Altered Calcium Sensitivity of Isometric Tension in Myocyte-Sized Preparations of Porcine Postischemic Stunned Myocardium

P.A. Hofmann, W.P. Miller, and R.L. Moss

Postischemic ventricular myocardial dysfunction, termed stunning, is characterized by a persistent but ultimately reversible depression of contractile function. The present study was undertaken to investigate the possibilities that reduced contractile force in stunning is due to a decrease in maximal tension-generating capability or to a decrease in the Ca\textsuperscript{2+} sensitivity of the myofilaments. The experiments combine an in vivo open-chest porcine heart model of stunning (n=5) with in vitro measures of myocyte myofilament calcium sensitivity from these same hearts. Regional myocardial function in the left anterior descending coronary artery (LAD) perfusion bed of porcine hearts was measured with transmural ultrasonic crystals. The protocol was 45 minutes of low-flow LAD ischemia at 40% of control flow, followed by 30 minutes of posts ischemic reperfusion at control aerobic flow. Percent systolic wall thickening decreased to 8±5% of control during ischemia (p<0.05) and returned to 38±8% of control in the postischemic stunned state (p<0.05). Serial endocardial biopsies were obtained from the preischemic and posts ischemic myocardium in the LAD perfusion bed and from the aerobically perfused myocardium in the circumflex bed. The biopsies were mechanically disrupted, and myocyte-sized preparations of permeabilized myocardium were attached to a force transducer and a length-changing device to allow for direct measurement of steady-state tension–pCa (i.e., –log[Ca\textsuperscript{2+}]) relations. The pCa for half-maximal activation of tension, i.e., pCa\textsubscript{50}, in LAD myocardium decreased from 5.88±0.05 before ischemia to 5.69±0.03 after ischemia (p<0.05); however, maximal Ca\textsuperscript{2+}-activated tension and the slope of the tension–pCa relation were unaffected by the ischemic episode. We conclude that the Ca\textsuperscript{2+} sensitivity of isometric tension is reduced and accounts at least in part for the depressed contractile function of stunned myocardium. (Circulation Research 1993;72:50–56)

KEY WORDS • myocardium • ischemia • tension • calcium

A wide variety of mechanisms has been proposed to account for the contractile dysfunction that is evident in post ischemic “stunned” myocardium.\textsuperscript{1,2} Stunning is characterized by depressed contractile function that reverses over a period of hours or days.\textsuperscript{3} Recent work suggests that there may be abnormalities in excitation–contraction coupling in stunned myocardium and possibly a decrease in the sensitivity of the myofilaments to Ca\textsuperscript{2+}, so that the tension-generating capability of the myocardium is reduced.\textsuperscript{2} In the context of the myofilamentary regulatory protein tropomyosin, changes in contractility result either from an alteration in the intracellular Ca\textsuperscript{2+} transient or from changes in myofilament sensitivity to Ca\textsuperscript{2+} or both.\textsuperscript{4} Available data generally suggest that the myoplasmic Ca\textsuperscript{2+} transient is unchanged relative to the control value during hypoxia, in early ischemia, and in posts ischemic stunned myocardium.\textsuperscript{2} By microinjecting aequorin into some cells of a papillary muscle preparation, Allen and Orchard\textsuperscript{5} found no change in the intracellular Ca\textsuperscript{2+} transient when the preparations were subjected to hypoxia. On the other hand, MacKinnon et al\textsuperscript{6} microinjected aequorin into cardiac myocytes and observed a reduction in the Ca\textsuperscript{2+} transient during hypoxia. In other studies, nuclear magnetic resonance spectroscopy was used in isolated ferret hearts to characterize the intracellular Ca\textsuperscript{2+} transient by monitoring the spectrum of 5F-BAPTA, i.e., fluorinated BAPTA.\textsuperscript{7,8} Under hypoxic conditions, peak intracellular Ca\textsuperscript{2+} was increased.\textsuperscript{7} Postischemic stunned hearts developed pressures that were depressed by 40% relative to preischemic control values, but peak intracellular Ca\textsuperscript{2+} during systole was actually greater than in the control hearts.\textsuperscript{8} These results suggest that the intracellular Ca\textsuperscript{2+} transient in reperfused postischemic stunned myocardium is preserved and that the decrease in developed pressure may be secondary to a reduction in myofilament calcium sensitivity.

Kusuoka et al\textsuperscript{9} observed a decrease in maximal calcium-activated pressure (measured during sustained contractions after exposure to the alkaloid ryanodine) as well as a shift in the relation between developed pressure and perfusate [Ca\textsuperscript{2+}] to higher levels of [Ca\textsuperscript{2+}] in stunned ferret hearts. These data suggest that there is
both a decline in maximal force-developing capabilities and a decrease in myofilament sensitivity to Ca\(^{2+}\). However, these results from isolated ferret hearts must be reconciled with results from in vivo working canine hearts.\(^{10,11}\) First, the positive response of myocardium to inotropic stimuli is preserved in regionally stunned canine hearts.\(^{10}\) Second, regional myocardial function (systolic shortening) of stunned myocardium increases to control levels with the intracoronary infusion of Ca\(^{2+}\) in canine hearts.\(^{11}\) If a decrease in the sensitivity of myofilaments to Ca\(^{2+}\) accounts for the decline in contractile force observed in isolated stunned hearts, then the in vivo contractile responses to inotropic stimuli that are mediated by increased intracellular Ca\(^{2+}\) should be depressed.

The purpose of the present study was to directly test the hypothesis that the sensitivity of the myofilaments to Ca\(^{2+}\) is reduced in postischemic stunned myocardium. By use of an in vivo open-chest porcine heart model to induce regional ischemia, single cell-sized preparations of permeabilized myocardium were obtained by mechanical disruption of endocardial biopsies from preischemic and postischemic stunned myocardium. Measurements of isometric tension as a function of free [Ca\(^{2+}\)] in these functionally skinned preparations showed that the Ca\(^{2+}\) sensitivity of tension, assessed as the [Ca\(^{2+}\)] for half-maximal activation of tension, was significantly depressed in stunned myocardium. There was no change in maximal Ca\(^{2+}\)-activated tension.

**Materials and Methods**

**In Vivo Porcine Heart Regional Stunning Model**

An open-chest porcine heart preparation of regional myocardial stunning, which has been previously described, was used.\(^{12-14}\) Adolescent pigs (n=5) of either sex weighing 44.7±2.9 kg were studied after anesthesia with pentobarbital (35 mg/kg i.v.) and establishment of controlled positive-pressure ventilation with supplemental oxygen. Additional pentobarbital was given as needed to ensure adequate anesthesia. Frequent determinations of the animals’ arterial pH, O\(_2\), and P\(_{\text{CO}_2}\) values were obtained throughout the study to ensure adequate oxygenation and a physiological acid–base balance. Anesthesia, surgery, and general care of the animals involved in these studies conformed in every respect to the ‘Guide for the Care and Use of Laboratory Animals’ of the National Institutes of Health, and the protocol was approved by the University of Wisconsin Animal Care Committee. The heart was exposed by bilateral thoracotomy with transthoratomy. A high-fidelity micromanometer-tipped catheter (Millar Instruments, Houston, Tex.) was advanced retrogradely from the right internal carotid artery into the left ventricle to measure left ventricular pressure, which was differentiated to obtain left ventricular dP/dt. Left ventricular transmural wall thickness was measured by use of ultrasonic crystals placed midway between apex and base in the left anterior descending coronary artery (LAD) bed.\(^{15}\) Regional left ventricular function in the LAD bed was assessed by percent systolic wall thickening.\(^{16}\) After treatment with heparin (20,000 units i.v.), an extracorporeal perfusion circuit was constructed. The cannulated left femoral artery was connected to the LAD by a cannula positioned near its origin. Flow to the LAD was controlled by a calibrated low-flow perfusion pump (Sarns). Control aortic flow in the LAD bed was set by adjusting the mean LAD perfusion pressure to the average aortic pressure corrected for perfusion line resistance. A small cannula was placed in the anterior cardiac vein to sample blood for oxygen saturation. Oxygen consumption in the LAD bed was measured using the Fick principle. LAD coronary blood flow was determined from the calibrated perfusion pump. Coronary blood flow and oxygen consumption were normalized to the wet weight of myocardium in the LAD perfusion bed, as determined by postmortem injection of colored dye at the conclusion of the experiment.\(^{17}\) A microcomputer (Zenith) was used to obtain measurements of both global and regional left ventricular function, measured from an average of 10 cardiac cycles, at 15-minute intervals throughout the protocol.

The protocol for inducing stunning and obtaining myocardium by biopsy was as follows (Figure 1): At time 0 during preischemia, indicated by the first B, in Figure 1, transmural needle (0.9-mm-i.d.) biopsies of myocardium (approximately 15 mg each) were taken separately from the areas of the ventricle perfused by the LAD and circumflex coronary artery (CFX).\(^{17}\) After 30 minutes of control flow, ischemia was then induced in the LAD perfusion bed by reducing LAD flow to 40% of the preischemic flow for 45 minutes. The LAD was then reperfused at preischemic flow rates, and needle biopsies were again taken separately from the LAD and CFX perfusion beds after 30 minutes of LAD reperfusion, indicated by the second B, in Figure 1.

**Isolation of Single Cell–Size Myocyte Preparations**

Biopsied ventricular tissue was placed in cold relaxing solution (pCa 9) and was then divided into epicardial
endocardial pieces. The endocardial samples were mechanically disrupted for 20 seconds by using a tissue homogenizer (Polytron, Kinematica) set at a low speed. This resulted in a suspension of small clumps of myocytes, single myocyte–sized preparations, and cell fragments. Single cell–sized preparations of mechanically disrupted myocardium were selected on the basis of size (100–150 μm long by 15–μm diameter) and uniformity of striation pattern. These preparations appeared to be permeabilized (“skinned”), since subsequent treatment with Triton X-100 had no effect on Ca2+-activated tension. The preparation was then attached with silicone adhesive to micropipettes extending from a force transducer (model 406, Cambridge Technology, Inc.) and a piezoelectric transducer (Physik Instrumente, Waldbronn, Germany) by using methods previously described.18,19 Once attached, the preparations were first bathed for 20 seconds in relaxing solution containing 0.2% ultrapure Triton X-100 (Pierce Chemical Co., Rockford, Ill.) to disrupt any remaining sarcoplasmic reticulum. In the typical sequence of tension measurements, the preparation was initially activated in a solution of pCa 4.5 to assess striation uniformity and the integrity of the attachment. The length of the preparation was then adjusted to achieve a sarcomere length of approximately 2.2 μm during subsequent activations, which was verified by photomicroscopy.  

### Tension Measurements

Mechanical measurements on the single cell–sized preparations were obtained as previously described.18,19 Tension–pCa relations were characterized at 22°C by first maximally activating the preparation in a solution of pCa 4.5 and subsequently in a series of solutions of higher pCa (i.e., lower [Ca2+]). At each pCa, a steady tension was allowed to develop, and the preparation was then slackened to obtain an accurate force baseline, after which it was returned to relaxing solution and reextended to its original length. Active tension at each pCa was determined as the difference between total tension measured by slackening the preparation during steady activation and passive tension measured by slackening the relaxed preparation. A final contraction was done in a solution of pCa 4.5 to determine whether the maximal tension-generating capability of the preparations had declined during the measurement protocol. Typically, tension declined by less than 5% between the initial and final contractions at pCa 4.5. Tension data from preparations in which maximal tension declined by more than 15% between the initial and final contractions at pCa 4.5 were not analyzed and are not included in the results reported here. The average number of activation–relaxation cycles was nine. Sarcomere length reported for any given preparation was determined by averaging the sarcomere lengths measured from all the photos of that preparation taken while immersed in solutions of pCa 9.0 and during steady activation at pCa 4.5. On average (22 myocytes), the sarcomere length of myocardial preparations while relaxed was 2.22±0.03 μm and during maximum activation was 2.14±0.04 μm. Preparation width was also obtained from the photos, and tension per unit cross-sectional area was calculated by assuming a circular cross section (Table 2).

### Solutions

Relaxing and activating solutions contained 4 mM MgATP, 1 mM free Mg2+, 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, pH 7.0, and sufficient KCl to adjust ionic strength to 180 mM. Free [Ca2+] was varied between 10−9 M (relaxing solution) and 10−4.5 M (maximally activating solution) and is expressed as pCa (−log[Ca2+]). The apparent stability constant for calcium EGTA was corrected to 22°C and an ionic strength of 180 mM.20 The computer program of Fabiato20 was used to calculate concentrations of each metal, ligand, and metal–ligand complex.

### Analysis of Data

Tension at each pCa was expressed as a fraction of maximal tension (P0) developed in the same preparation at pCa 4.5. In those cases in which a decline in maximal tension was observed between the beginning and end of the experiment, tension at each pCa was referenced to a P0 value obtained by linear interpolation.18,19 Ca2+ sensitivity of isometric tension was assessed by determining the pCa50 of the tension–pCa relation, i.e., the pCa at which tension was half maximal. The pCa50 and the slope (i.e., the Hill coefficient) of each tension–pCa relation were quantified by Hill plot transformation of the tension data.21 A straight line was fit to the transformed data by least-squares regression by using the following equation:

\[
\log(P_0/(1-P_0)) = n_H(\log([Ca^{2+}]) + \log k)
\]

where P0 is tension as a fraction of P0, nH is the Hill coefficient, and k is the pCa50. By using the constants derived from the Hill analysis, curves were fit by computer to the tension–pCa relation according to the following equation:

\[
P/P_0 = [Ca^{2+}]^n/(k^n + [Ca^{2+}]^n)
\]

where P is tension.

### Statistical Analyses

Paired t tests were done to determine whether measures of global and regional ventricular function in the
intact hearts were different from preischemic control values. An analysis of variance was done to test whether stunning had significant effects on $\text{pC}_50$, $\text{n}_H$, sarcomere length, or $P_\text{p}/\text{CSA}$, cross-sectional area measured in disrupted ventricular myocardial preparations (BMDF Statistical Software Package, BMDF Statistical Software, Inc., Los Angeles). When significant interactions were found, a Bonferroni $t$ test was used to determine the level of significance of mean differences. In all cases, a value of $p<0.05$ was considered significant. All values are reported as mean±SEM.

### Results

There were no changes in left ventricular global function between preischemic and postischemic heart rate (133±6 versus 159±12 beats per minute), left ventricular pressure (100±1 versus 97±3 mm Hg), or left ventricular maximum $dP/dt$ (2,112±175 versus 2,441±369 mm Hg/sec). Regional LAD flow, metabolism, and function in the porcine hearts are shown in Table 1. Ischemia was induced by reducing LAD coronary blood flow to 40% of the preischemic level. This resulted in a LAD perfusion pressure of 41±3 mm Hg and a LAD venous $\text{O}_2$ saturation of 16.1±1%, documenting ischemic levels of LAD perfusion.$^{22}$ Measurements of systolic wall thickening showed that the myocardium in the LAD perfusion bed was stunned with reperfusion after the ischemic episode (Table 1 and Figure 1). Systolic wall thickening was significantly reduced to near zero (8±5%, $p<0.001$) during ischemia. Reperfusion partially reversed this loss of function to 38±12% of control values measured in the same hearts, but the postischemic values remained significantly less than the control values ($p<0.05$). This degree of stunning was stable for at least 30 minutes during postischemic reperfusion, the time at which needle biopsies were again taken from the LAD and CFX perfusion beds.

Maximal activation of the mechanically disrupted myocardial preparations used in this study revealed no change in the maximal tension-generating capability of myocardium from the LAD perfusion bed after the induction of ischemia (Table 2). Although the specific tension of preparations from the CFX and LAD regions appeared to differ, our statistical analyses indicated that these values were not significantly different. In all cases, sarcomere length was well maintained during maximal activations of preparations from both preischemic and postischemic myocardium (Figure 2), and mean sarcomere lengths during activation of LAD or CFX postischemic myocardium did not differ significantly from the control values (Table 2).

Tension–pCa relations obtained from disrupted preparations show that the induction of ischemia significantly reduced the Ca$^{2+}$ sensitivity of isometric tension in myocardium from the LAD perfusion bed (Figure 3), as assessed by a decrease in $\text{pC}_50$ in the postischemic LAD myocardium (Table 2). Control values of $\text{pC}_50$ obtained from LAD and CFX myocardium were similar, and $\text{pC}_50$ of the aerobically perfused CFX myocardium was unaffected by inducing ischemia in the LAD perfusion bed. There were no differences in the slope of the tension–pCa relation, quantified as the Hill coefficient, under any of the conditions studied.

As a first attempt to determine a molecular basis for the observed decrease in Ca$^{2+}$ sensitivity in postischemic stunned myocardium from the LAD bed, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on samples from suspensions of disrupted ventricular myocardium obtained before and after the ischemic episode. There were no discernable differences in myofilament protein composition in samples of myocardium from the LAD and CFX perfusion beds and no changes in composition of myocardium from either source after LAD ischemia.

### Discussion

Stunning of the myocardium is characterized by a reversible depression of contractility after a brief period of ischemia, but the basis for this transient dysfunction is not understood. The present studies involved direct measurements of myofilament sensitivity to Ca$^{2+}$ in stunned myocardium. These data from different regions of the heart both before and after regional ischemia (i.e., in the LAD perfusion bed) indicate that the Ca$^{2+}$ sensitivity of tension is significantly reduced after ischemia. One functional consequence of reduced sensitivity to Ca$^{2+}$ is that isometric tension at a given submaximal concentration of myoplasmic Ca$^{2+}$ will be reduced. A secondary effect that could be predicted on the basis of reduced tension-generating capacity at low levels of [Ca$^{2+}$] is that the velocity of myocardial shortening against a given load will be reduced. Thus, reduced Ca$^{2+}$ sensitivity alone or in concert with other factors may be sufficient to explain reduced contractile capabilities of stunned myocardium, although our results do not specifically exclude the possibility of additional mechanisms.
FIGURE 2. Photomicrographs of detergent-treated mechanically disrupted ventricular myocardium obtained from the region perfused by the left anterior descending coronary artery. These micrographs were obtained while the preparation was relaxed (pCa 9.0) and also during steady activation (pCa 4.5). The preparation used in panels A and B is different from the one used in panels C and D. Sarcomere lengths were 2.11±0.05 μm (panel A), 2.06±0.09 μm (panel B), 2.28±0.06 μm (panel C), and 2.18±0.06 μm (panel D). Bar, 25 μm.

The present studies of mechanically disrupted myocardium combine an in vivo large animal model of stunning with in vitro measurements on myocyte-sized preparations from the same hearts. The advantages of this approach include the ability to perform serial measurements of myofilament Ca²⁺ sensitivity of tension over the course of the experiment and from different regions of the ventricle. The low-flow model of ischemia was chosen, rather than a no-flow model of myocardial stunning, because it has been shown previously in our laboratory to provide a reproducible, stable, large animal model in which to assess the function and metabolism of stunned myocardium.12-14 Although low-flow ischemia may differ from no-flow ischemia, both result in reproducible postischemic stunning. Finally, low-flow ischemia may be more clinically relevant because it occurs frequently in patients with coronary heart disease.

FIGURE 3. Graphs showing mean tension–pCa relations from disrupted ventricular myocardium obtained before and after the induction of ischemia in the perfusion bed of the left anterior descending coronary artery (LAD). CFX, circumflex coronary artery; P, tension; Po, maximal Ca²⁺-activated tension; Pr, tension as a fraction of Po. Myocardium was obtained by biopsy from the regions of the left ventricle perfused by the LAD and CFX before and after ischemia in the LAD bed. The CFX bed was aerobically perfused throughout the study. In the left panel, the data are plotted as relative tension–pCa relations; in the right panel, the same data have been transformed to yield a Hill plot. The pCa values for half-maximal activation and Hill coefficients for these relations are presented in Table 2.
The use of isolated permeabilized (skinned) myocyte-sized fragments allows for the direct measurement of maximal Ca\(^{2+}\)-activated tension and myofilament Ca\(^{2+}\) sensitivity. An advantage of this approach is that these cellular determinants of myocardial function can be assessed independent of non–myocyte-related mechanisms of stunning, such as abnormalities in coronary vascular reserve or changes in interstitial collagen. A possible concern is that the biopsy and isolation procedures may damage or modify the myocyte-sized preparations, thereby inducing artifactual changes in their mechanical properties. Though this cannot be totally discounted, it is unlikely for several reasons: First, the mechanical characteristics of myocyte-sized preparations obtained from biopsy specimens in this study are comparable to those obtained by mechanical disruption of isolated heart tissue.\(^{18,19}\) Second, myocyte-sized preparations were selected on the basis of size as well as uniformity of striation pattern; i.e., measurements were not performed on irreversibly damaged cells, which were contracted and therefore had abnormal morphology. Third, the shift in Ca\(^{2+}\) sensitivity found in this study occurred only in myocytes from the postischemic stunned LAD myocardium and not in myocytes obtained from serial biopsies from aerobic CFX myocardium.

Although the basis for reduced Ca\(^{2+}\) sensitivity is not known, the specific mechanism may involve reduced affinity of troponin C, the Ca\(^{2+}\)-binding subunit of the thin-filament regulatory protein troponin, for Ca\(^{2+}\). Such a mechanism is consistent with the finding that inotropic interventions that increase myoplasmic [Ca\(^{2+}\)]\(_{th}\) during systole can enhance function in stunned myocardium to near normal levels.\(^{10,11}\) Since postischemic stunning had no discernable effects on the slope of the tension–pCa relation in disrupted myocardium, it is unlikely that the molecular cooperativity of Ca\(^{2+}\)-activated tension was altered. The finding that the maximal tension-generating capabilities of myocardium were unchanged after stunning suggests that ischemia has no significant effects on myosin or actin or on the chemical equilibrium between tension-generating and non–tension-generating crossbridge states. Using isolated ferret hearts, Kusuoka et al.\(^{12}\) showed that maximal Ca\(^{2+}\)-activated pressure (measured during tetani after exposure to ryanodine) decreased in postischemic hearts relative to control hearts. Their data suggest that there is a decline in maximal force development in stunning, but this was not found in the present study, in which maximal tension was unchanged at the cellular level. Possible explanations for these discrepancies include preparation and species differences; e.g., the isolated buffer-perfused ferret hearts may differ from in vivo canine and in vivo porcine heart preparations. Also, different ischemic insults were applied to induce stunning in these different models.

The basis for an altered binding of Ca\(^{2+}\) by troponin C remains to be determined but could be the result of a direct effect of ischemia on troponin C or an indirect result of an effect on other regulatory proteins within the thin filament, such as possible alterations in phosphorylation of either myosin light chain 2, troponin I, or troponin T. Coronary occlusion for periods longer than those used in the present study resulted in disruption of regulatory protein complexes in canine hearts. More recently, an in vitro rat heart model showed degradation of troponin I and troponin T on SDS-PAGE after 60 minutes of total global ischemia.\(^{23}\) Possible agents for effects on regulatory proteins are proteases that may be activated by Ca\(^{2+}\) or free radicals that are generated during ischemia and reperfusion.\(^{1,2}\) In the present study, we observed no changes in protein content in our preparations of stunned myocardium, but SDS gels may not detect subtle changes in protein structure. The time course of recovery of stunning (i.e., from hours to days) suggests that there may be an irreversible effect on protein structure that occurs during ischemia and/or reperfusion and that normal function returns only when the affected protein is replaced by the process of protein turnover within individual cells.

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**References**


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