Cellular Mechanisms of Altered Contractility in the Hypertrophied Heart
Big Hearts, Big Sparks

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Abstract—To investigate the cellular mechanisms for altered Ca\(^{2+}\) homeostasis and contractility in cardiac hypertrophy, we measured whole-cell L-type Ca\(^{2+}\) currents (\(I_{\text{Ca,L}}\)), whole-cell Ca\(^{2+}\) transients ([Ca\(^{2+}\)]\(_i\)), and Ca\(^{2+}\) sparks in ventricular cells from 6-month-old spontaneously hypertensive rats (SHRs) and from age- and sex-matched Wistar-Kyoto and Sprague-Dawley control rats. By echocardiography, SHR hearts had cardiac hypertrophy and enhanced contractility (increased fractional shortening) and no signs of heart failure. SHR cells had a voltage-dependent increase in peak [Ca\(^{2+}\)], amplitude (at 0 mV, 1330±62 nmol/L [SHRs] versus 836±48 nmol/L [controls], \(P<0.05\)) that was not associated with changes in \(I_{\text{Ca,L}}\) density or kinetics, resting [Ca\(^{2+}\)], or Ca\(^{2+}\) content of the sarcoplasmic reticulum (SR). SHR cells had increased time of relaxation. Ca\(^{2+}\) sparks from SHR cells had larger average amplitudes (173±192 nmol/L [SHRs] versus 109±64 nmol/L [control]; \(P<0.05\)), which was due to redistribution of Ca\(^{2+}\) sparks to a larger amplitude population. This change in Ca\(^{2+}\) spark amplitude distribution was not associated with any change in the density of ryanodine receptors, calsequestrin, junctin, triadin 1, Ca\(^{2+}\)-ATPase, or phospholamban. Therefore, SHRs with cardiac hypertrophy have increased contractility, [Ca\(^{2+}\)], amplitude, time to relaxation, and average Ca\(^{2+}\) spark amplitude (“big sparks”). Importantly, big sparks occurred without alteration in the trigger for SR Ca\(^{2+}\) release (\(I_{\text{Ca,L}}\)), SR Ca\(^{2+}\) content, or the expression of several SR Ca\(^{2+}\)-cycling proteins. Thus, cardiac hypertrophy in SHRs is linked with an alteration in the coupling of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels and the release of Ca\(^{2+}\) from the SR, leading to big sparks and enhanced contractility. Alterations in the microdomain between L-type Ca\(^{2+}\) channels and SR Ca\(^{2+}\) release channels may underlie the changes in Ca\(^{2+}\) homeostasis observed in cardiac hypertrophy. Modulation of SR Ca\(^{2+}\) release may provide a new therapeutic strategy for cardiac hypertrophy and for its progression to heart failure and sudden death. (Circ Res. 1999;84:424-434.)

Key Words: spontaneously hypertensive rat ▪ cardiac hypertrophy ▪ Ca\(^{2+}\) transient ▪ Ca\(^{2+}\) spark

Cardiac hypertrophy is associated with marked changes in myocardial contractility. Peak active tension increases, and the rates of both contraction and relaxation are slowed. These contractile abnormalities are associated with alterations in the whole-cell calcium transient ([Ca\(^{2+}\)]\(_i\)). In the hypertrophied myocardium, the amplitude of [Ca\(^{2+}\)]\(_i\) increases, whereas in failing myocardium, the amplitude of [Ca\(^{2+}\)]\(_i\) decreases. In most animal models of hypertrophy and in failing human hearts, the duration of the whole-cell [Ca\(^{2+}\)]\(_i\), is also prolonged. However, the precise cellular mechanisms that are responsible for changes in contractility and alterations in [Ca\(^{2+}\)]\(_i\) are largely unknown.

Identification of the cellular mechanisms that underlie altered excitation-contraction coupling in cardiac hypertrophy and heart failure is complicated by several issues, including differences in experimental animal models and disease progression. In addition, it has only recently proved possible using confocal microscopy to measure local non-propagating elevations of Ca\(^{2+}\) (Ca\(^{2+}\) sparks) at the level of individual sarcomeres. The ability to measure Ca\(^{2+}\) sparks provides an opportunity to evaluate directly the role of sarcoplasmic reticulum (SR) Ca\(^{2+}\) release in muscle cells from animal models associated with cardiac hypertrophy.

In this study, we used laser scanning confocal microscopy and Ca\(^{2+}\)-sensitive fluorescent indicators to detect Ca\(^{2+}\) sparks evoked by electrical field stimulation in ventricular cells from normal rats and from spontaneously hypertensive rats (SHRs) with cardiac hypertrophy. By quantitative analysis of the kinetic characteristics of Ca\(^{2+}\) sparks, we identify enhanced SR Ca\(^{2+}\) release from hypertrophied SHR cells. In

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addition, we demonstrate that cardiac hypertrophy is associated with abnormalities in myocyte relaxation despite normal SR Ca\textsuperscript{2+} uptake and normal expression of several important Ca\textsuperscript{2+} cycling proteins. Our results suggest that alterations in SR Ca\textsuperscript{2+} release may be among the primary cellular mechanisms that underlie the enhanced contractility and increased [Ca\textsuperscript{2+}], associated with cardiac hypertrophy.

### Materials and Methods

**Animals**

Studies were performed on 6-month-old SHRs and age- and sex-matched Wistar-Kyoto and Sprague-Dawley control rats. There were no differences between Wistar-Kyoto and Sprague-Dawley in each of the experiments, and both groups were combined and denoted as control animals. Animals were obtained at 6 to 8 weeks of age and weighed between 180 and 280 g. The heart rates, blood pressure (BP), and respiratory rates of all animals were monitored at monthly intervals. BP was measured using the tail-cuff method. All measurements were obtained while the animal was resting comfortably and were repeated 3 times at each determination.

The clinical and echocardiographic characteristics, heart weight (wt)/body weight ratios, and cell capacitance of 6-month-old SHRs and control rats are shown in the Table. At the time of study, all SHRs were hypertensive with a mean systolic BP of 182 ± 6.8 mm Hg, whereas Wistar-Kyoto and Sprague-Dawley rats had a mean systolic BP of 121 ± 0.7 mm Hg (n = 78; P < 0.05 versus controls), and no animal exhibited any clinical signs of heart failure such as weight loss, tachypnea, resting tachycardia, or pleural and/or pericardial effusions.

In addition, a separate population of SHRs and controls was treated continuously with an angiotensin-converting enzyme inhibitor (lisinopril, 25 mg/(kg body weight \*z\textsuperscript{2})).

**Pathology and Histology Studies**

Animals were deeply anesthetized with sodium pentobarbital (170 mg/kg injected IP), and hearts were removed via midline thoracotomy. The heart was mounted on a Langendorff apparatus and perfused retrograde at 37°C with a physiological salt-containing solution (PSS, see below) containing 1 mmol/L CaCl\textsubscript{2} for 5 minutes, followed by Ca\textsuperscript{2+}-free PSS for 5 minutes to arrest the heart in diastole. The heart was then perfused with 25 mL of fixative solution consisting of 2.5% glutaraldehyde in 0.1 mmol/L sodium cacodylate buffer (pH 7.3). Cross sections of the heart were obtained at the level of the papillary muscles, and sample pieces of the LV were obtained from the anterior wall and papillary muscles. All samples were stored in fixative at room temperature (21°C to 23°C) for 48 hours and then transferred to cold (4°C) 0.2 mmol/L sodium cacodylate buffer. Some samples were embedded in paraffin, sectioned, and evaluated by standard histological techniques. Samples selected for electron microscopy were postfixed for 1 hour in 1% Os\textsubscript{4}O\textsubscript{4} in 0.1 mmol/L sodium cacodylate buffer (4°C), stained en bloc with 2% uranyl acetate, dehydrated in ethanol, and embedded in Epon. Thin sections were then stained with uranyl acetate and lead citrate and examined in a Phillips EM 201 transmission electron microscope.

For morphometric analysis of cell size, paraffin sections of papillary muscles cut in cross section were examined with a \times 40-objective lens in a Nikon Microphot microscope equipped with a Hitachi charge-coupled device camera connected to a Sony color video monitor. Morphometric analysis was performed with a commercially available software package (BioQuant, OS2 version 2.5; R&M Biometrics). Briefly, manual tracings of the perimeters of cardiac muscle cells were made from all cells visualized on a field (500 × 500 \mu\text{m}) projected on a video monitor. The area within the perimeter tracings was calculated using the BioQuant software. A total of 160 control and 156 SHR cells were measured from multiple sections taken from 3 control rats and 3 SHRs.

**Echocardiographic Studies**

Transthoracic echocardiographic studies were performed in rats using standard techniques. Briefly, rats were lightly anesthetized with sodium pentobarbital (10 to 15 mg/kg injected IP). Using a commercially available echocardiographic machine (Hewlett-Packard Sonos 2000) equipped with a 7.5-MHz transducer, a 2-dimensional short-axis view of the left ventricle (LV) was obtained at the level of the papillary muscles. M-mode tracings were recorded through the anterior and posterior LV walls. The internal dimensions of the LV cavity and the anterior and posterior wall thickness (end systole and end diastole) were measured using a modification of the leading-edge method from the American Society of Echocardiography from 3 consecutive cardiac cycles on the M-mode tracings. The analysis was performed offline (commercial software, Hewlett-Packard) by 2 echocardiographers (M.C. and J.M.B.), who were blinded to the strain of rat and prior results. The percentage shortening of the endocardium was calculated as (LVDD–LVSD)/LVDD \times 100, where LVDD = LV internal diastolic dimension and LVDS = LV internal systolic dimension.

**Whole-Cell L-Type Ca\textsuperscript{2+} Current (I_{\text{Ca,L}}, [Ca\textsuperscript{2+}]\textsubscript{o}, and Cell Shortening**

Single ventricular cells were isolated using a standard enzymatic dispersion technique. In both normal rats and SHRs, the cell isolation procedure yielded a single population of cells in which the distribu-
tion of cell capacitance was unimodal and fit by a single Gaussian function.

Single ventricular cells were studied using the whole-cell variation of the patch-clamp technique under conditions that eliminated Na+ and K+ currents that would interfere with the measurement of Ca2+ influx via L-type Ca2+ channels. The external solution was composed of the following (in mmol/L): NaCl 140, dextrose 10, HEPES 10, CsCl 10, MgCl2 1, and CaCl2 1, pH adjusted to 7.3 to 7.4 with NaOH at 25°C. The electrode-filling solution used for whole-cell recording and loading with the fluorescent indicator, Indo 1 (Molecular Probes), was composed of the following (in mmol/L): cesium glutamate 130, HEPES 10, MgCl2 0.33, Mg2-ATP 4, and Indo 1 0.05, pH adjusted to 7.1 to 7.2 with CsOH. The filled micropipette electrodes had resistances of 1.5 to 4.0 MΩ. The holding potential was −50 mV. All experiments were performed at room temperature (21°C to 23°C). Cell capacitance and whole-cell currents were recorded using standard methods. Current was filtered at 5 kHz and digitized at 12 kHz with 12-bit resolution. [Ca2+]i, calculated from the Indo 1 fluorescence through the use of “calibration parameters” obtained in situ and was corrected for the kinetics of Indo 1.29

In selected experiments, unloaded cell shortening was measured simultaneously with [Ca2+]i and [Ca2+]s, using a custom-made edge-detection system. Because high magnification was required for the peak amplitude. The fall time of a Ca2+ transient was monitored. The data from each experiment were normalized to the shortening measured at maximal depolarization to allow for comparison.

**Local SR Ca2+ Release: Ca2+ Sparks**

Cells were loaded with the membrane-permeant form of the Ca2+ indicator, Fluo 3 (Molecular Probes), by incubating the cells for 30 minutes at 21°C to 23°C in PSS containing Fluo 3-AM (10 mmol/L) and pleuronic acid (0.05% wt/vol). Nifedipine (10 μmol/L) was added to the external solution to reduce the probability but not the amplitude of single L-type Ca2+ channel currents. Cells were electrically field stimulated with a 50- to 100-mA pulse of 5 ms duration. A series of prepulses3–5 was given to ensure a constant section (30 pixels) of the background-subtracted image centered around the Ca2+ spark was extracted and smoothed (recursive boxcar average filter with a width of 3 pixels). The first 3 line scans of this area were averaged to determine the resting fluorescence. The peak amplitude of the Ca2+ spark was determined as the difference between the maximal fluorescence of the Ca2+ spark and the resting fluorescence before the peak of the Ca2+ spark. A threshold level was set at half the difference between the peak amplitude and the resting level. The rate of onset of a Ca2+ spark was defined as the time required for the fluorescence signal to increase from threshold to peak amplitude. The fall time of a Ca2+ spark was defined as the time required for the fluorescence to decrease from peak amplitude to below threshold. The following criteria were used to identify a rise in fluorescence as a Ca2+ spark. (1) The peak amplitude had to remain above threshold for at least 2 consecutive line scans (4 ms). (2) The fluorescence signal had to fall below threshold within the area analyzed. (3) The area analyzed contained only 1 peak of [Ca2+]s, elevation [Ca2+]s, was calculated from the Fluo 3 fluorescence, with a self-ratio method using an equation and calibration parameters given previously.27

**Assessment of SR Ca2+ Load**

The Ca2+ content of the SR was measured using the whole-cell variation of the patch-clamp technique coupled with Indo 1 (Molecular Probes) fluorescence. Briefly, eight 200-ms voltage-clamp steps to 0 mV were applied before data collection to achieve steady-state SR Ca2+ loading. After the prepulses, caffeine (20 mmol/L) was rapidly applied to the cell, eliciting both a [Ca2+]i, and an inward current that represents the activation of Na+/Ca2+ exchange by the Ca2+ released from the SR. SR load was assessed by both the amplitude of the evoked Ca2+ transient and the integral of the elicited current. The current was integrated offline using Origin (Microcal Software, Inc). All data were normalized to cell capacitance.

**Biochemical Methods**

Control and SHR hearts were obtained and arrested in diastole as described above. The hearts were then immediately placed in cold (0°C) Ca2+-PSS and stored at −70°C until analyzed.

LV samples (∼110 mg) were homogenized in 2 mL of 0.25 mol/L sucrose and 10 mmol/L histidine (pH 7.2), and [3H]ryanodine binding assays were conducted at saturating ryanodine concentration (15 nmol/L).26 Briefly, a sample of crude homogenate protein (0.38 mg) was incubated for 1 hour at 37°C in 200 μL of binding solution, which contained the following: 20 mmol/L MOPS, 1 mmol/L CaCl2, 600 mmol/L NaCl, and 15 mmol/L [3H]ryanodine (pH 7.1) with and without 10 μmol nonradioactive ryanodine for determination of nonspecific binding. After incubation, 2 mL of iced buffer containing the above solution was added to the mixture, and the samples were filtered through glass fiber type C filters using a cell harvester. The filters were rinsed twice with cold buffer, placed in glass scintillation vials with 7 mL of Altima Gold scintillation fluid, and counted. All experiments were performed in triplicate.

Quantitative immunoblotting of the SR Ca2+-ATPase, phospholamban, the ryanodine receptor, junctin, triadin, and calsequestrin were performed.33 The antibodies used were the following: for Ca2+-ATPase, monoclonal antibody 2A7-A1; for phospholamban, monoclonal antibody 2D12; for ryanodine receptor antibody, monoclonal antibody 1E9; for junctin, affinity-purified antibodies to residues 6 to 20 of canine junctin; for triadin, affinity-purified antibodies to residues 146 to 159 of mouse triadin; and for calsequestrin, affinity-purified antibodies to cardiac calsequestrin.33 121I-Protein A binding bands were quantified with the use of a GS-250 molecular imager (Bio-Rad). All experiments were done in triplicate.

**Statistical Analysis**

Results are expressed as mean±SD. Significance was determined using Student t test, with a value of P<0.05 considered to be statistically significant.

**Results**

**Characteristics of Cardiac Hypertrophy in SHRs**

At 6 months of age, SHRs have systemic hypertension and cardiac hypertrophy without any clinical signs of heart failure, including tachycardia, tachypnea, or edema. As shown in the Table, SHRs had a mean systolic BP of 182±21 mm Hg compared with controls (121±11 mm Hg, P<0.05). Concentric left-ventricular hypertrophy was revealed by 2-dimensional and M-mode echocardiography (Figure 1). Six-month-old SHRs had increased LV wall thickness (posterior wall, 1.9±0.4 mm [SHRs] versus 1.6±0.3 mm [controls], P=0.05) and decreased end-diastolic LV dimensions (5.7±0.8 mm [SHRs] versus 6.8±0.7 mm [controls], P<0.05). In addition, SHRs had increased fractional shortening (0.49±0.05 mm [SHRs] versus 0.42±0.06 mm [controls], P<0.05), consistent with an in-
crease in contractility. None of the 6-month-old SHRs had echocardiographic signs of heart failure, including LV chamber dilation or pleural or pericardial effusions.

To evaluate the possibility that the development of cardiac hypertrophy in SHRs was independent of the sustained increases in afterload from systemic hypertension, a cohort of SHRs (and controls) was studied after 18 months of treatment with an angiotensin-converting enzyme inhibitor (lisinopril) at a dose sufficient to restore the BP of SHRs to the levels of untreated controls. As shown in the Table, determinations of the posterior LV wall thickness and fractional shortening in treated SHRs were nearly identical to those in both the treated and 6-month-old controls. The end-diastolic diameters in treated SHRs were similar to those of treated controls, although they were both less than those of the 6-month-old controls, possibly because of treatment with the antihypertensive medication.

The in vivo findings of cardiac hypertrophy in 6-month-old SHRs were also confirmed by a significant increase in the heart weight (LV, wet)/body weight ratio (5.1 ±0.8 mg/g [SHRs] versus 3.8±0.5 mg/g [controls], P <0.05) and in membrane capacitance determined electrically in single cells (220±41 pA/pF [SHRs] versus 171±31 pA/pF [control], P<0.05).

Consistent with these findings, Figure 2 shows the pathological and histological changes indicative of cardiac hypertrophy. Cross sections (Figure 2A) taken from representative hearts at the level of the papillary muscles show slight concentric thickening of the walls of the LV in SHR hearts. From images of transmitted light microscopy (Figure 2D [SHRs] and Figure 2C [controls]), SHR cells are clearly hypertrophied (average cross-sectional area of cells, in μm² of transverse images, 296±78 [SHRs] versus 135±58 [controls], P<0.0001). These sections do not show any histological characteristics frequently seen in heart failure, including increased fibrosis and destruction of the myofibrillar structure with misalignment of the myofibrils. The images from transmission electron microscopy also do not show any alterations in sarcomere structure consistent with heart failure, including misalignment of the contractile bands.36 Therefore, 6-month-old SHRs have hypertension and cardiac hypertrophy that is remarkably similar to hypertensive heart disease in humans.

$I_{\text{CaL}}, [\text{Ca}^{2+}]_i$, and Cell Shortening

Figure 3 shows $I_{\text{CaL}}$ (middle tracings), [Ca$^{2+}$], (upper tracings), and cell shortening (lower tracings) in controls (Figure 3A, n=34) and SHRs (Figure 3B, n=24). When normalized to cell size (as determined from cell capacitance), the peak amplitude of $I_{\text{CaL}}$ in SHRs is nearly identical to that of controls (peak $I_{\text{CaL}}$ at 0 mV, 5.22±1.92 pA/pF [SHRs] versus 5.66±1.58 pA/pF [controls], P=NS) at all test potentials (Figure 3C). In addition, the time course of decay for $I_{\text{CaL}}$ from SHRs and controls was identical at all test potentials (time constants for decay at 0 mV, 19.2±4.8 and 160±35 ms [SHRs] versus 18.7±3.8 and 143±5 ms [controls], P=NS). Thus, there is no apparent change in the trigger for Ca$^{2+}$ release from the SR, namely $I_{\text{CaL}}$ in SHRs with cardiac hypertrophy.

In contrast, SHR cells had demonstrable changes in [Ca$^{2+}$], (Figure 3B). At several test potentials, SHRs had a significant elevation in the peak amplitude of [Ca$^{2+}$], (Figure 3C; peak [Ca$^{2+}$], at 0 mV, 1330±62 nmol/L [SHRs] versus 791±38 nmol/L [controls], P<0.001). These changes in peak [Ca$^{2+}$] were not associated with changes in the time to peak (89.8±5.8 ms [SHRs] versus 78.8±9.1 ms [controls], P=NS) or the kinetics of [Ca$^{2+}$] decline at 0 mV, time to 50% decay, 191.8±11.8 ms [SHRs] versus 193.4±9.7 ms [controls], P=NS; time to 90% decay, 496.1±28.9 ms [SHRs] versus 520.1±30.5 ms [controls], P=NS). Importantly, resting
[Ca\[^{2+}\]]\_i\] was unchanged in SHR cells (103±23 nmol/L [SHRs] versus 97±17 nmol/L [controls], \(P=NS\)). Therefore, peak [Ca\[^{2+}\]]\_i\] is significantly elevated in a voltage-dependent manner in SHRs with cardiac hypertrophy.

There was a marked increase in the duration of unloaded cell shortening in SHRs (Figure 3B), which was independent of voltage (time to 90% decay, at 0 mV, 770±130 ms [SHRs] versus 484±77 ms [controls], \(P<0.003\)). No significant difference was noted in the voltage dependence of normalized cell shortening (Figure 3E; normalized shortening at 0 mV, 0.032±0.006 [SHRs] versus 0.031±0.01 [controls], \(P=NS\)). The maximal extent and rate of unloaded cell shortening could not be reliably compared between SHRs and controls because of the variability in the fixed point of the cell.

**Ca\[^{2+}\] Sparks**

Since [Ca\[^{2+}\]]\_i\] is determined by multiple cellular processes, of which SR Ca\[^{2+}\] release is just 1, we exploited the enhanced spatial resolution of confocal microscopy to quantify directly the release of Ca\[^{2+}\] from the SR, visualized as Ca\[^{2+}\] sparks. Using this approach, 396 Ca\[^{2+}\] sparks were measured in 13 rat ventricular cells obtained from 5 control rats (Figure 4A). Both spontaneous and evoked Ca\[^{2+}\] sparks were included in the analysis, because previous studies have shown that spontaneous Ca\[^{2+}\] sparks are indistinguishable from Ca\[^{2+}\] sparks evoked by electrical stimulation on the basis of similar kinetic characteristics.\[^{18,19,22}\] In control cells, [Ca\[^{2+}\]]\_i\] rises rapidly with an overall mean Ca\[^{2+}\] spark amplitude of 109±64 nmol/L. However, the distribution of Ca\[^{2+}\] spark amplitudes suggests at least 2 distinct populations (Figure 4A). Whereas the majority (87%) of Ca\[^{2+}\] sparks had a mean amplitude of 88±67 nmol/L, a smaller number (13%) of Ca\[^{2+}\] sparks had significantly larger amplitudes (“big sparks”) with a mean elevation in [Ca\[^{2+}\]]\_i\] of 198±68 nmol/L. Additional
distributions could not be reliably detected because of the small number of observed Ca$^{2+}$ sparks of larger amplitudes.

The temporal and spatial size (area) of Ca$^{2+}$ sparks varied greatly, and there was no correlation between the amplitude of a Ca$^{2+}$ spark and its area, rise time, or fall time ($r^2 = -0.05, 0.06,$ and $-0.22$, respectively). There was a weakly positive correlation ($r^2 = 0.52$) between the area of the Ca$^{2+}$ spark and its fall time. If the variation in Ca$^{2+}$ spark amplitude were due to out-of-focus events, then there should be a negative correlation between Ca$^{2+}$ spark amplitude and duration. Thus, these results support the notion that the total variation in Ca$^{2+}$ spark amplitude is not due to the detection of out-of-focus events.$^{30,37}$

Five hundred sixty-two Ca$^{2+}$ sparks were observed in 17 ventricular cells obtained from 6 SHRs (Figure 4B). The mean amplitude of SHR Ca$^{2+}$ sparks was considerably greater than the mean amplitude of control Ca$^{2+}$ sparks (173±192 nmol/L [SHRs] versus 109±64 nmol/L [controls], $P<0.0001$). Similar to control cells (Figure 4A), SHR Ca$^{2+}$ spark amplitudes were not normally distributed and were fit better with 2 overlapping distributions. As shown in Figure 4B, these 2 distributions were nearly identical in amplitude to those identified in control cells and included a population with a mean amplitude of 105±74 nmol/L and a second population with a mean amplitude of 197±103 nmol/L. In contrast to control Ca$^{2+}$ sparks, the first distribution comprised only 57% of the population of SHR Ca$^{2+}$ sparks, while the second distribution contained the remaining 43% of SHR Ca$^{2+}$ sparks (big sparks). Therefore, the overall increase in Ca$^{2+}$ spark amplitude in SHR cells appears attributable to a redistribution of the Ca$^{2+}$ sparks to the larger amplitude population.

Finally, there was no correlation between the amplitude of SHR Ca$^{2+}$ sparks and the area of spread, rise time, or fall time ($r^2 = 0.02, 0.01,$ and $0.003$, respectively). In SHR cells, there was also a weak positive correlation between the area encompassed by the Ca$^{2+}$ spark and its fall time ($r^2 = 0.47$). Consistent with control Ca$^{2+}$ sparks, the variation in SHR Ca$^{2+}$ spark amplitude is unlikely to be due to detection of out-of-focus events.

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**Figure 4.** Ca$^{2+}$ sparks from control (A) and SHRs (B). In each panel, representative line-scan images of 2 Ca$^{2+}$ sparks are shown at top, with the corresponding 3-dimensional surface plots in the middle. Amplitude histograms of 244 Ca$^{2+}$ sparks from 4 control cells (A) and 400 Ca$^{2+}$ sparks from 8 SHR cells (B) are shown at bottom. Each histogram was best fit by the sum of 2 gaussian distributions.
SR Ca\(^{2+}\) Load

The increase in SHR Ca\(^{2+}\) spark amplitude could be due to an increase in the amount of Ca\(^{2+}\) accumulated in the SR.\(^{38}\) To evaluate this possibility, the SR Ca\(^{2+}\) load under these experimental conditions was determined by measuring both the amplitude of the [Ca\(^{2+}\)]\(_i\) and the inward current in response to the rapid application of caffeine to the cell (Figure 5). As shown in Figure 5, the caffeine-induced [Ca\(^{2+}\)]\(_i\) had a peak of 1200±200 nmol/L, and the total inward charge was 1.4±0.3 pC/pF in 5 normal cells. Similar values were obtained in 4 SHR cells (1300±300 nmol/L and 1.3±0.3 pC/pF, respectively). These results indicate that SR Ca\(^{2+}\) content is similar in SHR and control cells if the quantity of the SR is similar in each cell type. No information is yet

Figure 5. [Ca\(^{2+}\)]\(_i\) transients (upper) and inward Na\(^+\)/Ca\(^{2+}\) exchange current from representative control and SHR cells in response to caffeine. Note the similarity between the [Ca\(^{2+}\)]\(_i\) transients and Na\(^+\)/Ca\(^{2+}\) exchange current elicited in each cell type. See text for further details.
available regarding the amount of SR in control and SHRs at this age.

**Density of Ryanodine Receptors**

To evaluate the possibility that increased SR Ca\(^{2+}\) release in SHRs (both [Ca\(^{2+}\)]\(_{i}\) and Ca\(^{2+}\) sparks) was due to an increase in the numbers of SR Ca\(^{2+}\) release channels (ryanodine receptors), we measured ryanodine-receptor density in hearts from 3 SHRs and 3 control animals. Ryanodine binding at saturating ([\(^{3}H\)]ryanodine concentrations was equivalent in the 2 groups ([\(^{3}H\)]ryanodine binding, n = 3; 193 ± 12 fmol/mg [SHRs] versus 211 ± 11 fmol/mg [controls]). In other experiments, we observed that [\(^{3}H\)] ryanodine receptor binding density was also unchanged in 18-month-old SHRs (data not shown). In addition, quantitative immunoblotting did not show any change in contents of Ca\(^{2+}\)-handling proteins in free SR (Ca\(^{2+}\)-ATPase and phospholamban) and junctional SR (ryanodine receptor, calsequestrin, triadin 1, and junction; Figure 6). Therefore, the increase in SR Ca\(^{2+}\) release observed in SHR cells appears to reflect changes in the efficacy of the trigger signal to elicit SR Ca\(^{2+}\) release.

**Discussion**

The major finding of this study is that hypertrophic myocytes from SHRs have an increase in the amplitude of [Ca\(^{2+}\)]\(_{i}\) and in the mean amplitude of Ca\(^{2+}\) sparks. This increase in SR Ca\(^{2+}\) release occurs without observable changes in the Ca\(^{2+}\) trigger for SR Ca\(^{2+}\) release (I\(_{Ca,L}\)) or SR Ca\(^{2+}\) content. In addition, there was no alteration in the density of several SR Ca\(^{2+}\)-handling proteins, including those of the free SR and the junctional SR. Thus, these data suggest that the increased (or at least the preservation of) contractility observed with cardiac hypertrophy in SHRs most likely involves an alteration of the coupling of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels and the release of Ca\(^{2+}\) from the SR.

In addition, SHR myocytes exhibited a marked prolongation of the relaxation phase of unloaded contractions. However, the time course of [Ca\(^{2+}\)]\(_{i}\), in SHRs was identical to those from control cells, and no alterations in the expression of the Ca\(^{2+}\)-ATPase were found. Collectively, these results indicate that a change either in the Ca\(^{2+}\) affinity of the contractile proteins or in the ultrastructural proteins that maintain cell shape may be responsible, in part, for the diastolic dysfunction observed with hypertensive heart disease.

The observation of normal I\(_{Ca,L}\) in hypertrophied SHR cells is in good agreement with data from several animal models of cardiac hypertrophy. I\(_{Ca,L}\) density is unaltered in ventricular cells from SHRs\(^{39,40}\) rats with aortic banding\(^{41}\) cats with pulmonary artery banding\(^{42}\) cardiomyopathic hamsters\(^{43}\) and human ventricular cells from failing hearts\(^{12,44}\). This finding is not universal, however, and appears to be both model and experiment dependent. I\(_{Ca,L}\) increases in guinea pigs with aortic banding\(^{45}\) in rats with renal artery banding\(^{46}\) and in 1 report on SHRs\(^{47}\). In addition, I\(_{Ca,L}\) has been shown to decrease in ventricular cells from cats with aortic banding\(^{48}\) and ferrets with pulmonary artery banding\(^{49}\). Thus, it appears that the I\(_{Ca,L}\) is unchanged in most models of hypertension and heart failure.

The relationship between hypertrophy and [Ca\(^{2+}\)]\(_{i}\), amplitude also appears model dependent. In contrast to our findings in cardiac hypertrophy, the vast majority of studies of heart failure show both a decrease in the peak amplitude and a prolongation in the duration of the [Ca\(^{2+}\)]\(_{i}\). A decrease in the peak [Ca\(^{2+}\)]\(_{i}\), has been observed in myocytes from aortic-banded rats that worsened as heart failure developed\(^{50}\), aortic-banded cats\(^{51}\), rats with renovascular hypertension\(^{10}\), dogs with pacing-induced cardiomyopathies\(^{14}\), Dahl salt-sensitive rats\(^{15}\), a derivative population of SHRs that develop accelerated heart failure (spontaneously hypertensive-heart failure [SH-HF] rats)\(^{13}\) and patients with heart failure\(^{12,16,51,52}\). Only 2 groups have shown no alteration in peak [Ca\(^{2+}\)]\(_{i}\), in ventricular cells from failing human hearts\(^{5,53}\). These differences may be attributable, in part, to differences in temperature or stimulation frequency\(^{52,54}\). In addition, cardiac muscle from failing human hearts shows a decrease in active tension at stimulation rates >50 bpm\(^{52,54}\). Sipido et al\(^{54}\) showed that this negative force frequency curve is due to a decrease in the amplitude of [Ca\(^{2+}\)]\(_{i}\), at higher pacing rates (>0.5 Hz). This, in turn, may be due to the inhibition of the L-type Ca\(^{2+}\) current observed in failing human ventricular cells at high pacing rates, which would decrease both the trigger for SR Ca\(^{2+}\) release and the Ca\(^{2+}\) content of the SR. Similar to our data, Bing et al\(^{50}\) measured a 17% increase in [Ca\(^{2+}\)]\(_{i}\), amplitude in 16-month-old SHRs before the onset of heart failure, although this change did not achieve statistical significance. In addition, Brooksby et al\(^{50}\) noted a 34% increase in [Ca\(^{2+}\)]\(_{i}\), amplitude in 16-week-old SHRs in response to field stimulation, although no change was observed in voltage-clamp studies with depolarizations of short duration. Therefore, most models of cardiac hypertrophy with an accelerated progression to heart failure show either un-

![Figure 6](image-url)
changed or decreased [Ca\(^{2+}\)] amplitude. In SHRs, cardiac hypertrophy develops slowly and is associated with an increase in [Ca\(^{2+}\)] amplitude.

This increase in SHR [Ca\(^{2+}\)] amplitude is likely due to an increase in the amplitude of the Ca\(^{2+}\) sparks. If the probability of eliciting a Ca\(^{2+}\) spark is unchanged in SHRs, the 59% increase in Ca\(^{2+}\) spark amplitude could easily account for most of the 68% increase in the [Ca\(^{2+}\)] amplitude. These results are in contrast to those of Gómez et al., who noted no increase in Ca\(^{2+}\) spark amplitude in either Dahl salt-sensitive rats with hypertrophy or in SH-HF rats. In both of these animal models, the progression of cardiac hypertrophy is rapid, and [Ca\(^{2+}\)] amplitude was decreased. The SHRs we studied had mild cardiac hypertrophy with no clinical echocardiographic or histological signs of heart failure.

The increase in the amplitude of the Ca\(^{2+}\) sparks reflects a redistribution of Ca\(^{2+}\) sparks to a population in which the amplitude is larger. Initially, Ca\(^{2+}\) sparks were thought to reflect the “elemental” release of Ca\(^{2+}\) from the SR with a single distribution of amplitudes. The appearance of 2 populations of Ca\(^{2+}\) sparks in this study challenges this concept and is not due to out-of-focus events, as revealed by the absence of a correlation between Ca\(^{2+}\) spark amplitude and kinetics. Multiple populations of Ca\(^{2+}\) sparks have also been identified in rat right-ventricular trabeculae and guinea pig myocytes.

The shift in the Ca\(^{2+}\) sparks to a population of larger amplitude coupled with no change in \(I_{\text{Ca,L}}\) or the density of ryanodine receptors indicates a fundamental alteration in the Ca\(^{2+}\) sparks to a population of larger amplitude coupled with no change in \(I_{\text{Ca,L}}\) or the density of ryanodine receptors indicates a fundamental alteration in the coupling between the triggering and release of Ca\(^{2+}\) from the SR in hypertrophic cells. Gómez et al. reached a similar conclusion, although based on very different results. In their experiments, there was a decrease in the [Ca\(^{2+}\)] in Dahl salt-sensitive and SH-HF rats that was not accompanied by changes in the \(I_{\text{Ca,L}}\) or the Ca\(^{2+}\) sparks.

Taken together, these studies indicate that the contractile abnormalities seen in cardiac hypertrophy and its progression to heart failure might be explained, in large part, by alterations in the coupling between Ca\(^{2+}\) entry through the L-type Ca\(^{2+}\) channel and Ca\(^{2+}\) release from the SR. However, the big Ca\(^{2+}\) sparks seen in SHRs with cardiac hypertrophy cannot be understood in terms of an increase in “gain” per se. The relation between the Ca\(^{2+}\) influx and SR Ca\(^{2+}\) release has been defined previously at the macroscopic or whole-cell level as gain and quantified as the ratio of the peak influx of Ca\(^{2+}\) (determined from \(I_{\text{Ca,L}}\)) and the peak release of Ca\(^{2+}\) from the SR (calculated from whole-cell [Ca\(^{2+}\)], transients). Within the context of the individual or microscopic events of SR Ca\(^{2+}\) release (ie, Ca\(^{2+}\) sparks), gain has been further defined as the ratio of the peak L-type Ca\(^{2+}\) current and the peak number of evoked Ca\(^{2+}\) sparks. Defined in these terms, an increase in gain would be reflected macroscopically as an increase in the peak number of evoked Ca\(^{2+}\) sparks of similar amplitude and kinetics and macroscopically as an increase in the amplitude of the whole-cell [Ca\(^{2+}\)], transient. In other words, an increase in microscopic gain defined in this way could not account for the big Ca\(^{2+}\) sparks observed in our study. Alternatively, big sparks could be understood in terms of hypertrophy-related alterations in the following ways:

1. In the number of ryanodine receptors within a cluster that are recruited by Ca\(^{2+}\) entry through individual L-type Ca\(^{2+}\) channels, (2) in the size of the cluster of ryanodine receptors associated with an L-type Ca\(^{2+}\) channel, (3) in the number of adjacent clusters that are recruited by a given amount of L-type Ca\(^{2+}\) current, and (4) in the single-channel properties of the ryanodine receptor (possibly as a consequence of hypertrophyped-related changes in phosphorylation, for example). Although our study was not designed to discriminate between these possibilities, our results clearly show that SHRs with cardiac hypertrophy have enhanced contractility, increased [Ca\(^{2+}\)], transients, and big Ca\(^{2+}\) sparks that likely result from hypertrophy-mediated changes in the coupling of Ca\(^{2+}\) entry and SR Ca\(^{2+}\) release.

Finally, we observed a marked delay in relaxation in SHRs with cardiac hypertrophy. Similar results have been described in 12-month-old SHRs, guinea pigs with renal hypertension, and Dahl salt-sensitive rats with hypertrophy. This slowed relaxation is not due to a decrease in removal of Ca\(^{2+}\) from the cytoplasm by the SR Ca\(^{2+}\)-ATPase, since [Ca\(^{2+}\)] from SHRs and controls had virtually identical time courses (Figure 3) and there was no demonstrable increase in the expression of Ca\(^{2+}\)-ATPase in SHRs (Figure 5). The dissociation between the decline of [Ca\(^{2+}\)], and cell shortening indicates a change either in the Ca\(^{2+}\) affinity of the contractile proteins or in the characteristics of the protein(s) responsible for the restoration of resting cell shape. Several studies have shown no change in the Ca\(^{2+}\) sensitivity of the contractile proteins with the development of hypertrophy and heart failure. Therefore, it appears that the slowed relaxation (diastolic dysfunction) observed with mild cardiac hypertrophy may be better explained by an alteration in structure and/or function of intracellular structural proteins that restore the resting shape of rat ventricular cells after contraction.

Overall, our results show that cardiac hypertrophy is associated with an increase in the amount of Ca\(^{2+}\) released from the SR. Since prolonged increases in [Ca\(^{2+}\)], stimulate calcineurin, it is interesting to speculate that hypertrophy-mediated increases in cytosolic Ca\(^{2+}\) may underlie the recently reported calcineurin-dependent transcriptional pathway for cardiac hypertrophy. This speculation is supported by the observations that (1) calcineurin inhibitors, cyclosporin and FK506, prevented cardiac hypertrophy in transgenic mice predisposed to develop cardiac hypertrophy, and (2) cyclosporin prevented the increase in heart/body weight ratios in an abdominal-banded murine model of pressure-overload hypertrophy.

**Experimental Model of Hypertensive Heart Disease**

In our experiments, we used the SHR as the model for left-ventricular hypertrophy, because it may better mimic the clinical course of untreated or poorly controlled essential hypertension in humans. These animals have a stable and homogeneous expression of systemic hypertension with distinct and easily identifiable phases of cardiac involvement.

Initially, the animals develop hypertension without cardiac hypertrophy (<9 weeks of age). Over time (6 to 12 months of age), the animals gradually develop concentric cardiac hyper-
trophy, which eventually progresses to cardiac dilatation, heart failure, and sudden death (18 to 24 months of age). In addition, these animals have alterations in hemodynamics, renal function, peripheral resistance, and sympathetic tone that resemble changes seen in some patients with clinical hypertension.

This model does however, have limitations. The genetic variables that promote hypertension in the SHR model are probably polygenic. Whether the genetic variables are identical or overlap with those in hypertensive patients is unknown. In addition, the action potential in rats is typically shorter in duration that in humans. These differences can be attributed to changes in the outward K+ currents rather than to changes in the inward Ca2+ currents.64 It is also likely that data from SHR may not be applicable to models in which pressure overload is created by other means. Nevertheless, the SHR model offers an opportunity to study the cellular changes responsible for the contractile abnormalities that occur during development and progression of hypertensive heart disease.

Limitations

In this study, [Ca2+]i was determined from Fluo 3 fluorescence using the self-ratio method.17 Therefore, the absolute values of [Ca2+]i, depend heavily on the estimate of resting [Ca2+]i. In these experiments, [Ca2+]i was calculated using the resting [Ca2+]i, determined from separate experiments using the Ca2+ indicator, Indo 1, as we have done previously.28,29 Although it is possible that resting [Ca2+]i, was different in the Indo 1 experiments, the change, if any, would be expected to be similar in SHRs and controls, and thus would not alter the conclusions. In fact, resting [Ca2+]i, would have to increase by at least 60 nmol/L in SHRs to account for the overall increase in the amplitude of Ca2+ sparks that we observed. In addition, the similarities in the amplitudes of the different distributions of Ca2+ sparks in control (Figure 4A) and SHR cells (Figure 4B) also suggests that there is not a substantial difference in resting [Ca2+]i, between control and SHR cells.

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