Image-analysis-based assessment of the effects of the “Ca$^{2+}$-jump” technique on sarcomere uniformity

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Slawnych, M. P., L. Morishita, and B. H. Bressler. Image-analysis-based assessment of the effects of the “Ca$^{2+}$-jump” technique on sarcomere uniformity. J. Appl. Physiol. 85(3): 955–961, 1998.—A new image analysis-based technique was used to quantitatively examine the effects of the “Ca$^{2+}$-jump” activation protocol on the maintenance of fiber quality in skinned rabbit psoas muscle fiber segments. Specifically, contractions in pCa 4.6 were preceded by short-duration “preactivation” soaks in a solution in which EGTA was replaced with the low-Ca$^{2+}$ buffering capacity analog hexamethylenediamine-N,N′,N′′,N′′′-tetraacetate, which facilitated rapid Ca$^{2+}$ equilibration within the fiber segments. Fiber quality was assessed by examining the Fourier spectra of the muscle fiber images before, during, and after activation. Segment lengths were typically below 500 µm, thus allowing the majority of the sarcomeres to be visualized in the field of view (×200 and ×400 magnification). The preactivation protocol resulted in less deterioration of fiber quality with repetitive activation. In addition, there was also a significant reduction in the time required to reach the 50% level of maximum tension, with no significant change in the maximum tension level.

SKINNED MUSCLE FIBER preparations are widely employed in studies involving muscle mechanics (35). However, whereas these preparations provide a great deal of flexibility in terms of being able to control the intracellular fluid composition, problems can arise in maintaining the quality of the preparations, specifically the sarcomere length uniformity. This degradation can be at least partly attributed to the nonuniform activation of the entire fiber segment. By rapidly altering the free Ca$^{2+}$ concentration inside the skinned fiber preparation, more uniform activation can be achieved. Rapid alterations in internal Ca$^{2+}$ concentration can be accomplished by a variety of methods, including the following: 1) iontophoretically passing Ca$^{2+}$ into the solution in the region of small, skinned fiber segments (9); 2) using caged Ca$^{2+}$ compounds (2, 3); and 3) activating the preparation in a solution with a very high Ca$^{2+}$ buffering capacity, compared with that of the fiber as a whole (which includes the solution in the interfilament space as well as the myofibrillar proteins) (4, 5, 26).

The last method, which is commonly referred to as the “Ca$^{2+}$-jump” technique (33), is the method most commonly employed, as it can readily be incorporated into experimental protocols with minimal effort and expense. The basic premise of this method is as follows. Ordinarily, before activation, the fiber is resting in a relaxing solution that contains little Ca$^{2+}$ but a significant amount of EGTA, which replaces the majority of the fiber-segment Ca$^{2+}$ buffering capacity. By replacing the EGTA with a low-buffering capacity analog, such as hexamethylenediamine-N,N′,N′′,N′′′-tetraacetate (HDTA) (26), the buffering capacity of the fiber segment can be significantly reduced. (Table 1 compares the binding constants of these compounds.) The net result is that, when the fiber segment is exposed to the activating solution, contraction-related Ca$^{2+}$ binding is no longer in competition with the EGTA buffering system (Fig. 1).

Whereas it has been reported that the use of the Ca$^{2+}$-jump technique improves the quality of the striation pattern (17, 26, 27), this effect has not been quantified. Hence, the purpose of this work was to assess fiber quality with and without the Ca$^{2+}$-jump protocol. Whereas sarcomere length is commonly measured by methods based on laser diffraction, this technique is not well suited to the analysis of local sarcomere uniformity. Therefore, we have developed a novel image-analysis technique to perform this analysis (31). This method is based on the evaluation of twodimensional Fourier power spectra of muscle images. That is, we are capitalizing on the property that muscle fiber images can be considered as periodic patterns and, as such, the images can be represented in terms of Fourier spectra. This transformation can be carried out efficiently by using the fast Fourier transform (8). The magnitude of the transformed image represents the spectral energy density in the frequency domain. Assuming that the centroid frequency of the first-order spectral density peak represents the fundamental sarcomeric frequency, the mean sarcomere length is simply given by the reciprocal of this frequency.

METHODOLOGICAL APPROACH

Preparations. Fiber bundles ~2 mm in diameter and 3–4 cm long were obtained from psoas muscles of adult New Zealand White rabbits. The bundles were stored at ~18°C for up to 3 mo in relaxing solution containing 50% glycerol (vol/vol), as described by Goldman et al. (16). Single fiber segments, typically shorter than 500 µm, were isolated and treated with a nonionic detergent (Triton X-100, 0.5% vol/vol) for 15 min to disrupt remaining membrane fragments. The segments were then mounted horizontally between a semicon-
permitting the fiber to be visualized along the vertical axis. At 1°C, the chamber bottom was made of glass, the chamber walls were made of Plexiglas and contained cooling channels to facilitate temperature control. Experiments were carried out in air. The chamber volume was 200 µl. The chamber walls were made of Plexiglas and contained cooling channels to facilitate temperature control. Experiments were carried out at 10 ± 1°C. The chamber bottom was made of glass, permitting the fiber to be visualized along the vertical axis. The fiber segment could be rapidly transferred from one chamber to another, minimizing its exposure to air.

The chamber system and associated apparatus were placed on the stage of an inverted microscope (Diaphot 300, Nikon). The microscope was mounted on an air-suspension table system. Fiber segments were illuminated by a halogen light source filtered to 546 nm by using a green interference filter, which represents the best compromise between microscope resolution and charge-coupled device sensitivity (21). The segments were visualized by using ×40 (numerical aperture = 0.55, depth of field ~4 µm) and ×20 (numerical aperture = 0.40, depth of field ~6 µm) objectives in conjunction with a ×10 ocular, yielding total magnifications of ×400 and ×200, respectively. The condenser aperture was set at its full open setting. A charge-coupled device video camera (model 4910; Cohu, San Diego, CA) was connected to the video port of the microscope, which in turn was connected to both a video monitor and S-VHS video recorder.

Solutions. Solution compositions are listed in Table 2. All solutions were 200 mM total ionic strength, with propionate as the major anion. The solution pH was 7.0 in a MOPS buffer. All solutions contained 0.5 mM dithiothreitol to prevent protein degradation. Dextran T70 (5.6%) was also added to all solutions to restore the fiber diameters to preskinning levels (15, 18). HDTA was obtained from Aldrich (Milwaukee, WI). All other solutions were obtained from Sigma Chemical (St. Louis, MO). The solution compositions were determined by using a computer program initially developed by Godt (14).

Experimental protocol. The length of the fiber segment was adjusted to set the mean sarcomere length to 2.6 µm. (Whereas the resting sarcomere length was generally found to be in the range of 2.4–2.5 µm, in a few instances it was found to be >2.5 µm. Therefore, the initial sarcomere length was set to 2.6 µm to ensure that the fiber was not slack at the starting condition.) The fiber segment was then activated isometrically.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Cation</th>
<th>Average Apparent Binding Constant, [M]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA (mol mass = 380.4 kDa)</td>
<td>Ca²⁺</td>
<td>5 ± 0.5 × 10⁻⁶</td>
<td>Moisescu (25)</td>
</tr>
<tr>
<td>HDTA (mol mass = 348.4 kDa)</td>
<td>Mg²⁺</td>
<td>25 ± 5</td>
<td>Moisescu (25)</td>
</tr>
<tr>
<td>Troponin C (mol mass = 18.0 kDa)</td>
<td>Ca²⁺</td>
<td>6 ± 1.5</td>
<td>Farah and Reinach (11)</td>
</tr>
</tbody>
</table>

Values are means ± SE. HDTA, hexamethylenediamine-N, N', N''-tetraacetate.

Fig. 1. Basis for Ca²⁺-jump technique. Standard (A) and Ca²⁺-jump-based activations (B) are shown. A: in presence of relaxing solution, majority of Ca²⁺ buffering capacity of fiber segment is represented by EGTA within fiber. As a result, when fiber is exposed to activating solution, contractile proteins must compete with EGTA for incoming Ca²⁺. B: conversely, when fiber is preactivated in a solution containing little EGTA, there is no competition for incoming Ca²⁺ when fiber is activated. [Ca²⁺], Ca²⁺ concentration; HDTA, hexamethylenediamine-N, N', N''-tetraacetate.
Table 2. Solution compositions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Final MgATP, mM</th>
<th>Free Mg²⁺, mM</th>
<th>Total K, mM</th>
<th>CP, mM</th>
<th>CPK, mg/ml</th>
<th>CaCO₃, mM</th>
<th>EGTA, mM</th>
<th>HDTA, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxing</td>
<td>5.0</td>
<td>1.0</td>
<td>93.0</td>
<td>15.0</td>
<td>0.1</td>
<td>0</td>
<td>15.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Preactivating</td>
<td>5.0</td>
<td>1.0</td>
<td>93.0</td>
<td>15.0</td>
<td>0.1</td>
<td>0</td>
<td>14.9</td>
<td>14.9</td>
</tr>
<tr>
<td>Activating</td>
<td>5.0</td>
<td>1.0</td>
<td>93.0</td>
<td>15.0</td>
<td>0.1</td>
<td>14.9</td>
<td>15.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

CP, creatine phosphate; CPK, creatine phosphokinase.

1 In general, truncating a signal in the time domain (which is equivalent to multiplying the signal by a rectangular window function) leads to nonideal behavior in the frequency spectrum. Specifically, the magnitude of the power spectrum estimate at a given discrete frequency value (or “bin”) contains leakage from frequency components that can be several frequency bins away. The reason for this leakage is that, because the rectangular window function turns on and off very rapidly, its Fourier transform has substantial high-frequency components. This leakage can be reduced by multiplying the time domain signal by a nonrectangular window function that changes more gradually from its maximum value to zero (29). For this investigation, the Hanning window was employed, which is defined as

$$
W_j = \frac{1}{2}[1 - \cos(2\pi j/N)], \text{where } N \text{ is the number of data points and } j \text{ is the data point index.}
$$
reach 50% of final force. Conversely, there is no significant difference in the force levels (Table 3). A typical example of the force responses obtained with and without the preactivation step are shown in Fig. 5.

**DISCUSSION**

Sarcomere length uniformity plays a central role in studies involving muscle mechanics. In this paper, we showed that, by adopting a preactivation protocol in which the fiber segments are preactivated in a solution with minimal Ca\(^{2+}\) buffering capacity, improvements in the quality of the striation pattern, and hence sarcomere length uniformity, can be achieved. Specifically, sarcomere lengths measured from fiber segments that underwent the preactivation step exhibited less local variation when activated and were also more similar to the relaxed-condition sarcomere lengths than those obtained from fiber segments that did not undergo preactivation. This effect is most likely due to the rapid, uniform activation of the contractile proteins. Indeed, the rate of tension development was found to be significantly faster when the fibers were exposed to the preactivating solution. In contrast to the kinetic effects, we saw small increases in the amplitudes of the force responses obtained with preactivation, but the effect was not significant. Interestingly, in many of the contractions in which the fiber segments underwent preactivation, the isometric tension was associated with a small overshoot, as shown in Fig. 5. This phenomenon has also been observed by other investigators (32).

It should be noted that, when dealing with skinned fiber segments, as opposed to intact fibers, fiber radius plays a prominent role in terms of uniform activation (e.g., Fabiato and Fabiato (10)). Specifically, in skinned fibers, Ca\(^{2+}\) enters the system at the level of the surface of the fiber and then diffuses throughout the fiber. In intact fibers, however, Ca\(^{2+}\) release occurs at the A-band/I-band interface at the myofibrillar level, and hence the radial diffusion distance is significantly reduced. As a result, it has been proposed that experiments be carried out on single myofibrils, as opposed to single muscle fibers (1, 22), which would effectively reduce the...
Fig. 3. A and B show Fourier line spectra associated with image spectra in Fig. 2, A and B, respectively. x-Axis indicates frequency, ranging from 0 to \( f_s/2 \), where \( f_s \) is the sampling frequency, which is the reciprocal of the distance between successive pixels in the image (some corresponding sarcomere lengths are indicated in \( \mu m \)). y-Axes indicate relative amplitude on a logarithmic scale. Full, full spectrum; H1 and H2, half spectra, Q1–Q4, quarter spectra.

Fig. 4. Comparison of sarcomere length signals obtained from full muscle image and various subimages as a function of time. A: sarcomere lengths obtained from a segment that underwent HDTA preactivation. Fiber was transferred from HDTA solution to activation solution during time 1 (t₁) and returned to relaxing solution during time 2 (t₂). B: sarcomere lengths obtained from a segment that did not undergo HDTA preactivation. In this case, fiber was transferred from relaxing solution to activation solution during t₁ and returned to relaxing solution during t₂. Labels on y-axis indicate sarcomere length in \( \mu m \). Horizontal calibration line, 10 s.
It should be noted that a number of other methods have also been proposed to minimize sarcomere length heterogeneity. Iwazumi and Pollack (22) found that, by conditioning the fiber with an initial, slow activation step in which the Ca\(^{2+}\) concentration is increased from resting to full activation levels over a period of 5–10 min, subsequent contractions did not result in any significant deterioration in the striation pattern.

Brenner (6) found that cycling the fibers with brief periods of lightly loaded isotonic shortening stabilized the striation pattern during prolonged, maximal Ca\(^{2+}\) activation. Sweeney and colleagues (34) subsequently modified the technique by replacing the isotonic shortening with isovelocity ramps, hence eliminating the need for a force feedback system.

Hellam and Podolsky (20) and Julian (23) found that the striation pattern could be better maintained by minimizing the period of activation to as short a time as possible. Unfortunately, such an approach limits both the number and type of measurements that can be taken during each contraction.

Podolin and Ford (28) achieved rapid activation by exposing fibers to free Ca\(^{2+}\) for a 0.5- to 1.0-s period before introducing the EGTA-buffered solution through the use of an electronically controlled solution changer. The uniformity of the striation pattern can also be better maintained by reducing the force levels generated by the muscle fiber segments. This can be achieved in a number of ways, such as decreasing the temperature of the bathing solutions (30) and using submaximal levels of Ca\(^{2+}\) to activate the fiber segments (19, 24). However, it should be stated that the latter method only delays the deterioration of the striation pattern.

Instead of replacing EGTA with a low-Ca\(^{2+}\) buffering capacity analog, it can simply be eliminated from the preactivating solution (12). Whereas such an approach is somewhat inferior to the HDTA replacement protocol because it changes the ionic strength of the solution, the effects of such a change would be minimal.

Many of the above-mentioned methods can be used in conjunction with one another. Although the Ca\(^{2+}\)-jump protocol is presently being employed by a number of investigators, the results presented here warrant its more widespread use, particularly given that it can be incorporated into existing methodologies with minimal effort. Work is presently being conducted to extend this analysis to an examination of sarcomere length changes during activation as a function of radial distance from the central fiber axis.

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