated by changes in the ratio of intensities of the 1,1 and 1,0 equatorial X-ray reflections ($I_{1,1}/I_{1,0}$). Both myofilament calcium sensitivity and the $I_{1,1}/I_{1,0}$ parameter remained stable with further osmotic compression up to 6.7 kPa (6% Dextran T500), despite the fact that the myofilament lattice continued to shrink. These findings provide support for a switch-like mechanism that governs myofilament calcium sensitivity that is highly sensitive to changes in externally applied osmotic pressure.

**MATERIALS AND METHODS**

**Solutions**

The composition of all the solutions used is listed in Table 1. Dextran T500 (Sigma Chemicals) was added to obtain a concentration range between 1% and 12% (g/100 ml). For the 0.4% and 0.7% dextran solutions, appropriate amounts of the 0% and 1% Dextran T500 solutions were mixed; the dextran concentrations were converted to osmotic pressure using a polynomial fit to the data of Ref. 29. Activating solution and relaxing solution were mixed to obtain activating solutions containing between 0.64 and 46.8 μM [Ca$^{2+}$] (pCa, 6.2–4.3).

**Skinned Rat Cardiac Trabeculae**

All experiments were performed according to University of Illinois at Chicago institutional guidelines concerning the care and use of experimental animals and has been given an Institutional Review Board number of 03-107. Male rats (LBNF-1) of approximately 250–400 g received injections of 50 mg/kg pentobarbital sodium and 1.5 ml heparin (18). While the animal was under deep anesthesia, the heart was excised, transferred to a dissection dish, and perfused retrograde with a modified Krebs-Henseleit solution containing (in mM) 118.5 NaCl, 5 KCl, 2 NaH$_2$PO$_4$, 1.2 MgSO$_4$, 10 glucose, 26.4 NaHCO$_3$, and 0.2 CaCl$_2$, as well as 20,2,3-butanedione monoxime (BDM) to inhibit spontaneous contractions. This solution, when aerated at room temperature with a 95% O$_2$:5% CO$_2$ mixture had a pH of 7.4. Only single, isolated, unbranched trabeculae located along the exterior wall connecting the myocardial valve to the right ventricular wall were removed for further experiments. The muscles were placed into a dish containing standard relaxing solution with 1% (vol/vol) Triton X-100 added to chemically permeabilize all membranes so as to allow solutes to flow freely in and out of the myofilament lattice.

**Mechanical Experiments**

For mechanical studies, the muscles were incubated in the “skinning” solution for 14–18 h at 4°C and then transferred to fresh relaxing solution. Trabeculae (1–2 mm long and 150–250 μm wide) were attached to aluminum T-clips and then mounted on hooks between a silicon strain gauge (model AE801, SenSonor, Horten, Norway) and a servomotor (~1 ms 90% step response; Cambridge model 308). Sarcomere length was adjusted and maintained at 2.2 ± 0.04 μm during activation using the first-order diffraction band from an He-Ne laser (15). If the final maximal calcium activation in the run generated <90% of the force of the initial full activation, the data for that fiber were discarded; all experiments were performed at 15°C. Force-[Ca$^{2+}$] relationships were fit individually to a modified Hill equation as previously described (18):

$$ F_{rel} = [Ca^{2+}]^{nH}[EC_{50}^{m} + [Ca^{2+}]^{m}] $$

where $F_{rel}$ is force as a fraction of maximum force at saturating [Ca$^{2+}$] ($F_{max}$); $EC_{50}$ is [Ca$^{2+}$], where the $F_{rel}$ is half of $F_{max}$; and $n_H$ is the Hill coefficient.

**X-ray Diffraction Experiments**

X-ray diffraction of relaxed skinned trabeculae. For X-ray diffraction experiments with a separate group of skinned rat cardiac trabeculae, the muscles were incubated in skinning solution for no more than 12 h. We found that longer incubation times resulted in greater myofilament lattice disorder and lower quality X-ray diffraction patterns; however, control experiments showed no effect of skinning time between <12 h and overnight (>12 h) upon myofilament calcium sensitivity. In this study we wanted to examine a large number of muscles quickly and easily under relaxing (pCa, 9) conditions; that is, where simultaneous force and sarcomere length measurements are not necessary. The muscle was, therefore, attached to T-clips and mounted into a simple chamber that was designed to allow for both easy interchange of solutions and quick mounting and mounting of cells in the X-ray diffraction apparatus. Use of this simplified setup allowed a collection of a sufficient number of measurements to ensure statistically significant results in the limited instrument time available. Sarcomere length was adjusted to 2.20 ± 0.04 μm and checked after the experiment to ensure that the sarcomere length was maintained throughout the exposure using video microscopy as previously described (4). We have found that sarcomere length measurements assessed by either light diffraction or video microscopy are comparable; that is, within the resolution of either technique (4). A single batch of solutions was used for both mechanical and X-ray diffraction experiments.

In the present experiments, more detailed X-ray patterns were desired than those used by Irving et al. (13); that is, those showing at least five diffraction orders (out to the 3,0) on the equator. To this end, each muscle was exposed to X-rays only three to seven times [as opposed to 12–16 times in Irving et al. (13)], depending on the quality of the patterns that were obtained. The concentration of Dextran T500 in the relaxing solution was varied over the range between 0% and 12% to yield externally applied osmotic pressures between 0 and 31 kPa (Table 2). The exposure time for each skinned muscle was increased from 1 to 2.5–5 s (with an incident flux of $~1 \times 10^{12}$ photons/s) to increase the intensity of the outer orders. The X-ray dose was spread over the muscle by translating it back and forth across the beam during the timed exposure so as to prevent degradation of the muscle due to radiation damage. Experiments were performed at room temperature (21°C). Previous experiments (18) showed that this small (15–21°C) temperature difference had no effect on the lattice spacings, consistent with previous results by others (20).

**Table 1. Composition of solutions used for cardiac mechanical and structural experiments**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Relaxation</th>
<th>Preactivation</th>
<th>Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic strength, mM</td>
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<td>180</td>
<td>180</td>
</tr>
<tr>
<td>pH</td>
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<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>pCa</td>
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<td>9</td>
<td>4.3</td>
</tr>
<tr>
<td>Free Mg$^{2+}$, mM</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mg$^{2+}$-ATP, mM</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>EGTA, mM</td>
<td>20</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>HDTA, mM</td>
<td>0</td>
<td>19.5</td>
<td>0</td>
</tr>
<tr>
<td>BES, mM</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>CrP, mM</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

Ca-EGTA was made by mixing equimolar amounts of CaCl$_2$ and EGTA. All solutions contained (in mM) 15 phosphocreatine (CrP), 40 N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), 0.1 PMSF, and 1 dithiothreitol. Free Mg$^{2+}$ and Mg-ATP concentrations were calculated at 1 and 5 mM, respectively (see Ref. 5). Relaxing and activating solutions were appropriately mixed to obtain a range of free [Ca$^{2+}$] by using an apparent Ca$^{2+}$ binding stability constant of 106.39 at 15°C. Preactivating solution with low Ca$^{2+}$ activity contained 1,6-diaminohexane-N,N,N,N-tetraacetic acid (HDTA).

Ionic strength of solutions was kept at 180 mM by adding an appropriate amount of potassium propionate. pH was adjusted to 7.0 at 20.0° with KOH.
MYOFILAMENT Ca\(^{2+}\) SENSITIVITY

Table 2. X-ray diffraction data

<table>
<thead>
<tr>
<th>%Dextran</th>
<th>kPa</th>
<th>n</th>
<th>d_{1,0}</th>
<th>\sigma_4</th>
<th>\sigma_5</th>
<th>1.1</th>
<th>2.0</th>
<th>2.1</th>
<th>3.0</th>
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<tbody>
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<td>0.0</td>
<td>0</td>
<td>24</td>
<td>41.0±0.23</td>
<td>5.4±0.24</td>
<td>0.89±0.15</td>
<td>0.30±0.02</td>
<td>0.01±0.003</td>
<td>0.02±0.005</td>
<td>0.034±0.004</td>
</tr>
<tr>
<td>0.4</td>
<td>0.12</td>
<td>18</td>
<td>40.7±0.23</td>
<td>4.8±0.13</td>
<td>0.60±0.06</td>
<td>0.31±0.01</td>
<td>0.02±0.002</td>
<td>0.02±0.002</td>
<td>0.032±0.002</td>
</tr>
<tr>
<td>0.7</td>
<td>0.24</td>
<td>6</td>
<td>39.6±0.11</td>
<td>4.0±0.08</td>
<td>0.61±0.06</td>
<td>0.30±0.01</td>
<td>0.02±0.004</td>
<td>0.03±0.005</td>
<td>0.034±0.006</td>
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<tr>
<td>1.0</td>
<td>0.38</td>
<td>21</td>
<td>38.7±0.20</td>
<td>4.7±0.15</td>
<td>1.22±0.27</td>
<td>0.42±0.04</td>
<td>0.04±0.009</td>
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<tr>
<td>2.0</td>
<td>1.02</td>
<td>22</td>
<td>38.0±0.24</td>
<td>5.1±0.18</td>
<td>1.25±0.21</td>
<td>0.46±0.04</td>
<td>0.03±0.007</td>
<td>0.03±0.003</td>
<td>0.037±0.003</td>
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<tr>
<td>3.0</td>
<td>1.95</td>
<td>17</td>
<td>36.5±0.11</td>
<td>4.7±0.14</td>
<td>0.87±0.16</td>
<td>0.45±0.04</td>
<td>0.03±0.009</td>
<td>0.04±0.006</td>
<td>0.041±0.006</td>
</tr>
<tr>
<td>4.0</td>
<td>3.17</td>
<td>19</td>
<td>35.4±0.10</td>
<td>4.7±0.16</td>
<td>0.60±0.10</td>
<td>0.44±0.03</td>
<td>0.03±0.007</td>
<td>0.04±0.005</td>
<td>0.043±0.006</td>
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<tr>
<td>5.0</td>
<td>4.68</td>
<td>17</td>
<td>34.6±0.08</td>
<td>4.5±0.12</td>
<td>0.93±0.24</td>
<td>0.47±0.04</td>
<td>0.04±0.008</td>
<td>0.04±0.006</td>
<td>0.041±0.004</td>
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<tr>
<td>6.0</td>
<td>6.62</td>
<td>17</td>
<td>33.8±0.09</td>
<td>4.4±0.13</td>
<td>0.78±0.23</td>
<td>0.46±0.03</td>
<td>0.03±0.007</td>
<td>0.04±0.007</td>
<td>0.044±0.006</td>
</tr>
<tr>
<td>7.0</td>
<td>9.06</td>
<td>22</td>
<td>32.9±0.11</td>
<td>4.8±0.11</td>
<td>0.71±0.20</td>
<td>0.43±0.03</td>
<td>0.03±0.006</td>
<td>0.04±0.008</td>
<td>0.058±0.019</td>
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<tr>
<td>8.5</td>
<td>13.90</td>
<td>4</td>
<td>31.9±0.10</td>
<td>5.4±0.24</td>
<td>0.98±0.22</td>
<td>0.44±0.05</td>
<td>0.01±0.007</td>
<td>0.03±0.008</td>
<td>0.003±0.002</td>
</tr>
<tr>
<td>10.0</td>
<td>20.06</td>
<td>4</td>
<td>31.7±0.10</td>
<td>5.4±0.36</td>
<td>0.95±0.08</td>
<td>0.37±0.05</td>
<td>0.02±0.009</td>
<td>0.04±0.005</td>
<td>0.007±0.004</td>
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<tr>
<td>12.0</td>
<td>30.90</td>
<td>4</td>
<td>30.7±0.32</td>
<td>5.8±0.23</td>
<td>1.62±0.28</td>
<td>0.39±0.02</td>
<td>0.01±0.007</td>
<td>0.08±0.024</td>
<td>0.055±0.032</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of fibers at each dextran concentration analyzed. X-ray diffraction data are reported as a function of Dextran T500 concentration, including range used for mechanical analysis. First 4 equatorial reflections are expressed as intensities normalized to 110 equatorial diffraction pattern as described previously (12). The pixel distances so measured were converted to d_{1,0} lattice spacing using Bragg’s law, which, for small angles, reduces to d = 2AL/\lambda where \lambda is the distance from the center to the reflection, L is specimen-detector distance (2 or 3 m), and \lambda is the wavelength of the X-rays (0.103 nm). The \(I_{1,1}/I_{1,0}\) intensity ratio can be used to estimate shifts of mass (presumably cross-bridges) from the region of the thick filament to region of the thin filament. The widths of the pseudo-Gaussian peaks representing the X-ray reflections, as used in the fitting procedure, will vary systematically with distance from the center depending on the relative amount of heterogeneity in interfilament spacing among the myofilaments (as estimated by the parameter \(\sigma_4\)) and the amount of paracrystalline (liquid-like) disorder (as estimated by the parameter \(\sigma_5\)) of the myofilaments in the hexagonal lattice (41). These parameters, \(\sigma_4\) and \(\sigma_5\), can therefore be used as a measure of changes in the relative amounts of each kind of disorder.

Statistical analysis and curve fitting. Data were analyzed by using ANOVA as implemented in the R statistical language (version 2.11.1) (31), and data were held to be significantly different at P < 0.05. Nonlinear curve fitting was performed using a Levenberg-Marquardt algorithm as implemented in the nls function of the R statistical language. Fisher ANOVA was used to compare linear models of data. This test compared the sums of squares and degrees of freedom for two candidate models, one simpler (more degrees of freedom) and one more complex (fewer degrees of freedom due to more parameters). Under the null hypothesis of no difference, the sums of squares should be distributed in proportion to the degrees of freedom. An F-ratio near 1 would give no evidence of lack of fit, and the simpler model would be preferred. Alternatively, an F-ratio much greater than 1 (P < 0.05) would provide evidence of the simpler models failure to fit the data, and the more complex model would be preferred.

RESULTS

Figure 1 shows average relative force-[Ca\(^{2+}\)] relationships obtained at increasing levels of applied osmotic compression (0–6% Dextran T500). As is apparent from these data, modest osmotic compression up to 0.7% dextran of the cardiac sarcomere was virtually without affect on myofilament calcium sensitivity. Further compression by 1% dextran then induced an abrupt, almost stepwise, increase in calcium sensitivity that was then unaffected by additional higher levels of applied osmotic compression.

This phenomenon is illustrated more clearly in Fig. 2, where myofilament calcium sensitivity, as indexed by the EC_{50} parameter, is plotted as a function of applied osmotic pressure. There is a dramatic decrease in EC_{50} over a narrow range of osmotic pressure (0.12–0.38 kPa; 0.4–1% dextran) with no further significant change in EC_{50} up to 6% Dextran T500 (an osmotic pressure of ~6.7 kPa). A one-way ANOVA showed a significant effect of osmotic pressure on EC_{50} (P < 0.01), and
Fig. 2. Average myofilament calcium sensitivity, as indexed by the EC_{50} parameter, plotted as a function of externally applied osmotic pressure over entire range studied and on an expanded scale (inset: 0–2 kPa). Between 0.24 and 0.38 kPa applied osmotic pressure, there was a marked and abrupt decrease in EC_{50}. Error bars represent means ± SE.

Tukey’s honest significant difference test with 90% confidence intervals showed two groups: 1) 0–0.24 kPa (0–0.7% dextran) and 2) 0.24–6.62 kPa (0.7–6% dextran). This indicates that there was no significant change in EC_{50} after the osmotic pressure exceeded 0.38 kPa (1% dextran). In addition, over the entire range of osmotic pressures studied, no significant changes in either passive or active tension were observed.

Table 2 displays the average intensities of the first five equatorial reflections from skinned trabeculae at various levels of osmotic compression (0 to ~31 kPa) along with the peak width parameters \( \sigma_d \) and \( \sigma_r \). Reflections past the 1,1 are much weaker than those observed in skeletal muscle (40) and do not vary much with osmotic compression. There is an appreciable amount of lattice spacing inhomogeneity (\( \sigma_d = \Delta d_{10}/d_{10} \)) that stays relatively constant between 0% and 6% dextran compression but increases at higher lattice compression. Likewise, \( \sigma_r \), which can be related to the amount of liquid-like disorder in the myofilament lattice (41), is relatively low (~1%) but also does not vary greatly between 0% and 6% dextran and increases at higher levels of lattice compression. If disorder is the cause of the weak outer reflections, the amount of disorder appears to be about the same over the range of osmotic pressure that yields a physiological lattice spacing, and thus this phenomenon is unlikely to be the cause of the observed changes in myofilament calcium sensitivity.

Figure 3 illustrates the relationship between myofilament lattice spacing and applied osmotic pressure over the entire range studied, with an expanded view of the relationship in the range between 0 and 2 kPa (0–3% Dextran T500) (Fig. 3, inset). Myofilament lattice spacing decreased upon an increase in applied osmotic pressure in a monotonous nonlinear fashion but with a discontinuity at similar osmotic pressures as in Figs. 2 and 4. The data between 1% and 10% Dextran T500 are well fit by an exponential function (Fig. 3, solid line). However, the data obtained with 0, 0.4, and 0.7% Dextran T500 clearly deviate from this exponential relationship, laying above the fitted curve. The deviation is more clearly seen in the inset where the data are plotted on an expanded scale.

Figure 4 summarizes the relationship between \( I_{1,1}/I_{1,0} \) intensity ratio and applied osmotic pressure. As was the case for EC_{50} (Fig. 2), the \( I_{1,1}/I_{1,0} \) intensity ratio increased abruptly by ~50% over a narrow range of applied osmotic pressure (0.12–0.38 kPa; equivalent to 0.4–1% Dextran T500). This elevation in intensity remained stable with applied osmotic compression up to ~9 kPa (7% Dextran T500). The intensity ratio decreased somewhat (~20%) at an applied osmotic pressure >13 kPa (Fig. 4, left). However, it should be noted that myofilament lattice spacing at such a large applied osmotic pressure is below 34 nm, the lattice spacing observed in intact rat myocardium at sacomere length of 2.2 \( \mu \)m (13).

**DISCUSSION**

Our data, obtained using X-ray diffraction to accurately measure interfilament lattice spacing, revealed that 1) the changes in myofilament calcium sensitivity are much better correlated with the changes in the disposition of the myosin heads, as assessed by the \( I_{1,1}/I_{1,0} \) intensity ratio, than with changes in myofilament lattice spacing (Fig. 5); and 2) the position of the myosin heads and EC_{50} change substantially and discontinuously over a narrow range of applied osmotic pressure. Surprisingly, the applied osmotic pressure where this occurs is much less than that required to restore in situ lattice spacing at this sarcomere length (2.2 \( \mu \)m). Another surprising finding was that both myofilament calcium sensitivity and \( I_{1,1}/I_{1,0} \) intensity ratio did not change as the dextran concen-
tration was increased from 1% to 6% dextran. Most previous studies on skinned muscle have used dextran in the range between 3% and 6%. Therefore, an important implication of our current findings is that application of dextran anywhere in this osmotic pressure range would have been more than sufficient to induce similar high values for the \( \frac{I_{1,1}}{I_{1,0}} \) intensity ratio coupled with high myofilament calcium sensitivity. In contrast, myofilament lattice spacing is a continuous inverse function of dextran concentration over that same concentration range. Our results are somewhat different from those of Martyn et al. (21), who found, in rat skinned myocardium at 5°C, a proportional increase in \( \frac{I_{1,1}}{I_{1,0}} \) intensity ratio concomitant with a proportional decrease in interfilament spacing upon osmotic compression by application of dextran concentration up to 5% at short sarcomere length; at a longer sarcomere length, however, their data appear consistent with a saturation of the impact of osmotic compression on the \( \frac{I_{1,1}}{I_{1,0}} \) intensity ratio. The reason for this discrepancy is unclear but may be related to the large difference in temperature between these studies.

Implicit in the lattice spacing hypothesis is the idea that it is necessary to achieve an optimal lattice spacing to return in vivo function to demembranated, striated muscle. As demonstrated
in previous experiments involving Dextran T500 (10, 11, 25, 34–36), the addition of Dextran T500 to the bathing media increases myofilament calcium sensitivity as well as maximum active tension, concomitant with similar changes in fiber width. In addition, these changes occur regardless of whether fiber width reduction was induced by applied osmotic compression or by an increase in sarcomere length. In skeletal muscle, myofilament filament spacing and fiber width have been shown to be well correlated (14). However, we have shown recently that myofilament lattice spacing and muscle width do not change proportionately in skinned isolated myocardium under applied osmotic compression (18). Hence, estimates of interfilament spacing based on muscle width are fraught with uncertainty. Moreover, we observed here that a small amount of myofilament lattice compression by application of 1% dextran (equivalent to 0.38 kPa applied osmotic compression) induced a change in the $I_{1,1}/I_{1,0}$ intensity ratio from $\sim0.3$ to $\sim0.45$ in relaxed, isolated skinned myocardium. Various measurements of this parameter have been made previously in intact, nonskinned myocardium by Matsubara and colleagues ($I_{1,1}/I_{1,0} = 0.375$) (23) and ($I_{1,1}/I_{1,0} = 0.581$) (24), as well as Yagi and colleagues ($I_{1,1}/I_{1,0} = 0.4$) (38) and ($I_{1,1}/I_{1,0} = 0.444$) (37). Of interest, these values are similar to those observed in this present study in skinned rat cardiac trabeculae in the high-myofilament calcium sensitivity state, that is, those muscles that were compressed by application of $>1.0\%$ dextran (0.38 kPa). Insofar that the $I_{1,1}/I_{1,0}$ ratio is a measure of the relative position of the myosin heads with respect to the thick and thin filaments, it is clear that the arrangement of myosin heads in uncompressed skinned myocardium differs from the in vivo situation but that this can be restored to the in vivo arrangement by a relatively small amount of applied osmotic compression. In fact, further osmotic compression did not further affect either the $I_{1,1}/I_{1,0}$ intensity ratio or myofilament calcium sensitivity. Hence, our data are consistent with the notion that moderately ($\sim1\%$ dextran) compressed skinned muscle corresponds to the physiological condition of the sarcomere with regard to the $I_{1,1}/I_{1,0}$ intensity ratio. Further compression, between 3% and 6% dextran, depending on the specific sarcomere length studied (13, 18), however, will be required to restore interfilament spacing to the in vivo condition. In any case, myofilament calcium sensitivity will be the same.

**Osmotic Pressure Sensitive Switch for Calcium Sensitivity?**

Our findings suggest that whereas myofilament lattice spacing varies continuously with external applied osmotic pressure, myofilament calcium sensitivity and $I_{1,1}/I_{1,0}$ intensity ratio vary discontinuously. Even though the $I_{1,1}/I_{1,0}$ parameter had a very high degree of linear correlation with $EC_{50}$, the data seem equally consistent with a binary distribution with one value of $EC_{50}$ and $I_{1,1}/I_{1,0}$ at applied osmotic pressure below 0.24 kPa (0.7% dextran) and another value in the range between 0.38 kPa (1% dextran) and 9.1 kPa (7% dextran). These results indicate the possibility of a pressure-sensitive system that induces the myosin heads to associate more around the thin filament under a modest degree of applied osmotic compression. This tendency was better correlated to the myofilament response to calcium than were changes in myofilament lattice spacing (Fig. 5). We found a significant ($P = 0.012$) lack of fit when comparing a lattice spacing only linear model with a model that had both the lattice spacing and $I_{1,1}/I_{1,0}$ intensity ratio. This indicated that the two-parameter model was better at explaining the data than lattice spacing alone. A similar comparison between the two-parameter model and a model that had only the $I_{1,1}/I_{1,0}$ intensity ratio showed no significant ($P = 0.409$) lack of fit, demonstrating that the simpler intensity ratio alone model could explain the data as well as the two-parameter model. The lattice spacing term could be dropped from the model. $I_{1,1}/I_{1,0}$ intensity ratio alone was sufficient to explain changes in myofilament calcium sensitivity. Furthermore, the apparent clustering of the data in Fig. 5B suggests that the relationship between $EC_{50}$ and $I_{1,1}/I_{1,0}$ intensity ratio is not linear; rather, it is dichotomous. A low-intensity ratio is predictive of a high $EC_{50}$, whereas a high-intensity ratio is predictive of a low $EC_{50}$.

What mechanism may underlie such a switch-like phenomenon (see Fig. 5) where both myofilament calcium sensitivity and $I_{1,1}/I_{1,0}$ intensity ratio change over a very narrow range of applied osmotic pressure? One possibility is that it is the myofilament lattice spacing that is the operative mechanism, as posited by the lattice spacing hypothesis, but that this effect is highly nonlinear [as required by our current data and those of Konhilas et al. (18)]. In this scenario there is indeed a critical
myofilament lattice spacing below which weak-binding cross-bridges are able to form in a highly cooperative, and thus nonlinear, manner. At an interfilament spacing larger than this critical distance, myosin heads are still able to reach the thin filament but do so less optimally, whereas at an interfilament spacing below this critical value cross-bridges would operate more or less the same regardless of myofilament lattice spacing. Only when the myofilament lattice is compressed beyond the physiological range (>7% dextran), which is where there is steric hindrance, would cross-bridge function diminish. Such a mechanism can explain the abrupt transitions that we observed in myofilament calcium sensitivity, $I_{1,1}/I_{1,0}$ intensity ratio, and interfilament spacing at low levels of applied osmotic compression. A difficulty with this view, however, is that it conflicts with recent studies (30, 39) that measured myofilament lattice spacing and $I_{1,1}/I_{1,0}$ intensity ratio in whole hearts. Both studies reported a diastolic (relaxed) $I_{1,1}/I_{1,0}$ intensity ratio of ~0.4, equivalent to the compressed patterns we observed in the skinned fibers, but the reported myofilament lattice spacing was very different between the two studies: ~42 nm (30) and ~37.5 nm (39). The interfilament lattice spacing reported by Pearson and colleagues (30) are equivalent to those reported here for skinned preparations in the absence of dextran at a sarcomere length of ~2.2 μm. Because $I_{1,1}/I_{1,0}$ intensity ratio is better correlated with myofilament calcium sensitivity than with myofilament lattice spacing, it is difficult to reconcile a single optimal myofilament lattice spacing for a cooperative transition with these data showing a similar $I_{1,1}/I_{1,0}$ intensity ratio but a very different myofilament lattice spacing.

Another possibility we have considered is that the putative switch mechanism involves compression of the myosin thick filament. Externally applied osmotic pressure by Dextran T500 could potentially affect all components of the sarcomere, depending on their relative compressibility, in addition to myofilament lattice spacing. The relatively loose structure of the thick filament, being composed of a parallel arrangement of myosin molecules aligned by electrostatic interactions (27), suggests that it may be more sensitive to osmotic compression than other sarcomere components, such as the thin filaments. Compression of the thick filament backbone could alter interactions with the myosin light chains or alter the relative mobility of the myosin heads. Either mechanism may be expected to affect myofilament calcium sensitivity. This hypothesis can be tested experimentally by using relatively low-molecular-weight polymers, such as Dextran T10 (6). These compounds are capable of entering the myofilament lattice, but they are excluded from the thick filament. Application of >0.4 kPa osmotic compression to skinned muscle by such an agent is expected to increase both the $I_{1,1}/I_{1,0}$ intensity ratio and myofilament calcium sensitivity but not myofilament lattice spacing. These experiments must await membrane osmometry studies to yield accurate osmotic pressure for a given concentration of Dextran T10.

**Limitations of Study**

A limitation of this study is that the mechanical measurements of EC50 were measured under contracting conditions, whereas the measurements of lattice spacing and intensity ratio were done under relaxing conditions. So although our findings establish a strong correlation between calcium sensitivity and $I_{1,1}/I_{1,0}$ intensity ratio measured under relaxed conditions, they do not address what might be happening to lattice spacing and intensity ratio under contracting conditions and what relation these quantities may have to calcium sensitivity. These relationships would need to be assessed in future experiments designed specifically for this purpose.

In addition, because our study was conceived as an attempt to understand the impact of dextran on myofilament calcium sensitivity, it was performed at a single sarcomere length (2.2 μm), whereas, ultimately, we would like to be able to explain changes in calcium sensitivity with changes in length vis-à-vis, the Frank-Starling relation. Application of 1% dextran at sacomere length of 2.0 μm in a previous study (18) on rat skinned myocardium did not affect myofilament calcium sensitivity. This result implies, therefore, that the amount of osmotic compression that is required to induce the apparent switch may depend on sarcomere length. In a recent study in isolated skinned murine myocardium, it was reported that much higher levels of dextran application (>2% dextran) were required to induce compression of the myofilament lattice (28). It should be noted, however, that no attempt was made in that study to limit activation of the PKA system. Our group (19) previously found marked expansion of the murine myofilament lattice by PKA-mediated phosphorylation. Phosphorylation was not controlled in either that study or the current study. Therefore, the question as to why murine skinned myocardium is more resistant to osmotic compression is unclear and requires further investigation. Likewise, Martyn et al. (21) reported in skinned rat myocardium at 5°C that application of 1% dextran was without affect on either interfilament spacing or $I_{1,1}/I_{1,0}$ intensity ratio at sacomere length of 2.0 and 2.35 μm. Hence, the exact amount of dextran required to compress the myofilament lattice may depend on species, temperature, and, possibly, phosphorylation status of the contractile apparatus.

Yagi et al. (37) presented results from intact, twitching papillary muscle that, although their data showed considerable scatter, demonstrated a small but significant increase in $I_{1,1}/I_{1,0}$ under diastolic conditions with increasing sarcomere length from 1.7 to 2.3 μm, supporting the notion that the underlying causes of changes in $I_{1,1}/I_{1,0}$ may be related to the changes in calcium sensitivity with changing length. However, it should be noted that the function of the cardiac sarcomere within the cell is sufficiently complex to warrant caution in extrapolating data obtained in skinned myocardium to the intact beating heart.

**“Lattice Spacing Hypothesis” Revisited**

Here we have shown that the position and orientation of myosin heads, as assessed by the $I_{1,1}/I_{1,0}$ intensity ratio, are a better predictor of myofilament calcium sensitivity than interfilament lattice spacing under conditions of applied osmotic compression. These data, along with other data from our laboratories (16–18), suggest that may we need to reexamine interfilament lattice spacing as a unifying hypothesis that explains all changes in myofilament calcium sensitivity. Recent experiments by Martyn et al. (21) may lend some credence to this concept. These investigators demonstrated that an increase in either sarcomere length or externally applied osmotic pressure induces an increase in both the $I_{1,1}/I_{1,0}$ intensity ratio and passive fiber stiffness, thus implicating an increase in the...
number of thin filament-associated cross-bridges under those conditions consistent with the aforementioned results of Yagi et al. (37). Martyn et al. (21), however, concluded that the observed changes in \( I_1/I_{1,0} \) intensity ratio were caused by changes in interfilament spacing. Those data and the current results suggest to us, rather, that the relative position of myosin heads in relation to the thin filament in resting muscle, as assessed by the \( I_1/I_{1,0} \) intensity ratio, is the relevant factor that determines myofilament calcium sensitivity upon activation.

Unraveling of the causal mechanisms that underlie changes in \( I_1/I_{1,0} \) intensity ratio upon osmotic compression or changes in sarcomere length, however, requires further studies, preferably in intact twitching myocardium.

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