Decreased Myofilament Responsiveness in Myocardial Stunning Follows Transient Calcium Overload During Ischemia and Reperfusion


The purpose of this study was to test the hypothesis that abnormal intracellular calcium handling characterizes myocardial stunning. Isolated, isovolumic, buffer-perfused ferret hearts were loaded with the bioluminescent calcium indicator aequorin for simultaneous measurement of individual calcium transients and left ventricular pressure. After 15 minutes of global ischemia and 20 minutes of reperfusion, left ventricular developed pressure was significantly reduced (75±7 versus 93±6 mm Hg, p<0.05). During ischemia, [Ca²⁺]i levels were significantly elevated compared with preischemic levels, both during systole (1.38±0.31 versus 0.88±0.2 µM, p<0.05) and end diastole (0.85±0.16 versus 0.38±0.13 µM, p<0.05). Early during reperfusion, [Ca²⁺]i was also significantly elevated during systole (1.63±0.44 versus 0.88±0.20 µM, p<0.05) and end diastole (0.75±0.15 versus 0.38±0.13 µM, p<0.05). After 20 minutes of reperfusion, myocardial stunning occurred, but [Ca²⁺]i was not significantly different from preischemic levels. Thus, myocardial stunning does not result from decreased levels of activator calcium. The force-pCa relation generated by the stunned hearts was shifted downward compared with that generated by the control hearts, consistent with a decrease in maximum calcium-activated force (Fₘₐₓ). At steady state during tetanus, the decrease in Fₘₐₓ was confirmed, but there was no significant difference in the slope of the force-pCa relation of the stunned hearts versus controls. Thus, we conclude that stunned myocardium is characterized by decreased Fₘₐₓ without desensitization of the myofilaments to [Ca²⁺]i. Elevations of [Ca²⁺]i during ischemia and reperfusion precede myocardial stunning and may relate to its pathogenesis. (Circulation Research 1992;71:1334-1340)

KEY WORDS • calcium • aequorin • ischemia • reperfusion • stunning

Myocardial stunning refers to the phenomenon of transient myocardial dysfunction occurring after brief periods of coronary ischemia and reperfusion. Although first demonstrated after short periods of total coronary occlusion in the dog, evidence of recovery of dysfunctional myocardium has been observed in a variety of clinical and experimental settings, including thrombolysis involving myocardial infarction and following cardiopulmonary bypass. Puglissi theories proposed to explain the etiology of myocardial stunning include depletion of high-energy phosphate stores, oxygen-derived free radical toxicity, abnormalities of interstitial collagen, and the no-reflow phenomenon.

Several investigators have demonstrated that abnormalities in [Ca²⁺]i modulation are associated with myocardial stunning. However, the precise derangements in [Ca²⁺]i that may occur during both ischemia and reperfusion have yet to be elucidated. Limiting investigation of calcium handling during stunning has been the inability to accurately measure intracellular calcium in the intact heart without significantly perturbing hemodynamic performance. Our laboratory has developed a method for loading the bioluminescent calcium indicator aequorin into left ventricular (LV) myocytes of the isolated buffer-perfused ferret heart. This technique allows for simultaneous measurement of isovolumic LV pressure and intracellular calcium transients on a beat-to-beat basis at baseline and during ischemia and reperfusion.

The purpose of this study was to test the hypotheses that 1) increases in diastolic and peak [Ca²⁺]i occur during coronary ischemia and reperfusion and 2) decreased myofilament responsiveness to [Ca²⁺]i characterizes myocardial stunning.

Materials and Methods

After the intraperitoneal administration of 1,000 units heparin, male ferrets 10–14 weeks of age were anesthetized with chloroform. Hearts were rapidly excised through a midline sternotomy incision and...
mounted on a perfusion cannula inserted into the ascending aorta. Retrograde aortic perfusion of the coronary arteries was maintained with a constant flow pump, and the coronary perfusion pressure, recorded by a Statham P23Db transducer, was initially set at 80 mm Hg. Coronary flow remained constant throughout the experiment except during ischemic periods, when coronary flow was reduced to zero. The hearts were perfused with a modified Krebs-Henseleit buffer composed of (mM) NaCl 118, KCl 4.7, KH2PO4 1.2, CaCl2 2.0, MgCl2 1.2, NaHCO3 23, and dextrose 5.4, saturated with a 95% O₂–5% CO₂ gas mixture to achieve a pH of 7.40 ± 0.02 at 22°C.

An intraventricular balloon catheter was inserted into the LV through the mitral valve, and isovolumic LV pressure was monitored via another Statham P23Db transducer. Thebesian venous return was emptied from the LV via a drain inserted transmurally into the LV. Coronary sinus effluent was drained through a pulmonary artery catheter. The heart was paced at 100 beats per minute via a platinum pacing wire inserted through the tricuspid valve into the right ventricle.

**Aequorin Loading**

Hearts were allowed to equilibrate for approximately 20 minutes before the injection of aequorin. A small region of the inferoapical surface of the LV was macroinjected with aequorin as previously described. Briefly, after cooling to 16°C and perfusing with a zero calcium solution, hearts became asystolic. After 15 minutes, coronary perfusion was reduced to maintain a perfusion pressure of approximately 20–25 mm Hg. Three to 5 µl of a solution containing aequorin (1.0 µg/ml) was injected with a glass micropipette into the interstitium of the epicardium of the inferoapical region of the LV.

After loading, hearts were repositioned in the organ bath with the aequorin-loaded region of the LV directed toward the cathode of the photomultiplier (model 9635QA, Thorn-EMI, Gencom, Inc., Fairfield, N.J.). The organ bath was enclosed in a light-occlusive photographic bellows designed for aequorin studies by Blinks and modified for whole heart studies by Kihara et al.

After loading, calcium was gradually added back to the coronary perfusate while the temperature and coronary flow rate were gradually increased to preload levels. Before beginning the experimental protocol, hearts were allowed to equilibrate until a steady baseline aequorin light level was reached.

**Experimental Design**

Experiments were performed on 28 hearts: 16 were subjected to stunning, and 12 served as nonischemic controls. Steady-state baseline recordings of LV pressure and aequorin light signals were obtained after aequorin loading. Global coronary ischemia was then imposed by abruptly lowering coronary flow to zero, which caused coronary perfusion pressure to fall to zero. After 15 minutes of no-flow ischemia, coronary perfusion was reestablished at a flow rate identical to preischemic levels. Within 1–2 minutes, LV contractions returned. After 20 minutes of reperfusion, a new steady state of LV function and aequorin light signals was established. Throughout the duration of ischemia and reperfusion, individual LV pressure tracings and aequorin light signals were simultaneously recorded. For purposes of calculating [Ca²⁺], and LV pressure, tracings were obtained at the following times: “baseline,” immediately before ischemia; “ischemia,” 1 minute after the start of no-flow ischemia; “reperfusion,” immediately after reestablishment of coronary flow; and “stunning,” 20 minutes after reperfusion.

**Ca²⁺ Dose–Response Relations**

After completion of the ischemia/reperfusion protocol, the effects of 1, 2, 4, and 8 mM perfusate calcium concentrations on LV performance were assessed in six hearts. Before assessing force–calcium relations, phosphate was removed from the perfusate to avoid precipitation of calcium salts at the higher calcium concentrations. Recordings of LV developed pressure were made 20 minutes after the addition of the new dose of perfusate Ca²⁺ to allow for a new steady-state equilibrium to be recorded. Calcium dose–response relations were assessed in a similar manner in 16 control hearts not subjected to global ischemia. Developed pressure was plotted as a function of measured perfusate calcium.

**Steady-State Pressure–pCa Relations**

Eight additional experiments (four stunned and four nonstunned hearts) were performed to assess pressure–pCa relations at steady state. This was accomplished by disabling the sarcoplasmic reticulum with ryanodine and then tetanizing the hearts, as described by Kusuoka et al. Briefly, 20 minutes after reperfusion, ryanodine (3×10⁻⁶ M) was added to the perfusate. After 20 minutes, tetanus was induced by pacing at 10 Hz with a pulse width of 60 msec at 2.0 times threshold. LV pressure and aequorin luminescence were measured during tetanus. Repeat tetanizations were performed at perfusate calcium concentrations of 1.0, 1.5, 2.0, 4.0, and 8 mM.

**Quantification of Intracellular Calcium**

Quantification of [Ca²⁺], by conversion of aequorin light signals was performed via the method of fractional luminescence. At the end of the experiment, a subset of hearts (n=5) was perfused with 8 mM calcium and 5% Triton X-100 to lyse the aequorin-loaded cells and expose all of the remaining aequorin to calcium, resulting in an instantaneous burst of light, which declined quickly to baseline. The area under the curve was integrated to obtain a value for the total amount of light emitted from the aequorin in the heart (Lmax). The ratio of the light signal (L) at any given point in the experiment to Lmax represents the fractional luminescence. By use of a calibration curve derived in vitro, L/Lmax was converted to [Ca²⁺]. To correct for aequorin consumption during ischemia, the integral of light recorded during the ischemic period was added to preischemia baseline values of the light signal.

**Statistical Analysis**

Data are reported as the mean±SEM of values at baseline versus ischemia, reperfusion, and stunning and were analyzed using a paired Student’s t test. A linear relation between pCa and LV pressure was tested with simple regression. A difference in the slopes and y
intercepts of these lines was tested by multiple regression.

Results

Table 1 lists mean LV systolic and diastolic pressures recorded immediately before ischemia and during stunning (i.e., the transient myocardial dysfunction occurring after brief periods of coronary ischemia and reperfusion). After 15 minutes of global ischemia and 20 minutes of reperfusion, a significant decrease in LV systolic pressure (LVSP) and an increase in LV end-diastolic pressure (LVEDP) were recorded. LV developed pressure, calculated as the difference between LVSP and LVEDP, was also significantly lower in the stunned hearts compared with baseline values.

Simultaneous unaveraged aequorin signals and LV pressure were obtained after the loading procedure and were similar to those reported by Kihara et al. 

Figure 1 shows continuous simultaneously recorded aequorin light signal, LV pressure, and coronary perfusion pressure at baseline, during ischemia, and during reperfusion. When coronary flow was interrupted, there was an abrupt fall in LVEDP and LVSP. Diastolic and peak systolic light increased during ischemia. After 20 minutes of reperfusion, diastolic and peak light returned to pres ischemic levels, but LV developed pressure did not, consistent with stunning.

Mean end-diastolic and peak systolic [Ca\(^{2+}\)]\(_i\) levels at baseline, during ischemia and early reperfusion, and during stunning are shown in Table 2 and Figure 2. During ischemia, diastolic [Ca\(^{2+}\)]\(_i\) rose from 3.8 \times 10^{-7} \text{ M} at baseline to 8.5 \times 10^{-7} \text{ M} and remained significantly elevated during early reperfusion. However, after 20 minutes of reperfusion, diastolic [Ca\(^{2+}\)]\(_i\) was not significantly elevated compared with baseline values. Similarly, peak [Ca\(^{2+}\)]\(_i\) rose significantly during ischemia and early reperfusion but also returned to baseline values during stunning.

To assess whether calcium handling is abnormal in stunned myocardium, calcium dose–response relations were examined in the stunned and control hearts. Table 3 shows the effect of varying [Ca\(^{2+}\)]\(_o\) on LV developed pressure. LV developed pressure was significantly lower for any given [Ca\(^{2+}\)]\(_o\) in the stunned hearts compared with control hearts (Figure 3). However, to accurately assess myofilament response to calcium, the pressure–pCa relation is expressed as a function of [Ca\(^{2+}\)]\(_i\) during tetanization (Figure 4). Simple regression demonstrated a linear relation between pCa 5.7–6.4 and absolute LV pressure. When a group effect was tested, control hearts were found to have a significantly higher LV developed pressure than stunned hearts for any given pCa (Figure 5). However, no significant difference in slopes was found.

Discussion

The phenomenon of myocardial stunning refers to transient myocardial dysfunction observed after isch-
emias and reperfusion. Obscuring an understanding of the cellular pathophysiology underlying stunning is the problem of ascertaining whether metabolic derangements occur during ischemia or reperfusion or both. Myocardial stunning was first described after transient coronary occlusion in the dog. Since the original description, the term stunning has been applied to numerous clinical and experimental models. Whether any or all of these models are phenomenologically similar to the original dog experiments is unknown. Therefore, caution must be emphasized when extrapolating findings from an isolated heart preparation to other models such as open-chested dogs or the post-thrombolysis patient. Nevertheless, transient myocardial dysfunction following coronary ischemia is common to all models. At the present time, the buffer-perfused isolated heart remains the only tenable model for simultaneously measuring [Ca\(^{2+}\)], transients and LV pressure. Given that stunning results from a complex interaction involving the coronary vasculature and myocardium, the whole heart provides a more physiological model for studying stunning than do muscle strips or isolated myocytes.

Using the isolated buffer-perfused ferret heart, we have shown that LV developed pressure is significantly decreased after 15 minutes of ischemia and 20 minutes of reperfusion. Although this 20% reduction in LV developed pressure is modest compared with the magnitude of regional dysfunction observed in early investigations, it is similar to that observed in other studies of stunning following global ischemia in the isolated heart. The concept of stunning requires the assumption that abnormalities of contractile function are due to reversible myocardial dysfunction rather than infarction. Using a similar isolated ferret heart model, Kusuoka et al. evaluated the stunned hearts for morphological evidence of damage. By light and electron microscopy, no significant contraction band necrosis was observed, and mild mitochondrial swelling was found in <1% of myocytes. Our protocol was similar, except our experiments were performed at 30°C rather than 37°C to reduce aequorin consumption. This relative hypothermia, although probably contributing to the lesser degree of stunning observed in our study, may also decrease the likelihood of irreversible myocyte damage.

Another confounding factor that must be considered when evaluating the changes in [Ca\(^{2+}\)], during stunning is whether the loading of a calcium indicator into the myocytes perturbs hemodynamic performance under physiological conditions. Kusuoka et al. loaded the calcium indicator 5F-BAPTA into the isolated ferret heart and, using nuclear magnetic resonance (NMR) spectroscopy, measured [Ca\(^{2+}\)] during myocardial stunning. However, after loading, LV developed pressure

**TABLE 3. Calcium Dose–Response Relations in Control and Stunned Hearts**

<table>
<thead>
<tr>
<th>Left ventricular developed pressure (mm Hg)</th>
<th>1 mM [Ca(^{2+})]</th>
<th>2 mM [Ca(^{2+})]</th>
<th>4 mM [Ca(^{2+})]</th>
<th>8 mM [Ca(^{2+})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88±6</td>
<td>119±5</td>
<td>135±8</td>
<td>130±4</td>
</tr>
<tr>
<td>Stunned</td>
<td>63±9*</td>
<td>88±10*</td>
<td>101±10*</td>
<td>100±6*</td>
</tr>
</tbody>
</table>

\([\text{Ca}^{2+}]_o\), perfusate calcium concentration. Values are mean±SEM. *p≤0.05 versus control hearts.

**FIGURE 2. Bar graphs showing mean end-diastolic (left panel) and peak systolic (right panel) [Ca\(^{2+}\)], values at baseline, during ischemia, and early reperfusion and stunning.**

**FIGURE 3. Absolute left ventricular (LV) developed pressure plotted against perfusate calcium for control and stunned hearts. At 1, 2, 4, and 8 mM perfusate calcium concentrations, developed pressure is significantly lower for stunned hearts versus control hearts.**
was only approximately 10 mm Hg in 2 mM [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{20} Perfusion calcium concentrations had to be increased to levels fourfold higher than observed in vivo to achieve physiological hemodynamics. Our laboratory has shown that there are no significant differences in LVSP and LVEDP or in the time course of contraction before and after aequorin loading in the isolated whole heart. Also, by measurement of creatine phosphokinase and light microscopy, no significant myocardial damage was found in the aequorin-loaded region.\textsuperscript{16} Another significant advantage of aequorin as a calcium indicator for the evaluation of intracellular calcium in the isolated stunned hearts is its ability to generate quantifiable light signals without the need for signal averaging. This allows for measurement of [Ca\textsuperscript{2+}]\textsubscript{i}, on a beat-to-beat basis during dynamic processes such as ischemia and reperfusion.\textsuperscript{16} Another potential problem in the measurement of [Ca\textsuperscript{2+}]\textsubscript{i} in whole hearts is the possibility of indicator loading into nonmyocytes, such as endothelial cells. Treatment with bradykinin does not appear to change the luminescence of aequorin-loaded hearts, suggesting that significant endothelial loading does not occur.\textsuperscript{21}

In both the aequorin and indo-1–loaded isolated heart, bioluminescence and fluorescence, respectively, are measured from epicardial myocytes. Whether [Ca\textsuperscript{2+}]\textsubscript{i}, measured from these cells is similar to that measured in myocytes from other regions, such as the subendocardium, cannot be determined from these experiments. Guarnieri\textsuperscript{22} loaded aequorin into the endocardium of the right ventricular septum and, using a similar stunning protocol, measured values for [Ca\textsuperscript{2+}]\textsubscript{i}, similar to ours. This suggests that in this model calcium modulation during ischemia, reperfusion, and stunning is similar in both the epicardium and endocardium.

**Figure 4.** Simultaneous recording of isovolumic left ventricular (LV) pressure (top panel) and [Ca\textsuperscript{2+}]\textsubscript{i}, (bottom panel) before and during tetanus (arrow).

**Figure 5.** Left ventricular pressure and pCa plotted during control tetanic stimulation with 3 × 10\textsuperscript{-6} M ryanodine in the bath (p = 3007 – 467.36x; \textit{r} = 0.81, p < 0.001) and with stunned hearts (p = 2440.9 – 385.59x; \textit{r} = 0.90, p < 0.001). The slope of the left ventricular pressure–pCa relation is not significantly different between groups (\textit{B} coefficient, 382.8; 95% confidence interval, 269.5–496), but the \textit{y} intercepts are significantly different (p < 0.001) in the pCa range of 6.4–5.7.
myocardial stunning does not result from a decrease in intracellular activator calcium but, rather, from a decreased myofilament responsiveness to \([Ca^{2+}]_i\).

Myofilament calcium responsiveness can be considered in terms of two primary determinants. The first is the maximal \(Ca^{2+}\)-activated force (\(F_{\text{max}}\)), which, in classical pharmacological terminology, defines the efficacy of calcium as an agonist. The second is the \(Ca^{2+}\) sensitivity, reflected by the position of the calcium concentration–response relation relative to the control. Sensitivity is usually expressed in terms of the effective dose producing some degree of activation between 15% and 85% of \(F_{\text{max}}\) (\(ED_{15-85}\)) and defines the potency of calcium as an agonist.28,29 In these terms, decreased myofilament responsiveness to \(Ca^{2+}\) may be characterized by 1) a decrease in \(F_{\text{max}}\) or efficacy, 2) a decrease in calcium sensitivity or potency, or 3) some combination of changes in both determinants. As shown in Figure 3, the pressure–\([Ca^{2+}]_i\) relation is shifted downward. However, the ideal method of assessing calcium–myofilament interaction is to measure \([Ca^{2+}]_i\), and LV pressure at steady state when sarcoplasmic reticular calcium cycling has been eliminated. After perfusion with ryanodine, Kusuoka et al13 tetanized the isolated ferret heart during stunning by high frequency pacing. We performed similar experiments in the aequorin-loaded isolated heart. However, as seen in Figure 4, we have expressed the tetanized pressure as a function of \([Ca^{2+}]_i\). Since cytoplasmic calcium levels and, therefore, calcium bound to troponin do not vary in direct proportion to perfusate calcium, measured \([Ca^{2+}]_i\) should provide a more accurate assessment of myofilament responsiveness to calcium.

The downward shift in the pressure–\([Ca^{2+}]_i\) relation is confirmed by a similar downward shift in the pressure–pCa relation during tetani, suggesting that stunning is associated with a decrease in \(F_{\text{max}}\). However, no significant differences in slopes were found, suggesting that myofilament desensitization (i.e., an increase in \(ED_{15-85}\) for \(Ca^{2+}\)) did not occur. The force–pCa relation is best described as a sigmoidal curve over the entire range of \([Ca^{2+}]_i\). However, unlike single cells or muscle strips, the performance of the isolated heart cannot be evaluated at extremely high or low perfusate calcium concentrations because of electrical instability, which is manifested by calcium oscillations. Therefore, the downward shift in the pressure–pCa relation as shown in Figure 4 cannot be ensured at pCa values >5.7 or <6.4.

Thus, these experiments indicate that myocardial stunning is characterized by a decreased myofilament responsiveness to calcium secondary to a decrease in \(F_{\text{max}}\) but not by a decrease in myofilament sensitivity to calcium. Kusuoka et al13 also found that \(F_{\text{max}}\) was depressed in myocardial stunning but that myofilament sensitivity to calcium was also depressed. This apparent discrepancy with our results regarding sensitivity may be explained either by differing definitions of sensitivity used by each group or type II error in our analysis, secondary to a lower signal to noise ratio inherent when using nonaveraged signals. Given that the same data were used in comparing group effects in the pCa–LV pressure and the slopes of these relations, we feel that the signal-to-noise ratios are sufficient to exclude the null hypothesis. Also, the narrow confidence intervals for the slope of the pCa–pressure relation suggest that a true difference can be excluded. Our definition of sensitivity is based on the pharmacological principle that a change in sensitivity is manifested by a shift in the position of the dose–response relation when data are expressed as a percent of the maximal response.20 Kusuoka et al13 defined sensitivity as a change in the slope of the absolute developed pressure as a function of \(Ca^{2+}\). At the molecular level, both the potency and efficacy of \(Ca^{2+}\) as an agonist may be affected by changes in the binding properties of troponin C, as well as by factors altering crossbridge attachment and cycling rates.

Why should \(F_{\text{max}}\) be depressed in the stunned myocardium? Accumulation of intracellular metabolites such as inorganic phosphate can depress \(F_{\text{max}}\). Intracellular phosphate levels rise during ischemia as phosphocreatine and ATP concentrations decrease. However, as measured with NMR spectroscopy, intracellular phosphate levels have returned to preischemic levels after 20 minutes of reperfusion.13 A decrease in \(F_{\text{max}}\) could be explained by a change in myofilament crossbridge kinetics or by a decrease in the relative force generation per attached crossbridge. The precise intracellular derangements responsible for these abnormalities are not known. However, as we have shown, calcium overload occurs during ischemia and reperfusion. Furthermore, others have shown that reperfusion with low calcium solutions34 or pretreatment with calcium channel blockers30,31 may improve functional recovery. It is possible that transient calcium overload may activate protein kinases, such as protein kinase C, which ultimately may affect troponin I or troponymosin and lead to altered crossbridge interaction.32,33 Finally, interactions between \(Ca^{2+}\) and the so-called “third messenger system” involving inositol-1,4,5-trisphosphate and diacylglycerol may also have an impact on the control of excitation–contraction coupling.34

References


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