Effect of Ischemia and Reperfusion on Sarcoplasmic Reticulum Calcium Uptake

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To investigate the mechanism underlying postischemic cardiac dysfunction (myocardial stunning), contractility and adenine nucleotide metabolism were studied in three groups of isolated perfused rabbit hearts (control, ischemic, and reperfused), whereas Ca\(^{2+}\) uptake by the sarcoplasmic reticulum (SR) was measured in homogenates obtained from them. The hearts were Langendorff-perfused under constant pressure with Krebs-Henseleit solution at 37°C. Global normothermic ischemia was produced by closing the perfusion line. In the reperfused group, after 15 minutes of ischemia, Krebs-Henseleit solution was perfused for 10 minutes. Developed left ventricular pressure (control, 104±6.3 mm Hg) and left ventricular dP/dt (2,063±256.6 mm Hg · sec\(^{-1}\)) were significantly decreased in reperfused hearts (left ventricular pressure, 78±5.9 mm Hg; left ventricular dP/dt, 1,339±216.3 mm Hg · sec\(^{-1}\)). Myocardial ATP content (control, 13.6±0.98 μmol/g dry wt) decreased during ischemia (4.5±1.23 μmol/g) but was restored to control level on reperfusion (11.8±0.68 μmol/g). Maximum velocity of Ca\(^{2+}\) uptake by the SR (V\(_{\text{max}}\)) (control, 49.3±2.54 nmol · min\(^{-1}\) · mg\(^{-1}\)) was significantly depressed by ischemia (36.3±1.94 nmol · min\(^{-1}\) · mg\(^{-1}\)) but was restored to the control value after a 10-minute reperfusion (45.3±0.79 nmol · min\(^{-1}\) · mg\(^{-1}\)). Apparent dissociation constant K\(_{\text{D}}\) and the Hill coefficient for Ca\(^{2+}\) uptake were not different between control, ischemia, and reperfusion. To test for the possible role of the SR Ca\(^{2+}\)-release channel in the effect of ischemia and reperfusion, we measured Ca\(^{2+}\) uptake after incubation of homogenates with 610 μM ryanodine. The changes in V\(_{\text{max}}\) caused by ischemia and reperfusion were qualitatively similar to those observed in experiments without ryanodine (76.3±5.08, 54.0±5.08, and 69.7±2.82 nmol · min\(^{-1}\) · mg\(^{-1}\) for control, ischemia, and reperfusion, respectively). These results suggest an effect of ischemia on the SR Ca\(^{2+}\) pumping without an effect on the Ca\(^{2+}\)-release process. The recovery of Ca\(^{2+}\) uptake during reperfusion indicates that neither an altered uptake of Ca\(^{2+}\) by the SR nor an abnormal function of the release channels is the major cause of myocardial stunning. (Circulation Research 1992;71:1123–1130)

KEY WORDS • sarcoplasmic reticulum • calcium • ischemia • reperfusion • isolated hearts

During reperfusion after short periods of myocardial ischemia, a condition called “stunning” occurs in which cardiac muscle is not irreversibly injured but its contractile function remains decreased for hours or days before complete recovery.\(^1\)\(^-\)\(^3\) The mechanisms underlying myocardial stunning remain unknown. At the cellular level, two major changes have been shown to occur in reperfused myocardium: 1) Intracellular levels of ATP decrease during ischemia (for review, see Reference 4), and although there is a resynthesis of the nucleotide during reperfusion, a decreased level of cellular ATP may persist for a long time in stunned myocardium.\(^5\)\(^-\)\(^7\) 2) Although earlier studies have indicated that the cellular content in Ca\(^{2+}\) increases during reperfusion,\(^7\)\(^-\)\(^9\) recent studies suggest that the intracellular free Ca\(^{2+}\) concentration increases during ischemia and returns to the control level on reperfusion.\(^10\)\(^11\) Changes of either or both ATP and intracellular free Ca\(^{2+}\) concentration have been invoked as being the major mechanism underlying postischemic myocardial dysfunction. The hypothesis that a decrease of ATP levels underlies stunning has been tested in a number of studies, and the conclusion has been reached that the ATP levels and the available free energy during reperfusion after short ischemia are sufficient to sustain the energetic needs of contraction.\(^12\) It is found that ATP levels can recover to near normal values without improvement in contractility. Conversely, contractility can be made to increase during stunning,\(^13\) even in the presence of decreased ATP levels. Finally, recent studies of mitochondrial function, including one from this laboratory,\(^14\) suggest that mitochondria isolated from stunned hearts retain normal function.

Thus, research efforts on the mechanisms of myocardial stunning now focus on a possible abnormality of the excitation–contraction coupling. Among the cellular organelles whose altered functions might account for the contractile deficiency in stunned hearts are the sarcoplasmic reticulum (SR), the contractile myofilaments, and the cell surface membrane. The discovery of a Ca\(^{2+}\) overload during ischemia and/or reperfusion suggests that a deficient cellular Ca\(^{2+}\) homeostasis may be the major characteristic of an abnormal excitation–contraction coupling in stunned myocardium.\(^15\)\(^16\) Because of its role in intracellular Ca\(^{2+}\) regulation and in excitation–contraction coupling, the SR has been sug-
gested to undergo damage on reperfusion after ischemia and to become incapable of assuming its Ca$^{2+}$ release and uptake functions. Evidence has been provided in various studies for a decrease of SR Ca$^{2+}$ uptake in ischemic hearts. \textsuperscript{17-23} Since the net Ca$^{2+}$ uptake in the SR is a result of the activity of Ca$^{2+}$-ATPase and of the SR Ca$^{2+}$-release channel, an abnormal Ca$^{2+}$ uptake may be the result of the dysfunction of either or both structures. The effect of ischemia on the SR Ca$^{2+}$-release channel remains unclear. In one study, the observed decrease of Ca$^{2+}$ uptake in ischemia has been associated with an enhanced Ca$^{2+}$ loss by the Ca$^{2+}$ release process;\textsuperscript{23} in another investigation, the ryanodine- or ruthenium red–induced Ca$^{2+}$ uptake was reduced by ischemia or reperfusion.\textsuperscript{24}

The effect of reperfusion on SR function has been less studied. If myocardial stunning is a posts ischemic process caused by SR dysfunction, then one would expect an aggravation of any ischemic SR damage during reperfusion. A number of studies indicate that during reperfusion there is an aggravation of ischemia-induced SR dysfunction\textsuperscript{22,25} (but see Reference 26). However, these last studies used ischemic times (>30 minutes) that may be so long as to result in irreversible damage. The results from studies using short ischemic periods indicate that SR function recovers to normal level during reperfusion.\textsuperscript{23,27,28} The present study was carried out 1) to investigate the changes in Ca$^{2+}$ uptake under conditions in which no irreversible damage occurs, as assessed by contractile measurements and analysis of nucleotide metabolism in the same hearts and 2) to assess the contribution of the Ca$^{2+}$–release process to any observed change of net Ca$^{2+}$ uptake during ischemia.

Materials and Methods

Contraction Studies

The preparation used was the isovolumetric, Langendorff-perfused rabbit heart. New Zealand White rabbits weighing 2–3 kg were premedicated with fluanisone (2 mg/kg) and fentanyl (0.04 mg/kg) injected intramuscularly and were anesthetized with sodium pentobarbital (25 mg/kg i.v.). Tracheotomy was performed under local anesthesia (with 1% s.c. lidocaine) to allow tracheal intubation. Mechanical ventilation was started after curarizing the animal with pancuronium (0.1 mg/kg i.v.). The blood was heparinized (250 units/kg i.v.), and via a left thoracotomy, the heart was excised and placed in cold (4°C) Krebs-Henseleit solution. After cannulation of the aorta, the heart was immediately flushed with a similar solution and then suspended within the perfusion system. The time elapsed between the excision of the heart and the beginning of perfusion was usually approximately 3 minutes.

A modified Langendorff isolated heart system was used in the study. The perfusate was pumped from a reservoir through a blood transfusion filter (pore diameter, 20 μm; Cobe Laboratories, Colorado) to the perfusion column. The heart was perfused via the cannulated aorta with a constant perfusion pressure of 60 mm Hg. To obtain isovolumetric contractions, a latex balloon filled with fluid was inserted in the left ventricular cavity via an opening in the left atrium. A catheter-tipped manometer (type MTC-HD, Dräger Medical Electronics, Best, The Netherlands) placed in the balloon served to measure the left ventricular pressure (LVP). LVP and its electronically obtained first derivative (left ventricular dP/dt [LV dP/dt]) were continuously recorded. A temperature probe (Ellab Instruments, Copenhagen) placed in the right ventricle in contact with the interventricular septum was used for continuous monitoring of myocardial temperature. The hearts were maintained at 37°C and were not paced during the perfusion. They were allowed to recover for 60 minutes before the control measurements. The Krebs-Henseleit buffer used for perfusing the heart had the following composition (mM): Na$^+$ 135, K$^+$ 5.6, Cl$^-$(pH 7.0), 0.5 mM KCl, and 5 mM sodium azide (Na$_3$N) using a Polytron homogenizer (Kinetica, Switzerland) with a PTA 10S probe at setting 6. A high concentration of KCl was used during homogenization to provide homogenates with stable and improved Ca$^{2+}$ uptake activity.\textsuperscript{29} The homogenate was filtered through two layers of cheesecloth and immediately assayed for Ca$^{2+}$ uptake. Ca$^{2+}$ uptake assays were completed within 50 minutes after homogenization. Within this time, there was only a slight decrease in uptake (average, 4–8%; n = 3) both in the absence and presence of ryanodine.

Calcium uptake by SR vesicles was measured at 37°C with a filtration technique essentially similar to the one described by Solaro and Briggs.\textsuperscript{30} The final reaction medium contained 100 mM KCl, 20 mM imidazole (pH 7.0), 10 mM potassium oxalate, 5 mM MgCl$_2$, 10 mM Na$_2$ATP, 5 mM MgCl$_2$, 0.5 mM EGTA, various amounts of NaCl (0.5 μCi/mg 45CaCl$_2$) required to produce the desired free-Ca$^{2+}$ concentration, 430 μM ryanodine, when added, and 0.6–0.7 mg/ml protein. To obtain maximal stimulation of Ca$^{2+}$ uptake by ryanodine,\textsuperscript{31} the homogenate was preincubated for 8 minutes at 37°C in a medium containing KCl, imidazole, and Na$_2$ATP, with or without 610 μM ryanodine. The Ca$^{2+}$ uptake reaction was started by the addition of ATP, MgCl$_2$, 45CaCl$_2$, EGTA, and oxalate from a stock solution preincubated at 37°C. Aliquots of the reaction medium (60–70 μg protein) were taken every minute up to 3–4 minutes of reaction; they were filtered by suction on a manifold through a filter (0.45-μm pore size, Millipore Corp., Bedford, Mass.) and washed with 100 mM KCl, 20 mM imidazole (pH 7.0), 1 mM EGTA, and 5 mM MgCl$_2$. Radioactivity trapped on the filters was determined by liquid scintillation spectroscopy. The velocity of Ca$^{2+}$ transport was determined by linear regression analysis from slopes of lines relating calcium uptake to time. Free Ca$^{2+}$ concentrations were calculated by a computer program (CABUF, generously provided by Dr. G. Droogmans, Department of Physiology, University of Leuven [Belgium]) using dissociation constants de-
scribed by Fabiato.\textsuperscript{32} Parameters of Ca\textsuperscript{2+} transport—maximum velocity ($V_{\text{max}}$), the apparent dissociation constant for Ca\textsuperscript{2+} ($K_{C_a}$), and the Hill coefficient ($n$) — were calculated by nonlinear least-squares fitting of the experimental data to the following equation:

$$v = \frac{V_{\text{max}}}{1 + (K_{C_a}/[\text{Ca}^{2+}])^n}$$

where $v$ is rate of Ca\textsuperscript{2+} uptake, and [Ca\textsuperscript{2+}] is the free calcium concentration.

Protein was determined by a modified Lowry method that included sodium dodecyl sulfate, with bovine serum albumin as standard. There were no significant differences between control, ischemic, and reperfused hearts with respect to protein yield (75.6±1.37, 79.5±3.61, and 72.6±1.82 mg protein/g tissue wet wt, respectively).

Biochemical Analysis

Ventricular transmural tissue specimens were taken at the end of each contraction experiment (after control perfusion, after 15 minutes of ischemia, or at the end of the reperfusion). The tissues were cooled immediately in liquid nitrogen and stored therein (at < -180°C) until the time of analysis. After lyophilization, neutralized perchloric acid extracts of the tissues were prepared and were used for the determination of ATP and its metabolites; high-performance liquid chromatography was used as described previously.\textsuperscript{14}

Experimental Protocol

Hearts were divided into three groups. In the non-ischemic control hearts (group 1), perfusion with Krebs-Henseleit solution was carried out for 90 minutes without ischemia. In group 2, the normal perfusion lasted 75 minutes and was followed by 15 minutes of ischemia. In group 3, normal perfusion was carried out for 65 minutes and was followed by 15 minutes of ischemia plus 10 minutes of reperfusion. Thus, a total perfusion time of 90 minutes was allowed in all groups to ensure that any change observed after ischemia or reperfusion was not the result of a simple run-down of the preparation with time. Global total ischemia was produced by closing the perfusion line with a manual three-way valve. During ischemia, the heart was maintained at 37°C by enclosing it in a water-jacketed air chamber, the top of which was sealed with Parafilm (American National Can, Greenwich, Conn.). Some solution kept in the lower part of the chamber (without immersing the heart) saturated the air with humidity and prevented cooling of the heart by evaporation.

Drugs

Chemicals used for making the perfusate were obtained from Merck-Belgolabo, Overjise, Belgium. Fenanyl was from Janssen Pharmaceutica, Beerse, Belgium; lidocaine was from Astra Pharmaceuticals, Brussels; pentobarbital was from Ceva, Brussels; heparin was from Novo Nordisk, Bagsevaerd, Denmark; and pancuronium was from Organon, Oss, The Netherlands. Ryanodine was obtained from Calbiochem Corp., La Jolla, Calif. 45CaCl\textsubscript{2} was from Amersham International, Buckinghamshire, England. All other chemicals for Ca\textsuperscript{2+} uptake measurements were obtained from Sigma Chemical Co., St. Louis, Mo., or from Merck Sharp & Dohme, West Point, Pa.

Statistical Analysis

All data are expressed as mean±SEM. One-way analysis of variance for repeated measures was used to detect significant effects. Between the individual groups, comparisons were made using paired and unpaired t tests with the Bonferroni correction. A value of $p<0.05$ was considered statistically significant.

Results

Mechanical Changes

Myocardial contractility and coronary flow rate tended to decrease spontaneously with time during normal perfusion. This spontaneous run-down was most pronounced during the early 30–45 minutes of perfusion. There was little additional run-down between 60 and 90 minutes of perfusion. After 90 minutes of perfusion with Krebs-Henseleit solution (without ischemia), LVP and LV dP/dt decreased to 83.0% and 95.1% of their initial values, respectively (Table 1). The decrease of coronary flow was of a similar magnitude (to 84.3% of the initial value, $p<0.05$ by paired t test). This prompted us to select 60 minutes as the stabilization period and to use the same total perfusion time (90 minutes) for all protocols to exclude the time effect from comparisons between different groups of hearts. Table 1 also shows that, at comparable times before ischemia, functional parameters, including heart rate, coronary flow rate, LVP, and LV dP/dt, were similar between the three groups ($p>0.05$ when comparing any two groups for each parameter). Figure 1 illustrates the time course of change in developed and diastolic LVP during ischemia and reperfusion. During 15 minutes of ischemia, developed LVP declined to zero because of a fall of systolic LVP. With this duration of ischemia, diastolic pressure did not change significantly compared with the control level. On reperfusion, mechanical function resumed. After a transient “overshoot” of developed LVP to near control level immediately after the start of reperfusion, there was a progressive decline. After 3–10 minutes of reperfusion, developed LVP was significantly decreased compared with preischemic values in the same hearts and also compared with hearts normally perfused for the same total time (Table 1). The decrease in developed LVP was due to both a fall in systolic pressure and a slight rise in diastolic pressure. Similarly, both maximum positive and negative values of LV dP/dt were decreased. In the same preparations, the heart rate was not significantly different from preischemic values, whereas the coronary flow rate was still increased compared with preischemic levels because of a very slow rate of decay of reactive hyperemia.

Adenine Nucleotide Metabolism

Purine metabolism was investigated in the same preparations used for contraction and Ca\textsuperscript{2+} uptake studies (Table 2). ATP levels declined during ischemia, as expected, but were restored to near control values with 10 minutes of reperfusion. ADP levels were unchanged during ischemia but were decreased during reperfusion, probably as a result of increased rate of ATP resynthesis. The other substances, the concentrations of which changed during ischemia, include AMP, adenosine, inosine, hypoxanthine, and GTP. The concentrations of AMP, the two bases, and hypoxanthine were increased
during ischemia, whereas GTP levels were decreased. After 10 minutes of reperfusion, the levels of all the last substances were normal. These results on adenylate metabolism agree with data published by this laboratory\(^4\) and by others (see Reference 4) showing that stunning occurs even though ATP levels are restored to near control values. The decrease in GTP during ischemia has received little attention in previous studies, and its functional significance remains unknown.

\(Ca^{2+}\) Uptake

Figure 2A presents the rates of \(Ca^{2+}\) uptake, in the absence of ryanodine, in homogenates from control, ischemic, and reperfused hearts as a function of \(Ca^{2+}\) concentration in the incubation medium. \(Ca^{2+}\) uptake was negligible at pCa 7 (i.e., \(Ca^{2+}\) concentration=0.1 \(\mu\)M) and increased progressively with the increase in \(Ca^{2+}\) concentration. The SR from hearts subjected to 15 minutes of ischemia showed a decreased \(Ca^{2+}\) uptake rate, especially at higher \(Ca^{2+}\) concentrations (0.5–5.0 \(\mu\)M). \(Ca^{2+}\) uptake in SR from reperfused hearts had a tendency to be decreased compared with the uptake in control hearts, but changes were not significant at any \(Ca^{2+}\) concentration tested.

Data on kinetic parameters of \(Ca^{2+}\) uptake obtained by nonlinear regression analysis of \(Ca^{2+}\) dependence of \(Ca^{2+}\) uptake rates using the Hill equation are presented in Table 3. Maximum velocity of \(Ca^{2+}\) uptake after ischemia was decreased to 74% of control value. However, \(K_{cs}\) and Hill coefficient \(n\) were different from control values. Thus, the changes during ischemia were due to a decrease of maximal \(Ca^{2+}\) uptake without a change in the ability of \(Ca^{2+}\) to activate this process. In reperfused hearts, \(V_{max}\) for \(Ca^{2+}\) uptake was slightly and not significantly decreased, and values of \(K_{cs}\) and \(n\) were identical with control values.

To test for the contribution of a ryanodine-sensitive fraction to the total SR \(Ca^{2+}\) uptake and to assess the possible role of the \(Ca^{2+}\)-release channel in the effect of ischemia, we measured \(Ca^{2+}\) transport after the incubation of homogenates with 610 \(\mu\)M ryanodine to block the SR \(Ca^{2+}\)-release channel (Figure 2B, Table 3). \(Ca^{2+}\) uptake in homogenates incubated with ryanodine was higher compared with the uptake in the absence of ryanodine. The changes caused by ischemia and reperfusion were qualitatively similar to those seen in untreated homogenates.

\(V_{max}\) of \(Ca^{2+}\) uptake in ischemic hearts was decreased to 71% of the control value, a change practically identical to the one observed in experiments without ryanodine. Values of \(K_{cs}\) and Hill coefficient \(n\) were different from control values. As in experiments without ryanodine, \(V_{max}\) of \(Ca^{2+}\) uptake in reperfused hearts was only slightly decreased, and there was no change in \(K_{cs}\) or \(n\) as compared with control values.

Since the difference between \(Ca^{2+}\) uptakes in the absence and presence of ryanodine probably results

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**TABLE 1. Cardiac Function Before Ischemia and During Reperfusion**

<table>
<thead>
<tr>
<th>Group</th>
<th>Before ischemia*</th>
<th>After ischemia†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (bpm)</td>
<td>CF (ml · min⁻¹)</td>
</tr>
<tr>
<td>Group 1 (n=8)</td>
<td>0 Minutes: 170±7.9</td>
<td>70±7.4</td>
</tr>
<tr>
<td>Group 2 (n=8)</td>
<td>90 Minutes: 185±12.9</td>
<td>59±7.9</td>
</tr>
<tr>
<td>Group 3 (n=7)</td>
<td>150±15.9</td>
<td>55±12.7</td>
</tr>
<tr>
<td>Group 3 (n=7)</td>
<td>143±14.9</td>
<td>58±3.7</td>
</tr>
<tr>
<td></td>
<td>123±15.7</td>
<td>66±3.4</td>
</tr>
</tbody>
</table>

HR, heart rate; bpm, beats per minute; CF, coronary flow rate; sLVP, systolic left ventricular pressure; dLVP, diastolic left ventricular pressure; +LV dP/dt, maximum rate of pressure development; −LV dP/dt, maximum rate of relaxation; group 1, nonischemic control; group 2, 15 minutes of ischemia; group 3, 15 minutes of ischemia plus 10 minutes of reperfusion. Values are mean±SEM.

*In groups 2 and 3, data before ischemia were collected just before closing the perfusion line.
†In group 3, data after ischemia were collected at the end of the 10-minute reperfusion. For group 1 (nonischemic control), there were no data (hearts were not submitted to ischemia); for group 2 (ischemia), there were no data (hearts were isolated at the end of ischemia).

\(\ast p<0.05\) by paired \(t\) test when comparing parameters during reperfusion to those before ischemia in the same hearts.
Table 2. Effect of Ischemia and Reperfusion on ATP and Energy Metabolism

<table>
<thead>
<tr>
<th>Tissue contents (μmol/g dry wt)</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>ADO</th>
<th>INO</th>
<th>HYP</th>
<th>GTP</th>
<th>NAD</th>
<th>T+S+B</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (n=8)</td>
<td>13.6±0.98</td>
<td>4.6±0.40</td>
<td>1.5±0.41</td>
<td>0.1±0.06</td>
<td>0.2±0.04</td>
<td>0±0.0</td>
<td>0.7±0.05</td>
<td>1.6±0.08</td>
<td>17.9±2.36</td>
<td>0.8±0.031</td>
</tr>
<tr>
<td>Group 2 (n=8)</td>
<td>4.5±1.23*</td>
<td>4.5±0.48</td>
<td>4.7±1.00*</td>
<td>1.3±0.42*</td>
<td>3.0±0.50*</td>
<td>0.5±0.06*</td>
<td>0.3±0.04*</td>
<td>1.2±0.13</td>
<td>18.8±1.34</td>
<td>0.47±0.069*</td>
</tr>
<tr>
<td>Group 3 (n=7)</td>
<td>11.8±0.68</td>
<td>2.7±0.17*</td>
<td>0.7±0.11*</td>
<td>0.1±0.01</td>
<td>0.1±0.01</td>
<td>0.0±0.01</td>
<td>0.6±0.03</td>
<td>1.9±0.12</td>
<td>15.4±0.72</td>
<td>0.87±0.013</td>
</tr>
</tbody>
</table>

ADO, adenosine; INO, inosine; HYP, hypoxanthine; T, nucleotides; S, nucleosides; B, purine bases; E, energy charge=(ATP+ADP)/(ATP+ADP+AMP); group 1, nonischemic control; group 2, 15 minutes of ischemia; group 3, 15 minutes of ischemia plus 10 minutes of reperfusion. Values are mean±SEM and were determined by high-performance liquid chromatography.

*p<0.05 vs. control values.

from the loss of Ca\textsuperscript{2+} when SR Ca\textsuperscript{2+}-release channels are not blocked, it is possible to use this difference to assess the effect of ischemia and reperfusion on the SR Ca\textsuperscript{2+}-release process. Stimulation of Ca\textsuperscript{2+} uptake by ryanodine depended on free Ca\textsuperscript{2+} concentration (Figure 3). There were no statistically significant changes in the ryanodine-sensitive Ca\textsuperscript{2+} uptake in ischemic and reperfused hearts when compared with control hearts at any Ca\textsuperscript{2+} concentration. The relative increase in $V_{\text{max}}$ of Ca\textsuperscript{2+} uptake by ryanodine was 57±13.8%, 50±13.6%, and 54±6.9% for control, ischemia, and reperfusion, respectively.

Discussion

The purpose of this study was to test the hypothesis that damage to the SR occurs during ischemia and reperfusion and mediates postischemic contractile dysfunction. The 15-minute ischemic period used in the present study has been shown to result in significant postischemic depression of cardiac function during reperfusion of hearts perfused according to the working model.\textsuperscript{14} The present results in isovolumetric hearts confirm this finding, since contractile parameters at 10 minutes of reperfusion remained significantly low compared with preischemic or control levels. No morphological studies were carried out in the present study to ascertain the lack of irreversible cellular damage. However, after 10 minutes of reperfusion, the content of ATP, which had decreased markedly during ischemia, was restored to values similar to control levels. This is consistent with previous studies showing recovery of metabolism on reperfusion (for review, see Reference 4) and suggests that under our experimental conditions the cardiac tissue did not undergo irreversible damage.

The persistence of contractile dysfunction in the presence of quasi-normal ATP levels and of a normal mitochondrial function suggests that the energy supply is not a limiting factor in stunning.\textsuperscript{12} As stated in the introduction, the existence of an abnormal Ca\textsuperscript{2+} homeostasis in stunned myocardium has led to the suggestion that the SR function may be deficient during reperfusion. In the present study, we compared the Ca\textsuperscript{2+} uptake capability of the unfractonated homogenates obtained from normal hearts from preparations subjected to 15 minutes of ischemia and from hearts reperfused for 10 minutes after the 15-minute ischemic period. For the characterization of SR function, we have used the measurement of Ca\textsuperscript{2+} uptake in heart homogenates at conditions that restrict Ca\textsuperscript{2+} transport to SR vesicles.\textsuperscript{30} The validity of this method has been confirmed in several studies.\textsuperscript{21,23,28,34} Moreover, the use of homogenates may have some advantage over that of isolated SR vesicles, since Rapundalo et al\textsuperscript{21} have shown that SR isolated from ischemic myocardium is not representative of all SR because of a loss of greater portions of undamaged SR during subcellular fractionation after ischemia. The results show a decrease of Ca\textsuperscript{2+} uptake during ischemia. This decrease was observed in the homogenates incubated in the absence or presence of ryanodine (see Figure 2). The ryanodine concentration used should be sufficient to block all SR Ca\textsuperscript{2+}-release channels, and this block probably accounts

![Figure 2](http://circres.ahajournals.org/)

**FIGURE 2.** Graphs showing calcium dependence of oxalate-supported calcium uptake by homogenates from control (open circles), ischemic (filled circles), and reperfused (filled squares) hearts. Panel A: Homogenates incubated in the absence of ryanodine. Panel B: Homogenates incubated in the presence of 610 μM ryanodine. Values are mean±SEM (n=5 in each group). *p<0.05 vs. control hearts.
for the increased Ca\(^{2+}\) uptake in ryanodine-treated homogenates.\(^{31}\) The results suggest an effect of ischemia on the Ca\(^{2+}\) uptake process (i.e., on Ca\(^{2+}\)-ATPase) without an effect on the release process.

Studies carried out to test the effect of ischemia on SR Ca\(^{2+}\) uptake have used different species and models of ischemia, as well as various ischemic durations. Despite these differences in experimental conditions, all studies have shown a decrease of Ca\(^{2+}\) uptake during ischemia.\(^{18,19,21-24,27,28}\) The decrease becomes evident even for ischemic durations as short as 7–10 minutes.\(^{18,21,23,24,28}\) However, most studies have been carried out at a fixed Ca\(^{2+}\) concentration in the incubation medium, usually at a concentration high enough to achieve saturation of the Ca\(^{2+}\) uptake mechanism. At such high concentrations, an inhibition of Ca\(^{2+}\)-ATPase may start to develop. A kinetic analysis of Ca\(^{2+}\)-ATPase activity by Krause and Hess\(^{18}\) suggests a decrease, during ischemia, of the maximal enzymatic activity with no change in Ca\(^{2+}\) sensitivity. Although detailed kinetic analysis was not done in other studies that have used wide ranges of Ca\(^{2+}\) concentrations,\(^{24}\) they have reached a similar conclusion. In the present study, we analyzed the Ca\(^{2+}\) uptake data by fitting them with the Hill equation. Such an analysis indicated that the decrease in \(V_{\text{max}}\) of Ca\(^{2+}\) uptake during ischemia was obtained with unchanged \(K_{\text{Ca}}\). Hill coefficient \(n\) of Ca\(^{2+}\) uptake, reflecting the number of Ca\(^{2+}\) ions that bind on the Ca\(^{2+}\)-ATPase molecule, was close to 2 and was not modified by ischemia or reperfusion. Hill coefficient \(n\) close to 2 was obtained by Krause and Hess\(^{18}\) for Ca\(^{2+}\)-ATPase activity and was little or not modified by ischemia. Thus, the results suggest a decrease, by ischemia, of the number of active Ca\(^{2+}\)-ATPases without a change in their properties. The observations that these changes persisted after the removal of the heart and its processing for Ca\(^{2+}\) uptake measurements and that the changes with ischemia were obtained despite incubation conditions similar to those of control indicate that the observed decrease of Ca\(^{2+}\) uptake is not simply due to an eventual altered modulation of Ca\(^{2+}\) uptake by cytoplasmic changes. On the other hand, it is unlikely that the decrease in Ca\(^{2+}\) uptake by ischemic homogenates is the result of an irreversible denaturation of Ca\(^{2+}\)-ATPase molecules, since 10 minutes of reperfusion was sufficient to restore Ca\(^{2+}\) uptake to the control level. Such a recovery would be faster than expected if protein resynthesis were needed for restoration of SR function.

Only few other studies have assessed the role of the Ca\(^{2+}\)-release channel during ischemia.\(^{23,24,27,28}\) Blockade of the SR Ca\(^{2+}\)-release channel has been achieved by the use of ryanodine or ruthenium red in the Ca\(^{2+}\) uptake incubation medium, and it results in an increase in the rate of Ca\(^{2+}\) accumulation in the SR. Feher et al.\(^{23}\), using rat homogenates, reported that the increase in Ca\(^{2+}\) uptake on blockade of the Ca\(^{2+}\)-release channel was more pronounced in ischemic compared with control myocardium. A recent study from the same research group has confirmed this finding.\(^{28}\) This result suggests the presence of a higher background activity of the release channel in ischemia. On the contrary, Limbruno et al.\(^{24}\) also using rat preparations, obtained lower ryanodine- or ruthenium red-induced increases in SR Ca\(^{2+}\) uptake in ischemic or reperfused myocardium. This last result is consistent with a decreased Ca\(^{2+}\) loss through the SR Ca\(^{2+}\)-release channel during ischemia or reperfusion. A decreased SR Ca\(^{2+}\)-release channel activity is obtained in the presence of reactive free radicals and has been proposed to occur in stunned myocardium.\(^{35}\) The results from the present study do not confirm either of the above findings and are similar to those recently obtained by Rehr et al.\(^{27}\) who also observed no change in the absolute magnitude of the ruthenium red-induced increase in Ca\(^{2+}\) uptake in dogs. Although it is possible that part of the difference obtained in the various studies is due to the use of

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**Table 3. Kinetic Parameters of Ca\(^{2+}\) Uptake**

<table>
<thead>
<tr>
<th>Group</th>
<th>(V_{\text{max}}) (nmol · min(^{-1}) · mg protein(^{-1}))</th>
<th>(K_{\text{Ca}}) (μM)</th>
<th>(n)</th>
<th>(V_{\text{max}}) (nmol · min(^{-1}) · mg protein(^{-1}))</th>
<th>(K_{\text{Ca}}) (μM)</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>49.3±2.54</td>
<td>0.56±0.011</td>
<td>2.2±0.18</td>
<td>76.3±5.08</td>
<td>0.68±0.069</td>
<td>2.0±0.11</td>
</tr>
<tr>
<td>Group 2</td>
<td>36.3±1.94*</td>
<td>0.53±0.023</td>
<td>2.2±0.22</td>
<td>54.0±5.08*</td>
<td>0.58±0.073</td>
<td>2.1±0.35</td>
</tr>
<tr>
<td>Group 3</td>
<td>45.3±0.79</td>
<td>0.58±0.027</td>
<td>2.2±0.23</td>
<td>67.9±2.82</td>
<td>0.74±0.059</td>
<td>1.9±0.29</td>
</tr>
</tbody>
</table>

\(V_{\text{max}}\), maximum velocity of Ca\(^{2+}\) uptake; \(K_{\text{Ca}}\), apparent dissociation constant for Ca\(^{2+}\); \(n\), Hill coefficient; group 1, nonischemic control; group 2, 15 minutes of ischemia; group 3, 15 minutes of ischemia plus 10 minutes of reperfusion. Values are given as mean±SEM (\(n=5\) in each group).

\(^{*}\)p<0.05 vs. group 1.

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![Graph showing calcium dependence of ryanodine-induced increase in calcium uptake by homogenates from control (open circles), ischemic (filled circles), and reperfused (filled squares) hearts. The ryanodine-induced increase was obtained as the difference between calcium uptake in the presence (Figure 1B) and absence (Figure 1A) of 610 μM ryanodine. Values are mean±SEM (\(n=5\) in each group).](image_url)
different animal species, it remains unclear why the published studies in the rat\textsuperscript{23,24,28} have obtained opposite results.

Ca\textsuperscript{2+} uptake in SR from reperfused myocardium has also been studied after ischemia of various durations, but nearly all studies of short ischemia agree in showing a recovery, partial or complete, of Ca\textsuperscript{2+} uptake during reperfusion.\textsuperscript{23,27,28} Thus, changes in Ca\textsuperscript{2+} uptake are in the opposite direction to what is expected for reperfusion-induced injury, where more extensive damage should be obtained in reperfused myocardium. A reduced Ca\textsuperscript{2+} uptake in reperfused rabbit myocardium was obtained in other studies,\textsuperscript{36,37} but data from ischemic hearts were not given. In the present study, near complete recovery of Ca\textsuperscript{2+} uptake function was obtained despite a persisting decrease in contractile function. This implies that a simple decrease in Ca\textsuperscript{2+} uptake by SR with a subsequent decrease in Ca\textsuperscript{2+} release cannot be invoked as mechanism underlying myocardial stunning. However, this does not exclude other changes in the SR during stunning, because the methods we used did not allow us to assess other determinants of SR function, such as the Ca\textsuperscript{2+}-binding proteins and the transport of Ca\textsuperscript{2+} from the uptake to the release compartment.

Recent studies have measured intracellular Ca\textsuperscript{2+} transients using luminescence, fluorescence, or nuclear magnetic resonance techniques and have suggested an increase, instead of a decrease, of Ca\textsuperscript{2+} transients in ischemic\textsuperscript{10,38,39} and reperfused\textsuperscript{11} stunned myocardium. Decreased contraction in face of increased Ca\textsuperscript{2+} release was taken as an indication of decreased Ca\textsuperscript{2+} sensitivity and/or responsiveness of contractile myofilaments in stunned myocardium.\textsuperscript{11,40} Results from Ca\textsuperscript{2+} uptake studies do not allow us to explain such an increase in intracellular Ca\textsuperscript{2+} transients, since there is no increase in Ca\textsuperscript{2+} uptake during reperfusion. One has then to suppose an increase in sarcoplasmic Ca\textsuperscript{2+} influx or an increase in the fraction of SR Ca\textsuperscript{2+} load that is released by each contraction. On the other hand, as emphasized by Davis et al.,\textsuperscript{28} changes of mechanical function are evaluated under in situ conditions, whereas Ca\textsuperscript{2+} uptake is measured in the SR system in vitro. The SR function in vivo is modulated by physiological factors, including the cAMP-dependent phosphorylation state of phospholamban and the activity of calmodulin. The properties of these proteins could be modified by ischemia and reperfusion. The use of standard conditions for comparing control, ischemic, and reperfused myocardium may mask this altered modulation.

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