The Sarcoplasmic Reticulum and the Na\textsuperscript{+}/Ca\textsuperscript{2+} Exchanger Both Contribute to the Ca\textsuperscript{2+} Transient of Failing Human Ventricular Myocytes

Konstantina Dipla, Julian A. Mattiello, Kenneth B. Margulies, Valluvan Jeevanandam, Steven R. Houser

Abstract—Our objective was to determine the respective roles of the sarcoplasmic reticulum (SR) and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in the small, slowly decaying Ca\textsuperscript{2+} transients of failing human ventricular myocytes. Left ventricular myocytes were isolated from explanted hearts of patients with severe heart failure (n=18). Cytosolic Ca\textsuperscript{2+}, contraction, and action potentials were measured by using indo-1, edge detection, and patch pipettes, respectively. Selective inhibitors of SR Ca\textsuperscript{2+} transport (thapsigargin) and reverse-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity (No. 7943, Kanebo Ltd) were used to define the respective contribution of these processes to the Ca\textsuperscript{2+} transient. Ca\textsuperscript{2+} transients and contractions induced by action potentials (AP transients) at 0.5 Hz exhibited phasic and tonic components. The duration of the tonic component was determined by the action potential duration. Ca\textsuperscript{2+} transients induced by caffeine (Caf transients) exhibited only a phasic component with a rapid rate of decay that was dependent on extracellular Na\textsuperscript{+}. The SR Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin abolished the phasic component of the AP Ca\textsuperscript{2+} transient and of the Caf transient but had no significant effect on the tonic component of the AP transient. The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange inhibitor No. 7943 eliminated the tonic component of the AP transient and reduced the magnitude of the phasic component. In failing human myocytes, Ca\textsuperscript{2+} transients and contractions exhibit an SR-related, phasic component and a slow, reverse-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange–related tonic component. These findings suggest that Ca\textsuperscript{2+} influx via reverse-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange during the action potential may contribute to the slow decay of the Ca\textsuperscript{2+} transient in failing human myocytes.

Key Words: calcium transient ■ isolated myocyte ■ heart failure ■ Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger ■ sarcoplasmic reticulum

Numerous processes contribute to the rise and fall of the intracellular free Ca\textsuperscript{2+} that induces contraction and relaxation in cardiac myocytes. Ca\textsuperscript{2+} influx through sarcolemmal, voltage-dependent Ca\textsuperscript{2+} channels and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (reverse mode), together with Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR), is thought to be primarily responsible for the rapid increase in cytosolic free Ca\textsuperscript{2+} (upstroke of the whole-cell Ca\textsuperscript{2+} transient). Ca\textsuperscript{2+} efflux via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (forward mode) and the sarcolemmal Ca\textsuperscript{2+}-ATPase, together with Ca\textsuperscript{2+} uptake by the SR Ca\textsuperscript{2+}-ATPase and the mitochondrial Ca\textsuperscript{2+} uniporter, is responsible for the time course of the decay of the Ca\textsuperscript{2+} transient. The respective contribution of these Ca\textsuperscript{2+} transport processes to the Ca\textsuperscript{2+} transient is species dependent. In small mammals with fast heart rates and short-duration action potentials (ie, mice and rats), the size and shape of the Ca\textsuperscript{2+} transient are almost entirely determined by SR release and uptake. In larger mammals with slower heart rates and long action potential durations, transsarcolemmal ionic fluxes, particularly through the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, appear to play a significantly greater role.

In human heart failure Ca\textsuperscript{2+} homeostasis is disturbed, as indicated by the smaller and more slowly decaying Ca\textsuperscript{2+} transients recorded from failing versus nonfailing myocytes. These Ca\textsuperscript{2+} transient derangements are thought to be largely responsible for the depressed contractility of the failing heart. The role of specific Ca\textsuperscript{2+} transport processes to the deranged Ca\textsuperscript{2+} transients of failing human myocytes has not been well established. There do not appear to be significant changes in the L-type Ca\textsuperscript{2+} current density in human heart failure. However, several investigations imply that the slow decay of the Ca\textsuperscript{2+} transients is related to reduced SR Ca\textsuperscript{2+}-ATPase levels (protein and mRNA). Unfortunately, other studies have been unable to demonstrate any changes in the abundance of SR Ca\textsuperscript{2+}-ATPase proteins in failing human hearts. Therefore, the role of altered SR...
function in contractile disturbances of the failing heart is still not resolved.

Studies of the Na\(^+\)/Ca\(^{2+}\) exchanger in human heart failure have produced more consistent results. These studies have primarily shown that the abundance of exchanger mRNA and protein is increased in heart failure.\(^{20,21}\) However, because the Na\(^+\)/Ca\(^{2+}\) exchanger is the principal Ca\(^{2+}\) efflux mechanism in mammalian myocytes, it has been suggested that increased activity of the exchanger in the failing heart could help compensate for the associated decrease in SR Ca\(^{2+}\) uptake.\(^{20}\) However, because the Na\(^+\)/Ca\(^{2+}\) exchange can also transport Ca\(^{2+}\) into the cell, increased exchanger activity could produce increased Ca\(^{2+}\) influx during the action potential. This notion has not been examined in detail to date.

Recently, we developed an improved technique to isolate high-quality myocytes from failing human hearts. These myocytes were used in the present experiments to study the Ca\(^{2+}\) transport processes responsible for the small magnitude and the slow decay of Ca\(^{2+}\) transients in failing human left ventricular myocytes. We specifically explored the roles of the SR Ca\(^{2+}\) -ATPase and the Na\(^+\)/Ca\(^{2+}\) exchanger in the decay of the Ca\(^{2+}\) transient in these cells. Our experiments suggest that the small size of the Ca\(^{2+}\) transient results from reduced SR Ca\(^{2+}\) stores and that the slow decay of the transient is in part due to Ca\(^{2+}\) influx via reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange during the action potential.

**Materials and Methods**

**Myocyte Isolation**

Human ventricular myocardium was obtained from 18 patients with severe heart failure undergoing orthotopic cardiac transplantation. Heart failure was secondary to ischemic (n=12) or nonischemic (n=6) cardiomyopathy. Our protocol was reviewed by the Temple University Institutional Review Board and was determined to be exempt in accordance with paragraph 4 pertaining to research involving pathological specimens. Patient characteristics are presented in Table 1.

To ensure myocyte preservation during surgery, the coronary arteries were perfused with cold, blood-containing cardioplegic solution in vivo at the time of aortic cross-clamping. This technique was used to minimize subsequent ischemia/reperfusion damage during myocyte isolation. Explanted hearts were then transported (within 5 minutes) from the operating suite to the laboratory in cold, Ca\(^{2+}\)-free Krebs-Henseleit (KH) solution containing (in mmol/L) glucose 12.5, KCl 5.4, lactic acid 1, NaH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, and sodium pyruvate 2 (pH 7.4, with NaOH).

On arrival in the laboratory (<5 minutes), the heart was weighed and rinsed in KH, and a small catheter was placed into the lumen of an artery that supplied a noninfarcted free-wall region of the left ventricle. The perfused myocardial segment was cut from the heart and rinsed for 30 minutes with a nonrecirculating KH solution containing 10 mmol/L taurine. This tissue was then perfused for 30 minutes with 200 mL of KH containing 180 U/mL collagenase, 20 mmol/L 2,3-butanedione monoxime (BDM), 20 mmol/L taurine, and 0.05 mmol/L CaCl\(_2\). This solution was recirculated. The collagenase-containing solution was washed from the tissue for 10 minutes with 500 mL KH containing 10 mmol/L taurine, 20 mmol/L BDM, and 0.2 mmol/L CaCl\(_2\). The cardiac tissue was then removed from the cannula, and only midmyocardial tissue was minced. The resulting cell sus-

<table>
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<th>TABLE 1. Patient Characteristics</th>
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<td><strong>Patient</strong></td>
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<td>18</td>
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<tr>
<td>Mean±SEM</td>
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CHF indicates congestive heart failure; BW, body weight; HW, heart weight; Cl, cardiac index; LVEF, LV ejection fraction; Isch, ischemic cardiomyopathy; Dob, dobutamine; ACE, angiotensin-converting enzyme inhibitor; N, nitrate; Dg, digoxin; Am, amiodarone; BB, β-adrenergic blocker; Du, diuretic; Hy, hydralazine; Valv, valvular cardiomyopathy; M, milrinone; Idio, idiopathic cardiomyopathy; AB, α-adrenergic blocker; ARB, angiotensin receptor blocker; Faml, familial cardiomyopathy; and CaB, calcium channel blocker.
pension was filtered, centrifuged (25g for 1 minute), and resuspended in a KH solution (200 mL, KH, 1% weight/volume BSA, 10 mmol/L taurine, and 0.25 mmol/L CaCl2). All solutions were equilibrated with 95% O2 and 5% CO2. The temperature was kept at 37°C throughout the isolation. Initial yields of rod-shaped cells were between 7% and 70%. All experiments were conducted within 12 hours of cell isolation.

Myocyte Contraction and Ca2+
Transient Measurements

Myocytes were placed in a chamber mounted on an inverted microscope. The chamber (0.5 mL) was superfused at 1 to 2 mL/min with Tyrode’s solution (in mmol/L): NaCl 150, KCl 1.2, glucose 10, sodium pyruvate 2, and HEPES 5, pH 7.4 at 37°C. Myocytes were chosen on the basis of their morphology (rod shape, no hypercontracted areas) and absence of spontaneous contractions in 1 mmol/L Ca2+. Myocyte contractions were measured by using an edge-detection (Crescent Electronics) technique. Data were recorded and analyzed by using Axotape software (Axon Instruments). The maximal magnitude of contraction was normalized to resting cell length and expressed as percent shortening.

Indo 1 fluorescence was recorded from single myocytes as described previously.11 The excitation was at 350 nm, and the emission light was split through a 460-nm diachronic mirror. The emitted indo 1 fluorescence at 410 nm/480 nm (Ca2+ bound/ Ca2+ free) was recorded to represent the cytosolic Ca2+ transient. Myocytes were placed in KH solution containing 4.8 mmol/L indo 1-AM for 2 minutes and then rinsed in Tyrode’s solution for 10 minutes. With this loading technique, cytosolic indo 1-AM is almost completely hydrolyzed and there is minimal cellular compartmentalization. The amount of indo 1 in cellular compartments, such as mitochondria and the SR, was determined by “quenching” the cytosolic indo 1 signal with 50 µmol/L Mn2+. Exposure to Mn2+ abolished the indo 1 transient, and the remaining fluorescence was not significantly different from cellular autofluorescence (n=5 myocytes). These data therefore show that indo 1 is located primarily in the cytoplasm. As an additional control, twitch contractions were measured in myocytes with and without indo 1 to ensure that the loading procedure did not cause any significant buffering of the Ca2+ transient and contraction. Only those myocytes without any significant buffering were used. Although our technique uses small amounts of cytosolic indo 1 to minimally perturb cellular Ca2+ buffering, the signal-to-noise characteristics are acceptable only when photon counting techniques are used.22

Myocytes were field stimulated at 0.5 Hz with electrodes on the sides of the experimental chamber until the Ca2+ transients and contractions reached a steady state. The quantity of the Ca2+ stored in the SR was determined by rapidly applying caffeine (termed a caffeine “spritz”) to the myocyte for either 100 ms or 10 seconds through a large-bore micropipette placed just above the cell. Caffeine-induced SR Ca2+ release was induced in place of normal electrical stimulation. Caffeine (10 mmol/L) was used because it induces full SR Ca2+ release, as verified by failure of a subsequent caffeine spritz to cause a Ca2+ transient.

Selective inhibitors of SR Ca2+ transport (thapsigargin, 0.1 µmol/L),23 and reverse-mode Na+/Ca2+ exchange [No. 7943; (2-2-4-(4-nitrobenzoylxyloxy)phenyl)ethyl]isothiourea methanesulfonate, 1 µmol/L]24 activity were used to define their respective contributions to the Ca2+ transient. In the thapsigargin experiments, failure of a caffeine spritz to induce SR Ca2+ release was used as an indicator of SR unloading/emptying. Previous studies showed that 3 to 10 µmol/L No. 7943 is sufficient to block Na+/Ca2+ exchange-mediated Ca2+ influx without significantly altering the other ion transporters, such as the Na+/H+ exchanger, L-type Ca2+ channels, sarcocellular and SR Ca2+-ATPases, Na/K-ATPase, and passive Na+ permeability.24 The β-adrenergic agonist isoproterenol (1 µmol/L) was used to modify the SR component of the Ca2+ transient. Preliminary experiments showed that this concentration of isoproterenol induces maximal effects on failing myocytes without signs of toxicity.

Figure 1. Indo 1 transient (A) and length changes during contraction (B, shown as a downward deflection) from a representative failing human ventricular myocyte stimulated at 0.5 Hz. The contractions and Ca2+ transient exhibited both phasic and tonic components.

Action Potential Measurements

Action potentials were recorded as described in detail in numerous previous studies from this laboratory. In brief, an aliquot of cells was placed in a heated (37°C) chamber on the stage of an inverted microscope (Zeiss Axiovert 10). Normal Tyrode’s bath solution contained (in mmol/L): glucose 10, HEPES 5, KCl 1.2, NaCl 150, and sodium pyruvate 2 (pH 7.4 with NaOH). Low-resistance (1.5 to 4 MΩ) glass patch pipettes were used to gain electrical access to the cell interior. Fire-polished patch pipettes were filled with a standard solution that contained (in mmol/L): HEPES 20, KCl 130, NaCl 10, MgCl2 5, and K2 ATP 5 (pH 7.2, with KOH). Membrane potentials were recorded with an amplifier (Axoclamp 2A, Axon Instruments) connected to a personal computer. Passing current through the recording pipette induced action potentials. Data were analyzed with pCLAMP 6.0 (Axon Instruments) software.

Materials

Taurine, BDM, isoproterenol, thapsigargin, and albumin were obtained from Sigma Chemical Co. Collagenase (type II) was from Worthington Biochemical Co. Indo 1-AM was obtained from Calbiochem. Compound No. 7943 [2-(2-4-(4-nitrobenzoylxyloxy)phenyl)ethyl]isothiourea methanesulfonate was kindly provided by the New Drug Discovery Research Drug Laboratory, Kaneko Ltd, Osaka, Japan.

Statistical Analysis

All data in the text and tables are reported as mean±SEM. Differences between treatments on the same cell were assessed by 2-tailed, paired t tests. A probability level of P<0.05 was set as significant.

Results

Field Stimulation–Induced Ca2+ Transients and Contractions

Steady-state contractions (n=21) and Ca2+ transients (n=36) induced by electrical (field) stimulation were measured in failing human ventricular myocytes. Data from a representative myocyte are shown in Figure 1, and the average data are listed in Table 2. In these experiments, Ca2+ transients and contractions exhibited both phasic and tonic components in every cell studied (0.5–Hz stimulation rate). These findings suggest that 2 processes may make significant contributions to the Ca2+ transient of failing human ventricular myocytes.

Relationship of Contraction and Action Potential Duration

In a recent report from this laboratory, we showed that when contractions are induced with depolarizing voltage-clamp
steps, the phasic portion of the contraction decayed during depolarization while the secondary tonic component was maintained until the membrane potential was repolarized. The results of this previous study suggested that the level and duration of the plateau phase of the action potential may determine the magnitude and duration of the tonic component of contraction. To examine this idea further, we measured action potentials and associated contractions in 15 failing human ventricular myocytes. These experiments showed that at a slow rate of stimulation (0.5 Hz), the phasic component of contraction occurs during the action potential plateau and that the tonic component is maintained until final repolarization occurs (Figure 2A). Fluctuations in action potential duration at a fixed, slow rate of stimulation were associated with simultaneous changes in the tonic component of the contraction while the phasic component was not markedly changed. In every myocyte studied, we found a linear relationship between contraction and action potential duration (Figure 2B).

Both the action potential duration and the phasic and tonic components of contraction were influenced by the frequency of stimulation. Increasing the stimulation frequency from 0.5 to 1.5 Hz caused the action potential duration to decrease (Figure 3, top). Additionally, this increase in stimulation frequency also caused a decrease in the magnitude of the phasic component of contraction and the shortening and eventual elimination of the tonic portion of the contraction (Figure 3, bottom). These results strongly support the idea that voltage-dependent processes determine the tonic component of contraction and influence the phasic component of contraction.

Caffeine-Induced Ca$^{2+}$ Transients

The magnitude of the phasic component of the Ca$^{2+}$ transients in failing human myocytes is smaller than what we have observed in normal myocytes from other species studied under identical experimental conditions. The idea that peak systolic Ca$^{2+}$ is smaller than normal in failing human ventricular myocytes is also well supported by a number of studies from other laboratories. The reduced peak systolic Ca$^{2+}$ of failing human myocytes could result from defects in Ca$^{2+}$ influx, excitation-contraction coupling, and/or SR Ca$^{2+}$ loading. We studied the idea that the amount of Ca$^{2+}$ in the SR is abnormal by inducing full SR Ca$^{2+}$ release with a caffeine spritz that was substituted for an action potential.

### Table 2. Contraction and Ca$^{2+}$ Transient Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Electrical Stimulation Induced (0.5 Hz)</th>
<th>Caffeine Induced</th>
<th>(P)</th>
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<tbody>
<tr>
<td>Contractions</td>
<td>((n=21))</td>
<td>((n=21))</td>
<td></td>
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<tr>
<td>Shortening, % RCL</td>
<td>5.21 ± 0.61</td>
<td>6.65 ± 0.73</td>
<td>0.15</td>
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<tr>
<td>Time to peak, s</td>
<td>0.52 ± 0.04</td>
<td>0.63 ± 0.03*</td>
<td>0.01</td>
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<tr>
<td>Time to 50% relaxation, s</td>
<td>0.72 ± 0.30</td>
<td>0.41 ± 0.08</td>
<td>0.31</td>
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<tr>
<td>Time to 95% relaxation, s</td>
<td>2.53 ± 0.36</td>
<td>1.65 ± 0.17*</td>
<td>0.03</td>
</tr>
<tr>
<td>Myocyte length, (\mu m)</td>
<td>172.4 ± 22</td>
<td>172.4 ± 22</td>
<td></td>
</tr>
<tr>
<td>Indo 1 Ca$^{2+}$ transients</td>
<td>((n=28))</td>
<td>((n=28))</td>
<td></td>
</tr>
<tr>
<td>Diastolic ratio, arbitrary units</td>
<td>0.94</td>
<td>0.94</td>
<td></td>
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<tr>
<td>Peak ratio, arbitrary units</td>
<td>0.52 ± 0.04</td>
<td>0.53 ± 0.06</td>
<td>0.87</td>
</tr>
<tr>
<td>Time to peak, s</td>
<td>0.24 ± 0.02</td>
<td>0.57 ± 0.04†</td>
<td>0.000</td>
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<tr>
<td>Time to 50% decay, s</td>
<td>0.59 ± 0.06</td>
<td>0.58 ± 0.05</td>
<td>0.83</td>
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<tr>
<td>Time to 95% decay, s</td>
<td>2.17 ± 0.14</td>
<td>1.76 ± 0.11*</td>
<td>0.005</td>
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</tbody>
</table>

*\(P<0.05\).
†\(P<0.001\).
RCL indicates resting cell length.

![Figure 2A](http://circres.ahajournals.org/Downloaded from http://circres.ahajournals.org/figure.png)

**Figure 2.** A. Three consecutive action potentials at 0.5 Hz and their associated contractions are shown. At slow rates of stimulation, beat-to-beat action potential variability is a common feature. The duration of the tonic phase of contraction changes with the action potential duration. B. Relationship between action potential duration at 90% repolarization and contraction duration at 90% recovery in 2 cells (open and closed symbols) at 0.5 (squares), 1.0 (circles), and 1.5 (triangles) Hz.

![Figure 3](http://circres.ahajournals.org/Downloaded from http://circres.ahajournals.org/figure.png)

**Figure 3.** Action potentials (top) and associated contractions (bottom) at 0.5, 1.0, and 1.5 Hz are shown. The action potential duration shortened as the stimulation frequency was increased. The phasic component of contraction decreased as the stimulation frequency was increased. The tonic component of contraction was shorter and smaller at faster stimulation rates. This behavior was observed in all other myocytes studied.
Previous studies from this and other laboratories have shown that caffeine-induced \(Ca^{2+}\) transients (Caf transients) usually have a higher peak \(Ca^{2+}\) and a slower rate of rise and decay than do the action potential-induced \(Ca^{2+}\) transients (AP transients). The reason that Caf \(Ca^{2+}\) transients are larger than AP transients appears to be that caffeine induces full SR \(Ca^{2+}\) release, whereas normal excitation-contraction coupling causes only a fraction of the SR \(Ca^{2+}\) stores to be released. The reason that the decay rate of Caf \(Ca^{2+}\) transients is slower than the decay rate of AP transients is thought to be that Na\(^+/Ca^{2+}\) exchange, which transports cytosolic \(Ca^{2+}\) at a slower rate than does the SR, is primarily responsible for the decay of the Caf transient, whereas the SR is primarily responsible for the decay of the AP transient.

We measured the differences in Caf and AP \(Ca^{2+}\) transients to determine the amount of \(Ca^{2+}\) stored in the SR, assess the fraction of this store that is released during excitation-contraction coupling, and determine the relative abilities of the SR and the Na\(^+/Ca^{2+}\) exchanger to lower cytosolic free \(Ca^{2+}\). Caf and AP \(Ca^{2+}\) transients were measured in 36 failing human myocytes. The average results are reported in Table 2, and a representative example is shown in Figure 4. The most apparent differences between these transients were that the Caf transients had no tonic portion and were shorter in duration than AP transients, as evidenced by a significantly shorter (\(P<0.05\)) time to 95% decay in the Caf (1.76 seconds) versus the AP (2.17 seconds) transients. AP and Caf \(Ca^{2+}\) transients had similar peaks (see Table 2), but the rate of rise of Caf transients was slower than those induced by APs (see Table 2). All other features of AP and Caf transients were similar. The similar size of AP and Caf \(Ca^{2+}\) transients suggests that in failing human ventricular myocytes, the action potential induces a large fractional SR \(Ca^{2+}\) release. The fact that Caf transients, which occur in the absence of an action potential, do not have a tonic component suggests that \(Ca^{2+}\) influx during the action potential plateau is responsible for inducing this component of action potential–induced contractions. The similar early decay rates of Caf and AP \(Ca^{2+}\) transients also suggest that under the conditions of our experiments, forward-mode Na\(^+/Ca^{2+}\) exchange and SR \(Ca^{2+}\) uptake can reduce cytosolic \(Ca^{2+}\) at similar rates.

Previous studies have shown that the peak of the Caf \(Ca^{2+}\) transients can be blunted by rapid \(Ca^{2+}\) extrusion by forward-mode Na\(^+/Ca^{2+}\) exchange activity. This can lead to significant underestimation of the SR \(Ca^{2+}\) load and fractional SR \(Ca^{2+}\) release. This effect could be especially relevant in the present study, because Na\(^+/Ca^{2+}\) exchange activity is thought to be increased in failing human ventricular myocytes. The possibility that \(Ca^{2+}\) efflux via forward-mode Na\(^+/Ca^{2+}\) exchange activity blunts the peak \(Ca^{2+}\) produced when SR release is induced by caffeine was tested by rapidly exposing myocytes to caffeine in an Na\(^+\)- and \(Ca^{2+}\)-free solution. This technique induces SR \(Ca^{2+}\) release and virtually eliminates \(Ca^{2+}\) transport by the sarcolemmal Na\(^+/Ca^{2+}\) exchanger. Under these conditions (\(n=13\)), the peak of the Caf \(Ca^{2+}\) transient was 29% greater than the AP transient (Figure 5A).

**Figure 4.** AP \(Ca^{2+}\) transients and a caffeine spritz (100 ms), applied in place of the normal stimulus, are shown. Note that the peak indo 1 fluorescence is similar in the 2 conditions but that the Caf transient has no distinct tonic component and is shorter in duration than the AP transients. Also note that in this myocyte the Caf \(Ca^{2+}\) transient had a faster rate of decay than did the AP transients. Similar results were observed in 27 other myocytes.

**Figure 5.** A, \(Ca^{2+}\) transient induced by an action potential was followed by a Caf \(Ca^{2+}\) transient induced by rapid application of a caffeine-containing, Na\(^+\)- and \(Ca^{2+}\)-free solution for 100 ms. The peak indo 1 ratio was greater when a caffeine-induced \(Ca^{2+}\) release was performed with an Na\(^+\)- and \(Ca^{2+}\)-free solution. Similar results were observed in 12 other myocytes. B, Conditions were the same as in A, except that the caffeine (Na\(^+\)- and \(Ca^{2+}\)-free solution) was applied for 10 seconds. The decay of the indo 1 transient was very slow under these conditions.
The decay of Caf Ca\(^{2+}\) transients is faster than that of the AP Ca\(^{2+}\) transient and is thought to be produced by forward-mode Na\(^+\)/Ca\(^{2+}\) exchange activity.\(^4\) To further document the idea that the decay of the Caf Ca\(^{2+}\) transient results from forward-mode exchange activity, myocytes were exposed to caffeine in an Na\(^+\)- and Ca\(^{2+}\)-free solution for 10 seconds (Figure 5B). Under these conditions, the decay of the Ca\(^{2+}\) transient was very slow. These results strongly support the idea that the decay of the Caf Ca\(^{2+}\) transient results from Ca\(^{2+}\) efflux via the Na\(^+\)/Ca\(^{2+}\) exchanger.

A caffeine spritz causes depletion of SR Ca\(^{2+}\) stores, as evidenced by the fact that a second spritz causes no additional Ca\(^{2+}\) release. Therefore, the first action potential–induced contraction after exposure to caffeine should not have a component mediated by SR Ca\(^{2+}\) release. We induced action potentials after a caffeine spritz in 36 myocytes and found that only the tonic component of the Ca\(^{2+}\) transient was present (Figure 6). With subsequent stimuli, the phasic component of the transient increased and reached a steady state within 20 beats. The tonic component of the Ca\(^{2+}\) transient decreased slightly in beat number and reached a steady state at the same time (Figure 6). These experiments provide additional evidence that the phasic and tonic components of the indo 1 transient of failing human ventricular myocytes result from different processes, ie, Sr Ca\(^{2+}\) release and Ca\(^{2+}\) influx, respectively.

**Inhibiting the SR Ca\(^{2+}\)-ATPase**

Thapsigargin, an inhibitor of SR Ca\(^{2+}\)-ATPase, eliminated the phasic component of the Ca\(^{2+}\) transient but had no significant effect on the tonic component (n=9 myocytes). Representative results are presented in Figure 7, and average data are listed in Table 3. Failure of a caffeine spritz to elicit a Ca\(^{2+}\) transient after exposure to thapsigargin verified Sr Ca\(^{2+}\) depletion. The peak systolic indo 1 ratio of the AP transient was significantly (P<0.05) reduced after thapsigargin treatment. Thapsigargin also delayed the time-to-peak transient, time to 50%, and time to 95% decay; however, these data were not statistically significant. These findings provide further strong support for the idea that the phasic component of the indo 1 transient and contraction result from Sr Ca\(^{2+}\) release and reuptake.

**Inhibiting Reverse-Mode Na\(^+\)/Ca\(^{2+}\) Exchange**

One possible source of Ca\(^{2+}\) for the tonic component of contraction is Ca\(^{2+}\) influx via reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange. We tested this idea by measuring indo 1 transients before and after exposure to a putative reverse-mode-exchange inhibitor (No. 7943, Kanebo, Ltd). Application of No. 7943 eliminated the tonic component of the Ca\(^{2+}\) transient (n=9 myocytes). Representative raw data are presented in Figure 8, and average data are listed in Table 4. No. 7943 also significantly (P<0.05) reduced the peak of the Ca\(^{2+}\) transient (control, 0.47 versus No. 7943, 0.43) and abbreviated (P<0.05) the time to 95% decay (control, 2.15 seconds

### Table 3. Ca\(^{2+}\) Transient Characteristics Before and After Thapsigargin Treatment

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<td>Diastolic ratio, arbitrary units</td>
<td>0.96±0.06</td>
<td>0.89±0.04</td>
<td>0.4</td>
</tr>
<tr>
<td>Peak ratio, arbitrary units</td>
<td>0.34±0.04*</td>
<td>0.24±0.04*</td>
<td>0.04</td>
</tr>
<tr>
<td>Time to peak, s</td>
<td>0.26±0.08</td>
<td>0.32±0.07</td>
<td>0.4</td>
</tr>
<tr>
<td>Time to 50% decay, s</td>
<td>0.63±0.13</td>
<td>0.80±0.13</td>
<td>0.4</td>
</tr>
<tr>
<td>Time to 95% decay, s</td>
<td>1.48±0.08</td>
<td>1.79±0.33</td>
<td>0.35</td>
</tr>
</tbody>
</table>

\(^*P<0.05.\)

**Figure 6.** Ca\(^{2+}\) transients induced by action potentials after depletion of Sr Ca\(^{2+}\) stores with caffeine are shown. The tonic component of the Ca\(^{2+}\) transient was always present in the first stimulus. The phasic component was not seen in the first beat but rose to a steady state during the next 20 beats.

**Figure 7.** Ca\(^{2+}\) transients before (solid line) and after (dashed line) thapsigargin treatment are superimposed. Thapsigargin eliminated the phasic component of the Ca\(^{2+}\) transient.

**Figure 8.** Ca\(^{2+}\) transients before and after No. 7943 treatment are superimposed. No. 7943 eliminated the tonic component of the Ca\(^{2+}\) transient.
versus No. 7943, 1.36 seconds). These results suggest that Ca\(^{2+}\) enters the cell during the action potential through reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange, loads the SR, and directly elevates cytosolic Ca\(^{2+}\). Ca\(^{2+}\) influx via reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange activity during the action potential would antagonize SR Ca\(^{2+}\) uptake and contribute to the slow decay of the systolic Ca\(^{2+}\) transients of failing human ventricular myocytes.

**Effect of β-Adrenergic Stimulation on the Ca\(^{2+}\) Transients**

β-Adrenergic stimulation is known to increase SR Ca\(^{2+}\) uptake and increase SR Ca\(^{2+}\) loading in cardiac myocytes.\(^{30}\) To further explore the basis of the 2 components of the Ca\(^{2+}\) transient, failing human ventricular myocytes were exposed to isoproterenol, a nonspecific β-adrenergic agonist. Isoproterenol decreased the diastolic indo 1 ratio, increased (P<0.05) the peak of the phasic component of the Ca\(^{2+}\) transient, but had no significant effects on the tonic component of the Ca\(^{2+}\) transient (n=8 myocytes; see Figure 9 and Table 5). These experiments provide additional support that the phasic component of contraction in failing human myocyte results from SR Ca\(^{2+}\) release.

**Discussion**

The objective of this study was to explore the cellular and molecular bases of the reduced magnitude and slow decay of the systolic Ca\(^{2+}\) transient of ventricular myocytes from patients with severe congestive heart failure. In a recent study of these myocytes with voltage-clamp techniques, we showed that contractions can result from both SR Ca\(^{2+}\) release and, when the duration of the depolarizing voltage step was sufficiently long, from direct elevation of cytosolic Ca\(^{2+}\), apparently via reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange.\(^{25}\) In the present experiments, we primarily studied Ca\(^{2+}\) transients induced by extracellular field stimulation so that the native intracellular milieu would be undisturbed (no cytoplasmic dialysis via a micropipette). Experiments were performed in this fashion because the intracellular environment might be at least partly responsible for the behavior exhibited by failing human myocytes.

The most important observations of the present research were the following: (1) Action potentials elicited at low rates of stimulation induced contractions and Ca\(^{2+}\) transients with 2 distinct components (phasic and tonic). The peak systolic indo 1 ratio of the phasic component was smaller than those that we have observed in normal and hypertrophied/failing feline ventricular myocytes, but the tonic component was larger.\(^{26}\) (2) The phasic component of contraction and the Ca\(^{2+}\) transient was eliminated by thapsigargin and depletion of SR Ca\(^{2+}\) stores with caffeine but was increased by β-adrenergic stimulation. These findings strongly support the idea that the phasic component of the Ca\(^{2+}\) transient results from SR Ca\(^{2+}\) release and subsequent reuptake. (3) The tonic component of the Ca\(^{2+}\) transient was most apparent at slow rates of stimulation when the action potential duration was longest and was insensitive to thapsigargin or depletion of SR Ca\(^{2+}\) stores with caffeine but was blocked by the putative Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor No. 7943, suggesting that it is a direct result of Ca\(^{2+}\) influx via reverse-mode Na\(^+\)/Ca\(^{2+}\) exchange.

**Phasic and Tonic Components of Contraction and the Indo 1 Transient**

Contractions with both phasic and tonic components have been reported in many previous studies of cardiac myocytes.\(^{25,31}\) They are most often observed together when the duration of either the action potential or the voltage-clamp step is longer than the duration of the SR-mediated contraction. These conditions were present in our experiments because failing human ventricular myocytes have long-duration action potentials.\(^{28}\) In addition, we accentuated this feature by performing our experiments at low rates of stimulation.\(^{32}\) In large mammals, including humans,\(^{28}\) the action potential duration decreases as the beat frequency is increased. Therefore, as stimulation rate was increased, the tonic component of contraction became less obvious (Figure 3).

**TABLE 4. Ca\(^{2+}\) Transient Characteristics Before and After No. 7943 Treatment**

<table>
<thead>
<tr>
<th>Ca(^{2+}) Transient Characteristics</th>
<th>Control</th>
<th>No. 7943</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic ratio, arbitrary units</td>
<td>0.89±0.7</td>
<td>0.88±0.54</td>
<td>0.69</td>
</tr>
<tr>
<td>Peak ratio, arbitrary units</td>
<td>0.47±0.04</td>
<td>0.43±0.04*</td>
<td>0.04</td>
</tr>
<tr>
<td>Time to peak, s</td>
<td>0.19±0.02</td>
<td>0.20±0.02</td>
<td>0.76</td>
</tr>
<tr>
<td>Time to 50% decay, s</td>
<td>0.92±0.24</td>
<td>0.44±0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>Time to 95% decay, s</td>
<td>2.15±0.35</td>
<td>1.36±0.16*</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*P<0.05.

**TABLE 5. Ca\(^{2+}\) Transient Characteristics Before and After Isoproterenol Treatment**

<table>
<thead>
<tr>
<th>Ca(^{2+}) Transient Characteristics</th>
<th>Control</th>
<th>Isoproterenol</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diastolic ratio, arbitrary units</td>
<td>0.94±0.05</td>
<td>0.82±0.04*</td>
<td>0.04</td>
</tr>
<tr>
<td>Peak ratio, arbitrary units</td>
<td>0.34±0.04</td>
<td>0.64±0.04*</td>
<td>0.00</td>
</tr>
<tr>
<td>Time to peak, s</td>
<td>0.21±0.02</td>
<td>0.14±0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Time to 50% decay, s</td>
<td>0.63±0.20</td>
<td>0.34±0.06</td>
<td>0.34</td>
</tr>
<tr>
<td>Time to 95% decay, s</td>
<td>1.70±0.18</td>
<td>1.85±0.21</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*P<0.05.
Ca\(^{2+}\) transients with 2 distinct components (termed L1 and L2) have been observed in failing human myocardium in previous studies.\(^{33}\) However, the kinetics and pharmacological sensitivity of these transients are significantly different from those recorded with indo 1 in the present experiments. The reasons for these differences are not clear. However, the Ca\(^{2+}\) transients measured in voltage-clamped, failing human myocytes with fluorescent Ca\(^{2+}\) indicators are similar to those we report here.

It is also worth noting that the tonic component of the transient observed in our experiments was not due to secondary SR Ca\(^{2+}\) release resulting from cellular Ca\(^{2+}\) overload. If SR Ca\(^{2+}\) overload were responsible for the tonic component of the Ca\(^{2+}\) transient, then thapsigargin and caffeine spritzes would have abolished it, which they did not.

**Phasic Components**

The weakened contractility of failing human myocytes is thought to result in large part from reduced release of Ca\(^{2+}\) from the SR.\(^{34}\) The small phasic components of the indo 1 transients observed in the present experiments are consistent with this hypothesis. The mechanisms that produce these small, SR-mediated Ca\(^{2+}\) transients need additional study under more controlled experimental conditions. Possible explanations include reduced SR Ca\(^{2+}\) loading resulting from slowed Ca\(^{2+}\) uptake.\(^{34}\) This could be a consequence of reduced expression of SR Ca\(^{2+}\) pumps, as shown in some\(^{15,16}\) but not all\(^{17}\) previous studies of failing human hearts. Another possibility is abnormal triggering of SR Ca\(^{2+}\) release from a normally Ca\(^{2+}\)-loaded SR, as suggested by a recent study of failing rat ventricular myocytes.\(^{35}\) We began to explore these issues by inducing full SR Ca\(^{2+}\) release via rapid exposure to caffeine. We found that the caffeine-induced SR Ca\(^{2+}\) release was only slightly greater than the action potential-mediated release, even when caffeine-induced release was measured in the absence of Na\(^{+}\)/Ca\(^{2+}\) exchanger activity (Na\(^{+}\)- and Ca\(^{2+}\)-free extracellular solutions). These observations suggest that abnormal SR Ca\(^{2+}\) loading may be more important than abnormal excitation-contraction coupling in producing reduced peak systolic Ca\(^{2+}\) in failing human ventricular myocytes, at least under our experimental conditions. Similar results have been recently published by another group.\(^{27}\)

It is noteworthy that we were able to show that the phasic component of the Ca\(^{2+}\) transient (which is the major component) in our failing human myocytes was blocked by the SR Ca\(^{2+}\)-ATPase inhibitor thapsigargin. A previous report on failing human myocytes could not demonstrate significant effects of thapsigargin on contraction.\(^{36}\) The reasons for this discrepancy are not clear at present but could be the result of the differences in cell isolation techniques used in these 2 studies. It is worth pointing out that the magnitude of contraction we report here and in another recent study of failing human ventricular myocytes\(^{37}\) is significantly greater than previously reported in either normal or failing human myocytes. We believe that the improved performance of the myocytes we are using is the result of the in vivo cardioprotective techniques we used (see Methods and Reference \(^{37}\)). Our results suggest that many of the myocytes used in previous studies may have been significantly damaged by long periods of warm ischemia and subsequent reperfusion injury during the isolation process.

**Tonic Contractions**

Our experiments strongly support the idea that the tonic component of the Ca\(^{2+}\) transient and contraction in failing human ventricular myocytes results from Ca\(^{2+}\) influx during the action potential and that this influx occurs via reverse-mode Na\(^{+}\)/Ca\(^{2+}\) exchange activity. This conclusion is in large part based on the fact that a putative reverse-mode exchange inhibitor\(^{24}\) abolishes this tonic component (Figure 8). However, it is also supported by the fact that the inhibition of SR function with thapsigargin (Figure 7) and depletion of SR Ca\(^{2+}\) stores with caffeine (Figure 6) do not eliminate the tonic phase of contraction. The idea that the Na\(^{+}\)/Ca\(^{2+}\) exchanger might make a larger-than-normal contribution to contraction in failing human ventricular myocytes has been proposed in previous studies in which increased expression of exchanger mRNA and protein was observed.\(^{28}\) In those studies it was hypothesized that the exchanger might make an increased contribution to relaxation in failing human myocytes. Perhaps the most important aspect of the present study is that our results suggest that the exchanger will contribute to relaxation only when decay of the SR-mediated component of the Ca\(^{2+}\) transient coincides with repolarization of the action potential to induce Ca\(^{2+}\) efflux via forward-mode exchange activity. Only under these conditions will the SR and the exchanger work in concert to lower cytosolic free Ca\(^{2+}\). Importantly, our results also suggest that as SR uptake begins to lower the free intracellular Ca\(^{2+}\) concentration during the plateau phase of the action potential, the Na\(^{+}\)/Ca\(^{2+}\) exchanger may add Ca\(^{2+}\) to the cytoplasm via reverse-mode exchange activity to slow the fall in cytosolic free Ca\(^{2+}\). As hypothesized above, the critical issue in failing human myocytes appears to be whether the exchanger and the SR are acting in concert to lower cytosolic free Ca\(^{2+}\) or whether their respective actions are opposed. These ideas can only be adequately addressed by using more controlled experimental conditions than applied in the present study.

Our experiments in which SR Ca\(^{2+}\) release was induced with caffeine strongly support the idea that forward-mode Na\(^{+}\)/Ca\(^{2+}\) exchange activity is capable of transporting Ca\(^{2+}\) from the failing human myocyte at a rate comparable to SR Ca\(^{2+}\) uptake. This conclusion is based on the fact that the decay of the Ca\(^{2+}\) transient, which is mediated primarily by the Na\(^{+}\)/Ca\(^{2+}\) exchanger, is almost identical to the rate of decay of the phasic component of the Ca\(^{2+}\) transient that is eliminated by the SR Ca\(^{2+}\)-ATPase inhibitor thapsigargin. Our study did not measure the absolute levels of SR or exchanger function in failing myocytes. They do show that the relative balance of activities by these 2 Ca\(^{2+}\) transport systems is significantly different from what has been observed in normal myocytes in other species (wherein the Caf transient decays much more
slowly than does SR-mediated decay; see References 3 and 8).

Limitations

There are a number of important limitations to this study. The first is that the myocytes were derived from patients with different diseases and that these patients were taking different medications. Despite these facts, the results were uniformly observed. We have always been concerned about cell damage during myocyte isolation and its influence on the results of studies with single cardiac cells. However, we have developed an in vivo myocyte protection procedure that helps minimize these effects. There is also concern that myocyte behavior is heterogeneous in different regions of the heart. Because of the technically demanding nature of our experiments, we focused only on myocytes from the midmyocardial region of the left ventricular free wall. The idea that myocytes from other regions of the failing human heart have different contractile properties is always a possibility. In addition, we have had limited access to nonfailing human hearts. Therefore, we do not know whether the behavior we observed is limited to the failing human heart or is common to both normal and diseased human myocytes. However, our contention is that this issue has been thoroughly addressed in previous studies. Our key question is why do failing human cells behave the way they do?

Conclusions

The present experiments suggest that the phasic contraction of failing human ventricular myocytes primarily results from SR Ca\(^{2+}\) release and that the reduced magnitude of this contraction may result from abnormally low SR Ca\(^{2+}\) stores. We also show that when the action potential is sufficiently long, a tonic component of contraction results from reverse-mode Na\(^+/Ca\(^{2+}\)\) exchange. Our most important conclusion is that the slow decay of the Ca\(^{2+}\) transient in failing human myocytes (and thus, slow relaxation) may be related to Ca\(^{2+}\) influx via reverse-mode Na\(^+/Ca\(^{2+}\)\) exchange during at least the terminal phases of the action potential plateau. If these ideas are correct, then shortening the action potential duration in the failing human heart could augment relaxation and reduce diastolic defects.

Acknowledgments

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The Sarcoplasmic Reticulum and the \( \text{Na}^+/\text{Ca}^{2+} \) Exchanger Both Contribute to the \( \text{Ca}^{2+} \) Transient of Failing Human Ventricular Myocytes

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