Intracellular distributions of essential elements in cardiomyocytes

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Abstract

We describe the intracellular distributions of nine essential elements (P, S, Cl, K, Ca, Mn, Fe, Cu, and Zn) found in cardiomyocytes imaged using synchrotron X-ray induced fluorescence. Cardiomyocytes were isolated from rat hearts, flash frozen on Si 3N 4 windows, freeze-dried, and imaged with approximately 300 nm spatial resolution. Distinct longitudinal patterns in cardiomyocytes were most apparent for the elements Fe and Cu, which clearly colocalized. Transverse striations were apparent for P, S, Fe, and Zn, while those for Zn were consistently the most prominent (≈10 -3 M) and appeared with a periodicity in the range 1.63–1.75 μm, the expected length of a sarcomere. Transverse striations for high concentrations of P, Fe, and Zn and low concentrations of S colocalized and coincided with the I-band of the intact cardiomyocyte. Fluorescence microscopy using FluoZin-3 in intact cardiomyocytes suggests that Zn 2+ in flux is through sarcolemmal calcium channels and that significant stores of intracellular Zn 2+ may be released quickly (<1 s) into the cytosol. These data collectively suggest that Zn 2+ is buffered by structures associated near the T-tubules and/or in the sarcoplasmic reticulum and is found in relative abundance sufficient to act as a modifier of Ca 2+ regulation or as a possible signaling messenger for gene expression.

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1. Introduction

Cardiomyocytes are heart muscle cells specialized to repeatedly produce contractile force. The study of cardiomyocyte function has been compartmentalized roughly into areas of electrophysiology, force production, metabolism, and gene expression. Signaling mechanisms also exist within and between these functional compartments and govern overall cardiomyocyte performance and maintenance (Bers, 2001). Some signaling mechanisms are well characterized and are known to involve specific ions as signaling messengers. For example, membrane depolarization leads to a rapid rise in intracellular free Ca 2+ concentration, which directly activates force production (Bers, 2001).

Other currently uncharacterized signaling mechanisms, such as those modulating gene expression, may likewise involve other ions or metalloproteins directly activating transcription factors (Bird et al., 2000, 2003; DeMoor and Koropatnick, 2000; O’Halloran, 1993; Outten and O’Halloran, 2001) or more distal portions of transcription and/or translation pathways (Andrews, 2001; Finney and O’Halloran, 2003; Outten and O’Halloran, 2001; Pyle, 1993; Verhaegh et al., 1998).

Although the concentrations of several low atomic numbered elements (Na, Mg, P, S, Cl, K, and Ca) have been previously examined in cardiomyocyte myofilaments and organelles (Bond et al., 1994; Isenberg et al., 1996; Keller et al., 1995), detailed spatial distributions of these and other elements would further elucidate the structural availability of elements and their ionic forms as specialized signaling messengers in the cardiomyocyte. For example, high concentrations of Ca may be expected in the sarcoplasmic...
reticulum (SR) cistern, which buffers Ca\(^{2+}\) immediately before cardiomyocyte contraction (Bers, 2001; Isenberg et al., 1996) and whose release of Ca\(^{2+}\) is sensitive to stresses known to promote gene expression (Molkentin, 2000). Relatively high concentrations of Zn may also be expected in the cardiomyocyte nuclei, where zinc-finger transcription factors reside (Andrews, 2001; Molkentin, 2000; Wilkins and Molkentin, 2004). We hypothesized that distinct spatial distributions of essential elements normally exist in the cardiomyocyte and may serve as repositories of signaling messengers.

In the present study, we used X-ray induced fluorescence to examine the spatial distributions of several essential elements (P, S, Cl, K, Ca, Mn, Fe, Cu, and Zn) in cardiomyocytes. We paid particular attention to results for Ca, Fe, Cu, and Zn, which have been specifically implicated as signaling messengers for gene expression (Atar et al., 1995; DeMoor and Koropatnick, 2000; Duffy et al., 2004; Elsherif et al., 2004; Finney and O’Halloran, 2003; O’Halloran, 1993; Outten and O’Halloran, 2001). We report here that global concentrations of Ca, Fe, Cu, and Zn are on the order of 10\(^{-4}\)–10\(^{-3}\)M. While we did not detect conspicuous regions of differential Ca concentrations, we did observe high Zn concentrations distinctly localized to the sarcomere I-band and associated with the integrity of the sarcosomal and organellar membranes. These data, in conjunction with our observing rapid fluctuations of intracellular free Zn\(^{2+}\) concentrations in intact cardiomyocytes, lead us to conjecture that Zn is buffered by structures associated with the T-tubules and/or the SR.

2. Methods

2.1. Left ventricular cardiomyocyte isolation

Animal care and use were conducted under the guidelines accepted by the American Physiological Society and received prior approval from the Institutional Animal Care and Use Committee at the University of Vermont. Two male Dahl rats were obtained commercially from Harlan Sprague–Dawley (Indianapolis, IN), housed in a 12 h light/12 h dark cycle and given standard rat chow and water ad libitum. At 10 weeks of age left ventricular cardiomyocytes were obtained from the LV septal and free wall using methods described previously (Palmer et al., 2001). All chemicals and reagents were obtained from Sigma–Aldrich (St. Louis, MO) except where noted.

In brief, animals were heparinized (500 U, i.p.) and then anesthetized with sodium pentobarbital (90mg/kg, i.p.) (Abbot Laboratories, North Chicago, IL). Hearts were rapidly excised, the aorta was then cannulated and the coronary bed was perfused with a bicarbonate based Krebs–Henseleit buffer with nominal free Ca\(^{2+}\) (in mM: 113 NaCl, 4.7 KCl, 0.6 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 12 NaHCO\(_3\), 10 KHCO\(_3\), 20 glucose, 1% MEM amino acid, 10 Heps, 30 taurine, 2 carnitine, 2 creatine, and 15 BDM) and subsequently with a similar solution containing collagenase 196 U/ml (Worthington, Freehold, NJ) and trypsin 0.25mg/ml. All solutions were maintained at pH 7.4 and 37°C and bubbled with 95% O\(_2\) + 5% CO\(_2\) gas.

After the heart was palpably flaccid the atria and right ventricular free wall were removed and the left ventricular free wall and septum were minced in the solution containing collagenase and trypsin. Isolated cardiomyocytes were suspended in bicarbonate-base buffer, filtered through a nylon mesh to remove fibrous tissue and debris, washed three times by centrifugation and resuspended in bicarbonate buffer. Those cardiomyocytes prepared for measurements of intracellular free zinc ion concentration ([Zn\(^{2+}\)]) were gradually exposed to increasing Ca\(^{2+}\) concentrations and kept in loading buffer (in mM: 137 NaCl, 5.4 KCl, 1.2 CaCl\(_2\), 0.5 MgCl\(_2\), 10 Heps, 5.5 glucose, and 0.5 probenecid, pH 7.4) until use.

2.2. Measurement of spatial distributions of elements

Cardiomyocytes used in X-fluorescence imaging were suspended in nominally Ca\(^{2+}\)-free bicarbonate buffer, dropped onto 200 µm mesh Ni electron-micrograph grids or onto 500 nm thick Si\(_3\)N\(_4\) windows (Structure Probe, West Chester, PA) and quickly plunged into isopentane cooled with liquid nitrogen. Frozen cardiomyocytes were freeze-dried under vacuum for 2 h (Labconco, Kansas City, MO). Dried cardiomyocytes were shipped to the Experimental Facilities Division of the Advanced Photon Source (Argonne, IL) and stored in a standard laboratory desiccator until time of imaging.

Dried cardiomyocytes on grids or Si\(_3\)N\(_4\) windows were mounted on a kinematic sample holder and transferred to a visible light microscope (Leica DMXRE, Leica Microsystems, Wetzlar, Germany), equipped with a high spatial resolution motorized x/y stage (Ludl Bioprecision, Hawthorne, NY) with an encoder resolution of 0.1 mm, a repeatability of 0.75 mm and an accuracy of 3.0 mm overall a 77 x 51 mm travel range. Light micrographs were acquired using a 40×0.55NA N-PLAN long working distance objective and either a Leica DC 350F CCD camera (B/W) or a Qimaging Retiga EXi CCD camera (color with RGB filterwheel).

X-ray fluorescence dataset were acquired on the 2-ID-E beamline at the Advanced Photon Source (Argonne, IL). Incident X-rays of ~10keV were generated by using either a 5.5 or 3.3 cm periodicity undulator and monochromatized using a single bounce Si (111) monochromator. The X-rays were focused into a ~0.45 × 0.35 µm spot using a Fresnel zone plate with a Rayleigh resolution of 122 nm and a focal length of 25 cm (Xradia, Concord, CA) The sample was raster-scanned through the focal spot, characteristic X-ray fluorescence was detected using a 3-element energy dispersive Ge-detector (Canberra, Meriden, CT), and saved as a full spectrum at every scan position. Elemental maps were created by either spectral filtering (ROIs) or per-pixel-fitting, calibration to elemental area densities (µg/cm\(^2\)) was done by comparison of X-ray fluorescence signal strength.
from the sample to fluorescence from thin film standards NBS-1832 and NBS-1833 from the National Bureau of Standards (NBS/NIST, Gaithersburg, MD) using MAPS software (Vogt, 2003). Digitally recorded positioning information for both visible light and X-ray microscopes allowed the correlation of light micrographs and elemental maps acquired by the X-ray microprobe. Total time for scanning a single cardiomyocyte of ~30 μm x 100 μm dimension was approximately 8 h.

2.3. Measurement of \([Zn^{2+}]\), and sarcomere shortening dynamics

Cardiomyocytes were loaded with FluoZin-3, a fluorescent dye sensitive to Zn\(^{2+}\) with \(K_d\sim 15\) nM and not sensitive to physiological Ca\(^{2+}\) (Gee et al., 2002), by exposure to 3 μM FluoZin-3AM and 0.075% by volume DMSO for 20 min at room temperature (Molecular Probes, Eugene, OR) (Kay, 2003; Qian et al., 2003). Cardiomyocytes were dropped onto a heated flow-through chamber (Warner Instruments, Hamden, CT), which was placed on the stage of an inverted microscope (Nikon Diaphot) fitted with a 40× objective. Superfusion of the loading buffer with additional 50 μM Zn\(^{2+}\) or 0.3 μM \(N,N,N',N',N'-\text{tetrais}(2\text{-pyridylmethyl})\text{-ethylenediamine}\) (TPEN) (Kay, 2003) was maintained at 27°C, pH 7.4, and with an oxygen tension in equilibrium with ambient air. Cardiomyocytes were electrically paced at 2 Hz via field stimulation using platinum electrodes with stimulus duration of 0.5 ms and voltage of 1.5 times their threshold of stimulation (Grass Instruments, Boston, MA). Fluorescence of intracellular FluoZin-3 was simultaneously with whole-cell FluoZin-3 fluorescence at 240 Hz (Model Hyperswitch, Milton, MA).

2.4. Measurement of \([Ca^{2+}]\), and sarcomere shortening dynamics

Cardiomyocytes were loaded with Fura-2, a fluorescence dye sensitivity to both Ca\(^{2+}\) with \(K_d\sim 200 \text{nM}\) and Zn\(^{2+}\) with \(K_d\sim 10 \text{nM}\) (Atar et al., 1995), by exposure to 2 μM Fura-2 AM and 0.05% by volume DMSO for 20 min at room temperature (Molecular Probes, Eugene, OR) (Palmer et al., 2001). Cardiomyocytes were imaged as described above with fluorescence of intracellular Fura-2 monitored at 510 nm and excited at 485 nm (Chroma Technology, Rockingham, VT). Mean sarcomere length was calculated using a fast Fourier Transform of a video image (Model MyoCam, IonOptix, Milton, MA) recorded simultaneously with whole-cell Fura-2 fluorescence at 240 Hz (Model Hyperswitch, Milton, MA). The recorded cardiomyocyte fluorescent ratio (365/410 nm) and sarcomere shortening transients were analyzed to describe characteristics of systolic and diastolic \([Ca^{2+}]_{i}\) and sarcomere function.

3. Results

3.1. Distributions and concentrations of elements

Fig. 1A illustrates the projections of two-dimensional spatial distributions of nine essential elements (P, S, Cl, K, Ca, Mn, Fe, Cu, and Zn) in a single rat cardiomyocyte. Metal contamination artifact, like that from dust particles, caused the dots of high concentrations most notable in the Mn panel, but also seen in Ca, Fe, Cu, and Zn. Each element, except Mn, displayed a significant elevation (P, S, Cl, K, Ca, and Zn) or depression (Fe, Cu) of relative concentrations in or around a ~5 x 10 μm area highlighted by an arrow in the Zn panel of Fig. 1. We speculate that this ~5 x 10 μm area region corresponds to a cardiomyocyte nucleus, which is expected to be this size and to contain a high Zn concentration due to the aggregation of zinc-finger transcription factors in the nucleus.

Other distinct patterns in this cardiomyocyte were most apparent in Fe, Cu, and Zn. Fig. 1B illustrates the degree of colocalization of Fe and Cu using image addition of pseudo-colored images for Fe (red) and Cu (green). The colocalization of Fe and Cu was apparent due to the production of yellow and relative absence of red and green after image addition in Fig. 1B. Fig. 1C illustrates the longitudinal profile of the Zn striations, which arose with a highly regular periodicity of 1.65 μm. These findings suggest that the transverse striations of Zn represent a specific cellular structure that arises with the periodicity of the sarcomere.

Mean concentrations of the nine examined elements were determined in regions of interests no smaller than 10 μm x 10 μm (excluding dust artifacts and nuclei) and are reported in Table 1. Cellular concentrations of elements P, S, Cl, and K were estimated to be on the order of 10\(^{-1}\) M, while concentrations of Ca, Fe, and Zn were on the order of 10\(^{-3}\) M. Cellular concentrations of Cu and Mn were even lower and on the order of 10\(^{-4}\) M and 10\(^{-3}\) M, respectively. Our reported concentrations of these elements are similar to those reported previously by others using electron probe microscopy or atomic absorption techniques (Bond et al., 1994; Feng et al., 2004; Isenberg et al., 1996; Keller et al., 1995).

3.2. Locations of distinct distributions of elements

The transverse striations of Zn were the most conspicuous patterns to arise among the elements in almost all of the intact cardiomyocytes examined. Similar striations also often appeared for P, S, and Fe, although usually with less distinction. Fig. 2A depicts element images of a cardiomyocyte displaying relatively distinct striated patterns for P, S, Fe, and Zn. The superimposed pseudocolored images of P (green), S (yellow), and Fe (red) with Zn (blue) demonstrate the colocalization of transverse striations of P, Fe, and Zn in this cardiomyocyte. Interestingly, the prominent transverse striations of high Zn also appeared to correspond to distinct depressions in S concentration as illustrated in Figs. 2A and B. As also illustrated in Fig. 2B, the pattern of high Fe occurred with double the spatial frequency of the P, S, and Zn.
striations of high P, Fe, and Zn and low S appeared to correspond well with the I-band of the cardiomyocyte sarcomere imaged by light microscopy (Fig. 2A). The intermediate striations for Fe, therefore, must correspond with the A-band of the sarcomere.

3.3. Cardiomyocyte [Zn$^{2+}$]$_i$

Intracellular free Zn$^{2+}$ concentration ([Zn$^{2+}$]) and sarcomere shortening dynamics were measured in intact cardiomyocytes exposed to 50 µM external Zn$^{2+}$ using FluoZin-3 fluorescence microscopy. These cardiomyocytes loaded with FluoZin-3 and exposed to external Zn$^{2+}$ appeared normal and retained a rod shape throughout these procedures. Cardiomyocyte [Zn$^{2+}$], rose only while cardiomyocytes were electrically paced as shown in Fig. 3A and consistent with reports by Atar et al. (1995) and Turan (2003). The rate of rise in [Zn$^{2+}$], was also directly proportional to the pacing frequency (Fig. 3A), implying that Zn$^{2+}$ influx is through sarcolemmal ion channels and most likely
through the sarcolemmal calcium channels as suggested previously (Atar et al., 1995; Turan, 2003).

A number of cardiomyocytes (n = 24) underwent prolonged exposure to 50 μM external Zn$^{2+}$ while paced at 2 Hz frequency. Under these experimental conditions, including a temperature of 27 °C, cardiomyocyte [Zn$^{2+}$]$_i$ approached a steady-state in ~15 min (Fig. 3B). As cardiomyocyte [Zn$^{2+}$]$_i$ rose, diastolic sarcomere length was extended and peak sarcomere shortening was reduced (Fig. 3B). These data suggest that extracellular and/or intracellular Zn$^{2+}$ reduced myofilament sensitivity to Ca$^{2+}$ activation and/or suppressed [Ca$^{2+}$]$_i$ dynamics.

Fig. 3C illustrates the initial response observed in 18 of the 24 cardiomyocytes under these conditions: i.e., cardiomyocytes sarcomere shortening was immediately arrested upon exposure to 50 μM external Zn$^{2+}$. We speculate that the sudden exposure of external concentration of Zn$^{2+}$ transiently inhibited the inward calcium current and subsequent calcium-induced calcium release from the SR (Atar et al., 1995; Turan, 2003). The significant post-arrest contractions noted in Fig. 3C (indicated by the symbol ‘a’) suggest that the SR had not been activated to release Ca$^{2+}$ during these arrest periods and thus Ca$^{2+}$ accumulated in the SR. Therefore, we conjecture that extracellular Zn$^{2+}$ plays some role in modifying Ca$^{2+}$ regulation in part through its effect on the membrane potential.

Under conditions of no external Zn$^{2+}$ and during exposure to the membrane permeate Zn$^{2+}$-chelator TPEN cardiomyocytes demonstrated transient rises in intracellular Zn$^{2+}$ noted in Fig. 3C (indicated by the symbol ‘b’), suggesting spontaneous releases of internal stores of Zn$^{2+}$ into the cytosol. The presence of multiple arrest periods at times >1300 s furthermore suggests that the loss of intracellular Zn$^{2+}$ due to chelation by TPEN destabilizes Ca$^{2+}$ regulation under these experimental conditions. The relatively dramatic rise in intracellular free Zn$^{2+}$ observed at ~1420 s in Fig. 3C completely arrested cardiomyocyte contraction. Under the circumstances present at ~1420 s, when external Zn is non-existent and internal Zn is relatively high (~10 nM), inward calcium current may have been inhibited by intracellular rather than extracellular Zn$^{2+}$ (Atar et al., 1995; Turan, 2003). It is also possible that high [Zn$^{2+}$]$_i$ blocked the release of SR Ca$^{2+}$, perhaps by inhibiting Ca$^{2+}$-induced opening of SR Ca$^{2+}$ release channels (Wang et al., 2001). Based on our X-ray fluorescence images and our data from fluorescence microscopy, we speculate that there exist significant internal stores of Zn$^{2+}$ in organelles, probably in the SR, and that these stores of Zn$^{2+}$ modulate Ca$^{2+}$ regulation.

### 3.4. Cardiomyocyte [Ca$^{2+}$]$_i$ in response to [Zn$^{2+}$]$_i$

Fig. 4 illustrates sarcomere shortening transients and Fura-2 fluorescence ratio transients recorded simultaneously under experimental conditions equivalent to those presented in Fig. 3B. Figs. 4A and B demonstrate that diastolic sarcomere length was extended and peak sarcomere shortening was reduced after exposure to external Zn$^{2+}$ and during a rise in [Zn$^{2+}$]$_i$, as illustrated in first 600 s of Fig. 3B. Fig. 4C demonstrates that, after the removal of external Zn$^{2+}$, diastolic sarcomere length remained extended while peak sarcomere shortening was recovered. Sarcomere relaxation was also quantifiably improved by [Zn$^{2+}$]$_i$, as demonstrated in measures of time to 50% relengthening, which was 358 ms at minimal [Zn$^{2+}$]$_i$ (Fig. 4A) compared to 325 ms at elevated [Zn$^{2+}$]$_i$ (Fig. 4C). The extent of time at diastolic sarcomere length was noticeably prolonged with elevated [Zn$^{2+}$]$_i$ (Fig. 4C) compared to minimal [Zn$^{2+}$]$_i$ (Fig. 4A). In these cardiomyocytes loaded with Fura-2, we undertook to discern any effects of extracellular and/or intracellular Zn$^{2+}$ on [Ca$^{2+}$]$_i$ dynamics that may underlie the observed responses of sarcomere dynamics.

Figs. 4D and E demonstrate that exposure to external Zn$^{2+}$ led to a diminution in peak [Ca$^{2+}$]$_i$, which may explain in whole or in part the concomitantly reduced peak sarcomere shortening illustrated in Fig. 4B. Peak [Ca$^{2+}$]$_i$ was recovered after removal of the external Zn$^{2+}$, which would similarly explain the concomitant recovery of peak sarcomere shortening illustrated in Fig. 4C. These data demonstrate that external Zn$^{2+}$ inhibits systolic characteristics of cardiomyocyte [Ca$^{2+}$]$_i$ dynamics. The inhibition of calcium induced calcium release from the SR by external Zn$^{2+}$ may occur via the reduction in Ca$^{2+}$ influx through sarcolemmal calcium channels (Atar et al., 1995; Turan, 2003) or via reducing the probability of opening SR Ca$^{2+}$ release channels (Wang et al., 2001).

### Table 1

<table>
<thead>
<tr>
<th>Element</th>
<th>P</th>
<th>S</th>
<th>Cl</th>
<th>K</th>
<th>Ca</th>
<th>Mn</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atomic wt.</td>
<td>30.9</td>
<td>32.1</td>
<td>35.5</td>
<td>39.1</td>
<td>40.1</td>
<td>54.9</td>
<td>55.8</td>
<td>63.5</td>
<td>65.4</td>
</tr>
<tr>
<td>Mean (μg/cm$^2$)</td>
<td>3.60</td>
<td>11.27</td>
<td>13.28</td>
<td>9.88</td>
<td>177.7</td>
<td>1.7</td>
<td>144.1</td>
<td>22.5</td>
<td>62.8</td>
</tr>
<tr>
<td>SEM</td>
<td>0.39</td>
<td>2.08</td>
<td>4.71</td>
<td>0.97</td>
<td>0.066</td>
<td>0.4</td>
<td>24.9</td>
<td>3.2</td>
<td>12.2</td>
</tr>
<tr>
<td>Mean (ppm)</td>
<td>3600</td>
<td>11270</td>
<td>13280</td>
<td>9880</td>
<td>177.7</td>
<td>1.7</td>
<td>144.1</td>
<td>22.5</td>
<td>62.8</td>
</tr>
<tr>
<td>SEM</td>
<td>390</td>
<td>2080</td>
<td>4710</td>
<td>970</td>
<td>56.9</td>
<td>0.4</td>
<td>24.9</td>
<td>3.2</td>
<td>12.2</td>
</tr>
<tr>
<td>Mean (mM)</td>
<td>117</td>
<td>351</td>
<td>374</td>
<td>253</td>
<td>4.43</td>
<td>0.03</td>
<td>2.58</td>
<td>0.355</td>
<td>0.960</td>
</tr>
<tr>
<td>SEM</td>
<td>13</td>
<td>65</td>
<td>133</td>
<td>25</td>
<td>1.42</td>
<td>0.01</td>
<td>0.45</td>
<td>0.051</td>
<td>0.187</td>
</tr>
</tbody>
</table>

Approximated concentrations (ppm and mM) were calculated after assuming sample width of 10 μm and atomic weights shown.
In contrast, $[Ca^{2+}]_i$ relaxation dynamics were not significantly modified by external $Zn^{2+}$ or $[Zn^{2+}]_i$. Measures of time to 50% $[Ca^{2+}]_i$ decline were 185 ms during zero external $Zn^{2+}$ and minimal $[Zn^{2+}]_i$ (Fig. 4D), 197 ms during exposure to $50\mu M$ external $Zn^{2+}$ and moderate $[Zn^{2+}]_i$ (Fig. 4E) and 191 ms during zero external $Zn^{2+}$ and elevated $[Zn^{2+}]_i$ (Fig. 4F). Any influence of $[Zn^{2+}]_i$ on $[Ca^{2+}]_i$ dynamics was therefore not discernibly responsible for the improved sarcomere relaxation function observed in Fig. 4C. These data, however, are consistent with the idea...
that \([\text{Zn}^{2+}]_i\) may affect relaxation function by facilitating actomyosin ATPase (Edman, 1960) or by competing with Ca^{2+} at the troponin complex (Jin et al., 2000).

It is interesting to note that we observed a rise in diastolic Fura-2 fluorescence ratio at time points corresponding to elevated \([\text{Zn}^{2+}]_i\) (Figs. 4D and E). Because Fura-2 possesses a much higher binding affinity for Zn^{2+} than for Ca^{2+} (Atar et al., 1995), and because diastolic sarcomere length was extended rather than shortened during these times, we conclude that the rise in diastolic Fura-2 fluorescence ratio reflects the rise in \([\text{Zn}^{2+}]_i\) rather than a rise in diastolic \([\text{Ca}^{2+}]_i\).

4. Discussion

To the best of our knowledge, the distinct transverse striations of Zn observed in the isolated cardiomyocyte have not been previously described. These Zn striations appeared as altering concentrations of \(\sim 10^{-4}\) and \(10^{-3}\) M and occurred with a periodicity of the cardiomyocyte sarcomere. We found that these striations of high Zn colocalized with striations of high P and Fe and with striations of low S, all of which appeared to correspond to the I-band of the sarcomere. Although great care was taken to
superimpose correctly light micrographs with the X-ray fluorescence images, we must qualify the identification of high P, Fe, and Zn and low S in the I-band as our best fit without the help of unequivocal spatial or antibody markers. Nevertheless, our observing rapid releases of intracellular Zn$^{2+}$ stores are consistent with the likelihood that high Zn (and therefore high P and Fe and low S) are spatially associated with the I-bands and therefore with the structures, organelles and T-tubules found there. One candidate Zn$^{2+}$-binding protein would be calsequestrin (CS), which resides in the sarcoplasmic reticulum, an I-band organelle, and can bind up to 200 mol Zn$^{2+}$ per mol CS independently of binding up to 50 mol Ca$^{2+}$ per mol CS (Baksh et al., 1995). Considering also that [Zn$^{2+}$] can rise quickly and can influence Ca$^{2+}$ regulation, we speculate that the prominent striations of Zn reflect Zn$^{2+}$ bound to CS in the SR and may be regulated in part by those mechanisms handling Ca$^{2+}$ (Atar et al., 1995; Taylor et al., 1972; Turan, 2003; Wang et al., 2001).

It should be noted that our estimates for concentrations of Fe, Cu, and Zn represent underestimates of these concentrations in vivo due to the possible loss of these elements during the cardiomyocyte isolation process, which did not allow any possible replacement of Fe, Cu or Zn. The relative abundance of Fe, Cu, and Zn in the range of 10$^{-4}$ and 10$^{-3}$ M (and possibly higher in vivo) suggests the availability of these metals as second messengers for gene expression (Andrews, 2001; DeMoor and Koropatnick, 2000; Finney and O’Halloran, 2003; Outten and O’Halloran, 2001; Pyle, 1993). It has been shown previously that external Zn may activate gene expression in GH3 cells (Atar et al., 1995), that the expression of Zn$^{2+}$ regulating proteins is modified by changes in [Zn$^{2+}$], in other cell systems (Bird et al., 2000, 2003; Verhaegh et al., 1998) and that the expression of cardiac sarcomeric proteins may be downregulated due to a Zn deficient diet (Duffy et al., 2004). With the most recent findings that some zinc-fingers of zinc-finger transcription factors may be directly regulated by Zn$^{2+}$ availability (Andrews, 2001; Bird et al., 2000), it is tempting to speculate that Zn$^{2+}$ or Zn-carrying metalloproteins may serve as second messengers for transcription factor activation and therefore gene expression in response to stress. Of particular interest in the cardiomyocyte, our data suggest that Zn$^{2+}$ or Zn-carrying metalloproteins may be released from internal stores during mechanical stress and thereby signal mechanical stress to transcription/translation pathways (Knoll et al., 2003; Pikkarainen et al., 2003).

4.1. Other essential elements

The very low atomic numbered essential elements (H, C, N, O, and F) were not examined in this study due in part to technical limitations, but also due in part to the inherent difficulty that would arise in interpreting their ubiquitous distribution in water and organic compounds. Many low atomic numbered elements (Na, Mg, P, S, Cl, K, and Ca), on the other hand, play specialized roles in normal cardiomyocyte function and are found in relatively abundant
concentrations of $10^{-3}$ M or higher (Bond et al., 1994; Feng et al., 2004; Isenberg et al., 1996; Keller et al., 1995). Intracellular free K$^+$ concentration, for example, is on the order of $10^{-1}$ M and significantly contributes to establishing the cardiomyocyte resting membrane potential. Transmembrane movements of Na$^+$, K$^+$, and Ca$^{2+}$ produce the cardiomyocyte action potential, which travels quickly between cardiomyocytes and coordinates muscle activation in different regions of the heart (Bers, 2001). The high abundance of P is likely a reflection of its incorporation into several organic compounds; however, the particularly high abundance of P in the I-band may be due to phosphocreatine, which appears in high concentrations (>50 mM) in the I-band of the sarcomere (Bers, 2001). In contrast, S is likely depressed in the I-band of the sarcomere, because S reflects the total density of sulfur-containing amino acids, which are most abundant in the darker A-band.

Cardiomyocytes generate contractile force relative to intracellular free Ca$^{2+}$ concentrations over a range of approximately $10^{-2}$ M (relaxed) to $10^{-3}$ M (maximally activated), but the total Ca content is on the order of $10^{-3}$ M in myofilaments and in mitochondria (Bond et al., 1994; Feng et al., 2004; Isenberg et al., 1996; Keller et al., 1995). The beat-by-beat regulation of intracellular free Ca$^{2+}$ by transmembrane mechanisms and by intracellular buffers and organelles is considered by many to be the most important factor influencing cardiomyocyte contractile function (Shannon and Bers, 2004) and possibly also gene expression (Wilkins and Molkentin, 2004). We did not detect distinct patterns of total Ca concentrations in cardiomyocytes as was reported for high chelatable Ca concentrations reported in the SR using Fura-2 and Calcium Green-2 dyes (Isenberg et al., 1996). This may be due in part to Ca being mostly bound to proteins, which are ubiquitously distributed in cardiomyocytes. The technical limitation of distinguishing K and Ca concentrations (Vogt, 2003) also led to our having to tolerate a reduced spatial resolution for Ca.

Some intermediate atomic numbered elements, i.e., the transition metals (Mn, Fe, Cu, Zn, and Se), are also found in cardiomyocytes. Iron notably plays an essential role in the structure of heme, which is synthesized in the mitochondria (Ordway and Garry, 2004). Mitochondria are highly abundant in cardiomyocytes, and Fe regulation is proportionally vital to cardiomyocyte metabolic function (Lenka et al., 1998). Considering that Cu localizes to mitochondria as found recently by Yang et al. (2005), our finding that Fe colocalizes with Cu suggests that the prominent longitudinal patterns of Fe and Cu highlight the presence of cardiomyocyte mitochondria. The transverse patterns of Fe, which appeared in the I-band and A-band (Fig. 2B), may reflect the presence of myoglobin and mitochondria, respectively. It may be noteworthy that the elements Mn, Cu, Zn, and Se are all incorporated into activated forms of the antioxidant superoxide dismutase, which is cardioprotective against oxidative stress (Barandier et al., 1999).

In conclusion, further examinations of intracellular Zn$^{2+}$ regulation and its influence on Ca$^{2+}$ regulation and gene expression are warranted. Although distinct striations of high Zn concentrations appear at intervals of the sarcomere in the cardiomyocyte, the specific intracellular buffers of Zn$^{2+}$ have not been unequivocally identified or characterized. The valence, charge and abundance of Zn$^{2+}$ in the cardiomyocyte raise the possibility that intracellular Zn$^{2+}$ modifies intracellular Ca$^{2+}$ regulation including competing for divalent cation buffers. In addition, stores of Zn$^{2+}$ may be released spontaneously under stressful conditions and therefore may serve as repositories for Zn$^{2+}$ ion that could act as a second messenger for Ca$^{2+}$ regulation and/or gene expression to modify cardiomyocyte performance.

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