Myocardial contracture and accumulation of mitochondrial calcium in ischemic rabbit heart

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HENRY, PHILIP D., RAFAEL SHUCHLEIB, JAMES DAVIS, EDWARD S. WEISS, AND BURTON E. SOBEL. Myocardial contracture and accumulation of mitochondrial calcium in ischemic rabbit heart. Am. J. Physiol. 233(6): H677-H684, 1977 or Am. J. Physiol.: Heart Circ. Physiol. 2(6): H677-H684, 1977.—The relationship between myocardial contracture and cell calcium was studied in electrically paced, isolated perfused rabbit hearts. Isovolumic left ventricular dP/dt and end-diastolic pressure were utilized as indexes of contractility and ventricular stiffness. After 60 min of low flow (ischemia) without or with reperfusion at high flow for 10 min, calcium was measured in the mitochondrial fraction and used as an indicator of intracellular calcium. Low flow led to ventricular standstill and contracture, and reperfusion produced partial mechanical recovery with end-diastolic pressure remaining markedly elevated. Nifedipine (10⁻⁷ M), an antagonist of myocardial calcium uptake, prevented contracture and permitted nearly complete mechanical recovery without elevation in diastolic pressure. Increases in mitochondrial calcium paralleled the severity of contracture and the lack of diastolic relaxation after reperfusion. Mitochondrial calcium did not increase in hearts protected by nifedipine. Results demonstrate a close relationship between mechanical changes induced by ischemia and accumulation of intracellular calcium.

isolated perfused heart; mitochondrial calcium uptake; diastolic relaxation; ruthenium red; ventricular compliance; protection of ischemic myocardium; nifedipine

FACTORS MODULATING the energy metabolism of cardiac muscle appear to play an important role in determining the vulnerability of the myocardium to ischemia (15). Calcium may be one with particular importance since the intracellular concentration of free calcium controls major steps in the hydrolysis and synthesis of ATP. Thus, calcium activates muscular contraction (7), influences glycolytic flux (16) and oxidative phosphorylation in mitochondria (3, 14), and mediates or modulates adrenergic effects (16, 20). Myocardial injury induced by temporary coronary occlusion (25) or injection of catecholamines (6) has been found to be associated with accumulation of intracellular calcium, but the relationship between mechanical performance and intracellular accumulation of calcium in acute myocardial ischemia has not yet been investigated in detail. The present investigation was designed to examine the relationship between alterations in cardiac mechanics and accumulation of intracellular calcium in ischemic rabbit heart subjected to perfusions at low flow.

MATERIALS AND METHODS

Chemicals. Glutamic acid, malic acid, Trizma base, bovine serum albumin, adenine sulfate, and ruthenium red (lot R2751; purity ca. 20%; mol wt 786.5) were obtained from Sigma Chemical Company, St. Louis. Sucrose and mannitol were purchased from Schwarz/Mann, Orangeburg, N. J. Radioactive CaCl₂ (sp act, 20–30 Ci/g calcium), Protosol, and Aquasol were obtained from New England Nuclear Corp., Boston. Ampules of nifedipine (4-(2’-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine) (200 μg or of its vehicle (15% ethanol and 15% polyethylene glycol in water) were a gift from the Bayer Company (Delbay Division, Bloomfield, N. J.). Fluorescamine (Fluram) was purchased from Roche Laboratories, Nutley, N. J.

Isolated perfused heart preparations. Albino rabbits weighing between 2.0 and 2.5 kg were utilized. Hearts were isolated and perfused retrograde at 37°C through the aorta as previously described (10). The perfusate contained (mM) NaCl, 118; KCl, 2.8; KH₂PO₄, 1.2; CaCl₂, 2.5; MgSO₄, 1.2; NaHCO₃, 25; and glucose, 5. After equilibration at 37°C with a 5% CO₂-95% O₂ gas mixture, the pH of the perfusate was 7.38. The rate of perfusion was maintained at 22 ml/min with a roller pump (Ismatec model MP-4). A latex balloon mounted around a micromanometer (Konigsberg model P3.5) was inserted into the left ventricular cavity for measurement of left ventricular pressure and dP/dt, recorded with Brush amplifiers (models 02413 and 11 4307 00) and a Brush recorder (model 220). To monitor pulsatile pressure during recording at low paper speed (1 mm/min), phasic signals were continuously displayed on a Tektronix 7313 oscilloscope and intermittently recorded with a second Brush 220 recorder at a paper speed of 25 mm/s. The balloon was filled slowly with deaerated saline with the use of a micrometer syringe until end-diastolic pressure stabilized between 5 and 8 mmHg. A bipolar electrode was attached to the right ventricular free wall and the heart was paced at a frequency of 180/min with rectangular impulses (1 ms; voltage, 10% above threshold) provided by a Grass stimulator (model S4). Thus, in this preparation, major
determinants of cardiac performance including ventricular preload and afterload, cardiac frequency, coronary flow, and temperature were controlled.

All hearts were equilibrated for 30 min at a flow rate of 22 ml/min. Then, slow perfusion (0.2 ml/min; ca. 5 ml/100 g fresh myocardium·min⁻¹) with perfusate containing nifedipine or its vehicle was started and maintained for 60 min. This drug has been reported to inhibit the slow inward calcium current into myocardial cells ((6) and R. Kaufmann, personal communication) and was utilized to inhibit intracellular accumulation of calcium induced by ischemia. Nifedipine or its vehicle were infused with a syringe pump (Harvard model 975) into the perfusion system at a rate not exceeding 1% of the total flow. The concentration of the drug reaching the heart was calculated on the basis of its concentration in the syringe and the flow rates of the two pumps. To protect the light-sensitive nifedipine, the whole infusion system was covered with opaque materials. In some experiments, high flow (22 ml/min) without nifedipine was reinstituted ("reperfusion") at the end of the interval of slow flow. In other experiments, hearts at the end of perfusion were used to determine the ventricular wet weight-to-dry weight ratio. The large vessels and atria were removed; the ventricles were opened, blotted, weighed, and subsequently taken to a constant dry weight in an oven at 80°C.

Biomedical procedures. The calcium content of the mitochondrial fraction was utilized as an index of cell calcium, using copper as a mitochondrial marker. This approach is based on the findings that most of the calcium accumulating in ischemic myocardium is recovered in the mitochondrial fraction (26) and that copper in blood-free myocardium is an index of mitochondria and mitochondrial cristae (19). It should be emphasized that the purpose of preparing mitochondria was to obtain an index of cell calcium and the experiments were not designed to study mitochondrial metabolism in acute myocardial ischemia. The rationale underlying this approach will be discussed in more detail in the discussion.

At the end of the perfusions (or, in control experiments, immediately after quick removal of the heart from the animal’s chest), hearts were immersed in ice-cold isolation medium containing (mM) mannitol, 210; sucrose, 70; and Tris-HCl (pH 7.4), 10; and simultaneously perfused retrograde with 200 ml of the same medium. If not otherwise specified, ruthenium red, 500 µM, was added to all preparative solutions to inhibit calcium uptake by mitochondria during cell fractionation (28). In some experiments, nifedipine, 10⁻⁴ M, was included also. All preparative steps were performed at 0°C. Ventricular myocardium, 3–4 g, was minced in the isolation medium and homogenized with a motor-driven, tight-fitting glass-Teflon homogenizer with five strokes. The whole homogenate was centrifuged at 1,000 × g for 3 min, and the supernatant fraction was recovered and centrifuged for 6 min at 12,000 × g. The pellet was washed twice, suspended in 10 ml of isolation medium per gram of original mince, centrifuged for 1 min at 28,000 × g (24), resuspended in 10 ml/g, and centrifuged again at the same speed. The final pellet was resuspended in 2–3 ml of buffer. The entire preparative procedure was completed within ca. 30 min. Aliquots of the whole homogenates and of the resuspended pellets were assayed for protein, calcium, and copper as described below. In the present study, the terms mitochondria and mitochondrial fraction refer to resuspended pellets containing between 1.29 and 1.41 ng atom copper per milligram protein (Table 1). Similar values have been reported for mitochondria prepared from rat (19) and beef hearts (8).

The possibility that the mitochondrial fraction was losing or accumulating calcium during its preparation, thereby yielding spurious results, was considered. Accordingly, calcium uptake and discharge by the mitochondrial fraction at 0°C in the presence of ruthenium red, conditions mimicking those produced by the preparative procedure, were studied. For this purpose, three to four hearts were pooled and mitochondria were prepared without addition of ruthenium red. For uptake experiments, mitochondria were incubated at 0°C in a mechanically stirred mixture containing (mM) mannitol, 210; sucrose, 70; Tris-HCl (pH 7.4), 10; and mitochondrial protein, 3.5 mg/ml; in a final volume of 10 ml. The reaction was started by adding 400 nmoles of CaCl₂ per milligram protein (labeled with ⁴⁵Ca⁵⁺; final activity ca. 500,000 cpm/ml), producing an initial concentration of free calcium of 1.4 mM. A concentration of calcium in the millimolar range was chosen to mimic the concentration of calcium in the perfusate to which mitochondria were potentially exposed during cell fractionation. Other additions to the incubation medium are described in the legends to Figs. 2–6. Immediately before, and at timed intervals after addition of CaCl₂, 1-ml samples were removed and centrifuged promptly for 1 min at 28,000 × g. The pellet was washed twice, resuspended in 10 volumes of calcium-free incubation medium, and centrifuged again. The final pellet was assayed for protein, calcium, and copper as described below.

For assay of release of calcium from mitochondria, the organelles were preloaded with calcium by incubating them at 25°C for 30 min in a mixture containing (mM) mannitol, 210; sucrose, 70; Tris-HCl (pH 7.4), 10; Tris-glutamate, 5; Tris-malate, 5; Tris-succinate, 5; KH₂PO₄, 5; mitochondria; 3.5 mg/ml; and CaCl₂, 300 nmol/mg protein (labeled with ⁴⁵CaCl₂, final activity 500,000 cpm/ml). Loaded mitochondria were centrifuged for 1 min at 28,000 × g. The pellet was washed with incubation medium, then suspended in 10 volumes of the same medium and centrifuged again at the same speed. Mitochondria were resuspended in isolation medium and calcium release assayed promptly at 0°C in a mixture containing (mM) mannitol, 210; sucrose, 70; Tris-HCl (pH 7.4), 10; and mitochondrial protein, 3.5 mg/ml. Timed aliquots were collected and treated as in assays of calcium uptake.

Calcium and copper in whole homogenates and mitochondrial fractions were measured by atomic-absorption spectrophotometry in a Perkin-Elmer apparatus (model 303). Aliquots were treated by a wet ashing procedure (22) and read in the presence of lanthanum (1.2 mM) and EDTA (5.1 mM) at 422.7 and 324.7 nm, respectively.
For assay of radioactive $^{45}$Ca, aliquots were solubilized in Protosol and counted in Aquasol in a liquid scintillation spectrometer. Protein was measured fluorometrically with fluorescamine. Bovine serum albumin treated identically was used as the standard (27).

**Statistical analysis.** The significance of the difference between group means was evaluated by the student t-test for unpaired samples. The coefficients of correlation and regression equations were calculated by least-squares analyses.

**RESULTS**

**Effects of slow flow on cardiac mechanics.** A representative record of an isovolumic left heart preparation subjected to 60 min of low flow (0.2 ml/min) is illustrated in Fig. 1, upper panel. Throughout the experiment, the heart was paced at a frequency of 180/min. Peak systolic pressure, developed pressure (peak systolic minus end-diastolic pressure), maximum dP/dt, and minimum dP/dt ("peak negative dP/dt") declined precipitously. The simultaneously monitored phasic contractile activity revealed cardiac slowing (mean rate, ~180 min) after 6.96 min and complete ventricular standstill after 17.39 min. Reperfusion (22 ml/min) after 60 min of low flow produced partial recovery of cardiac mechanical activity and an irregular rhythm persisting for 20.83 min. Subsequently, during effective pacing, peak pressure and peak dP/dt stabilized at 78% and 38% of preischemic control, while end-diastolic pressure remained markedly elevated (40 mmHg, an eightfold increase compared to the preischemic control value). Figure 1, lower panel, shows the response of a heart subjected to exactly the same protocol, except that nifedipine, $10^{-7}$ M, was added to the perfusate during (but not before or after) low flow. The early decline in contractility, cardiac slowing after 7.31 min, and standstill after 16.53 min were similar to those observed in the control heart. However, no contracture developed after standstill. With reperfusion, there was a progressive recovery in mechanical activity, and a regular, paced rhythm appeared after 4.05 min. After 18 min of reperfusion, peak pressure and peak dP/dt were 87% and 85% of control, and end-diastolic pressure was increased by only approximately 2 mmHg. Thus, there was no change in myocardial stiffness as reflected by the contracture pressure during ischemic standstill or the end-diastolic pressure during reperfusion. Results of experiments performed as illustrated in Fig. 1 are summarized in Figs. 2 and 3. At the end of the low-flow period, nonpulsatile ventricular pressure differed significantly between control and treated hearts, averaging 56 ± 5 mmHg (mean ± SE; n = 15) and 7 ± 1 mmHg (n = 14; P < 0.001), respectively. After 20 min of reperfusion, end-diastolic and systolic developed pressures averaged 43 ± 5 mmHg and 29 ± 4 mmHg in control hearts (n = 15), and 7 ± 1 mmHg and 72 ± 5 mmHg in treated hearts (n = 15). These differences between the two

**FIG. 1.** Inhibition of ischemic contracture by nifedipine. Perfusion experiments with paced, isovolumic left-heart preparations were performed as described under METHODS. Standstill refers to no visible ventricular activity and no pulsatile pressure on tracings recorded with a 2nd recorder at a paper speed of 25 mm/s and a full scale of 100 mmHg. Low flow refers to a rate of perfusion of 0.2 ml/min. Fast and slow (black areas) segments of tracings were recorded at a paper speed of 25 mm/s and 1 mm/min, respectively.

**FIG. 2.** Ischemic contracture in isovolumic left heart preparation. Perfusions (n = 8) were performed as described in METHODS and illustrated in Fig. 1, upper panel. High and low flow refer to a flow of 22 and 0.2 ml/min, respectively. Buffer during low flow contained < 0.1% of vehicle of nifedipine (final ethanol and polyethylene glycol concentrations both less than 0.015%). Data points in this and in subsequent figures indicate means ± SE.
groups were highly significant \( (P < 0.001) \). Table 1 shows that changes in cardiac rhythm during the period of low flow were similar in control and treated hearts, cardiac slowing and ventricular standstill occurring after similar periods of low flow. On the other hand, cardiac rhythm during reperfusion differed between the two groups. Compared to the treated hearts, reappearance of a regular, paced rhythm was considerably delayed in the control hearts (Table 1).

Hearts perfused with balanced salt solutions containing no macromolecules are known to become edematous (2). To determine whether differences in myocardial stiffness were related to different accumulations of water in the tissue, the dry weight-to-wet weight ratios in fresh hearts and perfused hearts were determined. In fresh hearts not used for perfusion experiments, the dry weight-to-wet weight ratio averaged 0.213 \( \pm \) 0.001 (mean \( \pm \) SE, \( n = 7 \)). In hearts perfused for 60 min at high flow (22 ml/min) without nifedipine, the value was 0.178 \( \pm \) 0.003 (\( n = 5 \); \( P < 0.001 \)). In hearts perfused for 60 min at low flow (0.2 ml/min) without or with nifedipine, the ratios averaged 0.180 \( \pm \) 0.003 (\( n = 5 \)) and 0.179 \( \pm \) 0.002 (\( n = 6 \)), values that did not differ significantly from those of hearts perfused at high flow.

In the hearts perfused at low flow, nonpulsatile pressure at the end of perfusion was 43 \( \pm \) 4 mmHg in the untreated hearts, and 6 \( \pm \) 1 mmHg in the nifedipine-treated hearts. These pressures are almost identical to those illustrated in Figs. 2 and 3. Thus, the differences in myocardial stiffness observed in the present experiments did not appear to be related to differential accumulation of tissue water.

### Table 1. Cardiac rhythm during ischemia and reperfusion

<table>
<thead>
<tr>
<th>Control (( n = 15 ))</th>
<th>Nifedipine Treated (( n = 14 ))</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time from onset of slow flow to cardiac slowing, min*</td>
<td>5.81 ( \pm ) 0.52</td>
<td>6.11 ( \pm ) 0.52</td>
</tr>
<tr>
<td>Time from onset of slow flow to complete ventricular standstill, min†</td>
<td>16.32 ( \pm ) 1.81</td>
<td>17.46 ( \pm ) 2.32</td>
</tr>
<tr>
<td>Time from onset of reperfusion to onset of regular pacing, min‡</td>
<td>19.38 ( \pm ) 2.01</td>
<td>4.86 ( \pm ) 0.81</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE. Numbers in parentheses indicate number of hearts studied. *Values are based on t-tests for unpaired samples. Measurements are based on phasic recordings obtained during continuous oscilloscopic monitoring of pulsatile pressure. †Cardiac slowing defined as escape from ventricular pacing with frequency dropping below 150/min. ‡Complete ventricular standstill refers to no visible ventricular activity and complete absence of pulsatile pressure changes at a full scale of 100 mmHg. †‡Onset of regular pacing is defined as the appearance of a sequence of 20 or more paced ventricular systoles.

### Table 2. Calcium and copper in mitochondrial fraction

<table>
<thead>
<tr>
<th></th>
<th>Calcium, ng-atom/mg protein</th>
<th>Copper, ng-atom/mg protein</th>
<th>Calcium/Copper</th>
<th>Copper in Mitochondrial Fraction, %</th>
<th>Protein in Mitochondrial Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Perfusion*</td>
<td>-N(8) 6.2 ( \pm ) 3</td>
<td>1.37 ( \pm ) 0.0</td>
<td>4.53 ( \pm ) 2.3</td>
<td>10.9 ( \pm ) 4</td>
<td>4.01 ( \pm ) 2.2</td>
</tr>
<tr>
<td></td>
<td>+N(8) 6.3 ( \pm ) 3</td>
<td>1.36 ( \pm ) 0.0</td>
<td>4.85 ( \pm ) 2.4</td>
<td>11.4 ( \pm ) 5</td>
<td>4.12 ( \pm ) 2.5</td>
</tr>
<tr>
<td></td>
<td>-R(7) 6.8 ( \pm ) 4</td>
<td>1.34 ( \pm ) 0.0</td>
<td>4.83 ( \pm ) 9.9</td>
<td>10.7 ( \pm ) 4</td>
<td>4.10 ( \pm ) 3.8</td>
</tr>
<tr>
<td>60 min high flow†</td>
<td>-N(7) 7.6 ( \pm ) 4</td>
<td>1.30 ( \pm ) 0.0</td>
<td>5.81 ( \pm ) 4.1</td>
<td>11.9 ( \pm ) 5</td>
<td>4.21 ( \pm ) 2.5</td>
</tr>
<tr>
<td></td>
<td>+N(8) 7.4 ( \pm ) 4</td>
<td>1.34 ( \pm ) 0.0</td>
<td>5.52 ( \pm ) 4.3</td>
<td>12.0 ( \pm ) 7</td>
<td>4.00 ( \pm ) 2.8</td>
</tr>
<tr>
<td>60 min low flow</td>
<td>-N(10) 79.2 ( \pm ) 1.8</td>
<td>1.41 ( \pm ) 0.06</td>
<td>58.9 ( \pm ) 1.6</td>
<td>9.9 ( \pm ) 2</td>
<td>3.41 ( \pm ) 2.1</td>
</tr>
<tr>
<td></td>
<td>+N(10) 8.0 ( \pm ) 5</td>
<td>1.00 ( \pm ) 0.07</td>
<td>61.8 ( \pm ) 6.6</td>
<td>10.8 ( \pm ) 7</td>
<td>4.15 ( \pm ) 5.7</td>
</tr>
<tr>
<td>60 min low flow + 10 min reperfusion‡</td>
<td>-N(11) 55.5 ( \pm ) 4.8</td>
<td>1.28 ( \pm ) 0.09</td>
<td>43.6 ( \pm ) 3.9</td>
<td>6.8 ( \pm ) 4</td>
<td>2.02 ( \pm ) 2.1</td>
</tr>
<tr>
<td></td>
<td>+N(12) 10.0 ( \pm ) 6</td>
<td>1.36 ( \pm ) 0.0</td>
<td>7.52 ( \pm ) 6.5</td>
<td>9.4 ( \pm ) 7</td>
<td>3.50 ( \pm ) 2.4</td>
</tr>
<tr>
<td></td>
<td>-R(9) 58.4 ( \pm ) 6.0</td>
<td>1.24 ( \pm ) 0.0</td>
<td>47.73 ( \pm ) 4.8</td>
<td>6.9 ( \pm ) 8</td>
<td>2.31 ( \pm ) 3.0</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE. Numbers in parentheses indicate number of hearts studied. Preparations of mitochondria from ventricular myocardium and measurements of calcium, copper, and protein in whole homogenates and mitochondrial fractions were performed as described in METHODS. †Percent copper or protein in the mitochondrial fraction are expressed as percentages of the total myocardial copper or protein in the whole homogenates. *No perfusion hearts processed immediately after sacrifice of the animal without Langendorff perfusion, in these preparations, N and +N refer to the absence or presence of \( 10^{-7} \) M nifedipine in the preparative solutions, and -R indicates that ruthenium red was omitted from all preparative solutions. †High flow perfusion at \( 22 \text{ ml/min} \) in perfused hearts. ‡No +N and +N refers to the addition of vehicle alone, or vehicle + nifedipine (\( 10^{-7} \) M) to the perfusate during the experimental perfusion period (60 min), respectively. ‡Low flow perfusion at \( 0.2 \text{ ml/min} \). †Reperfusion. ‡R indicates that perfusion was carried out without nifedipine and that ruthenium red was omitted from all preparative solutions.

Calcium and copper in the mitochondrial fraction from perfused hearts. Results of measurements of calcium, copper, and protein from perfused hearts and from hearts processed immediately after sacrifice of the animal are summarized in Table 2. Preparations from hearts not used for perfusions exhibited a value of 6.3 \( \pm \) 0.3 ng-atom/mg protein. Values were not significantly different when preparative solutions without ruthenium red or nifedipine (\( 10^{-7} \) M) were utilized. In perfused hearts, perfusion at high flow for 60 min after a standard equilibration period produced a negligible increase in calcium. In contrast, perfusion at low flow for the same period resulted in a fourfold increase in calcium (28.2 \( \pm \) 1.9 ng-atom/mg protein). Reperfusion after low flow for 10 min further increased the value, calcium averaging 55.5 \( \pm \) 4.8 ng-atom/mg protein. Similarly elevated values were obtained when ruthenium red was omitted from the solutions. These increases in low flow perfusion without or with reperfusion did not
The presence of ruthenium red, 0.5 mM, net (nonisotopic) uptake was largely inhibited, both without or with substrate plus phosphate. The latter additions were made to test whether changes in calcium uptake induced by ruthenium red were modified by substrate and phosphate, as reported by Thakar et al. (28). As ruthenium red was not expected to completely block calcium uptake (3), uptake was further evaluated with the isotopic (45Ca) method. Results of measurements of net uptake by atomic absorption spectrometry and isotopic binding using rapid centrifugation for the separation of bound from free calcium (45Ca) are summarized in Table 3. All measurements were made in the same carefully resuspended final pellets. Ruthenium red inhibited net uptake, but did not prevent accumulation of radioactive calcium, suggesting that uptake by the isotopic method partly reflected isotopic exchange.

The possibility that the relatively high concentration of ruthenium red (0.5 mM; 143 nmol/mg protein) used in the present experiments promoted discharge of calcium from mitochondria was considered. Figure 5 shows that the release of calcium from calcium-loaded mitochondria was influenced little by ruthenium red and that it did not favor the retention of calcium. Results of these experiments indicated that a high concentration of ruthenium red largely inhibited accumulation of calcium at 0°C, but did not minimize its efflux.

Correlation between calcium in mitochondrial fraction and cardiac mechanics. The relationship between ischemic myocardial stiffness and accumulation of calcium in the mitochondrial fraction is depicted in Fig. 6. In hearts perfused at high flow or in hearts perfused at low flow without nifedipine, there was no contracture and no increase in calcium. In contrast, in hearts perfused at low flow without nifedipine, ischemic contracture was associated with graded increases in calcium. Thus, ischemic stiffness, the only readily measurable mechanical variable during standstill of the isovolumic ventricle, correlated positively with the accumulation of calcium. In reperfused hearts, end-diastolic pressure was also found to correlate with calcium in the mitochondrial fraction. In these hearts, other mechanical parameters at the end of reperfusion were tested for correlations with calcium. Correlation coefficients for peak

### Table 3. Net and isotopic calcium uptake by mitochondrial fraction

| No. of Preparations | Nonisotopic (Net) uptake \(\text{ng-atom/mg prot}\) | Isotopic (45Ca) Uptake \(\text{ng-atom/mg prot}\) |
|---------------------|-----------------------------------------------|
|                     | 30 min* | 60 min | 30 min | 60 min |
| Without ruthenium red | 5       | 16.60 ± 1.11 | 23.8 ± 1.90 | 24.04 ± 2.21 | 49.84 ± 4.06 |
| With ruthenium red*  | 5       | 5.81 ± 0.0  | 5.60 ± 0.30 | 15.76 ± 1.62 | 22.76 ± 2.78 |

Values are means ±SE of 5 assays with 5 different mitochondrial preparations. * Incubation times.
FIG. 5. Calcium efflux from calcium-loaded mitochondria. Loading with calcium (300 nmol/mg protein) and efflux experiments were performed as described in METHODS. Upper panel: without substrate. Lower panel: with substrate plus phosphate as described in legend to Fig. 4. Symbols: o-o, without ruthenium red; A-A, with ruthenium red. Data points represent means ± SE of 5 determinations with 5 different preparations.

FIG. 6. Relationship between ischemic contracture and accumulation of calcium in mitochondrial fraction. Perfusions were performed as described in METHODS. Mitochondrial fractions were prepared with solutions containing ruthenium red. • after a standard equilibration period, hearts were perfused at 22 ml/min for 60 min with buffer containing no nifedipine (n = 7); □ perfusions at low flow (0.2 ml/min) for 60 min with buffer containing nifedipine (10^{-7} M) and its vehicle (n = 9). □ after a 60-min period of low flow with nifedipine, hearts were reperfused at 22 ml/min for 10 min with buffer containing no nifedipine (n = 10). △ 60-min perfusions at low flow with buffer containing vehicle without nifedipine. △ 60 min of low flow with buffer containing vehicle without nifedipine, followed by 10 min of reperfusion. Data points are from the same experiments as those summarized in Figs. 2 and 3 and Table 2.

discussion

Results of the present study demonstrate that isolated hearts perfused at low flow undergo a progressive contracture which is associated with an accumulation of calcium in the mitochondrial cell fraction prepared from ventricular myocardium. In addition, nifedipine, a dihydropyridine derivative which antagonizes effects of calcium on cardiac and smooth muscle (6, 9, 29), prevents the myocardial contracture and the accumulation of calcium and promotes mechanical recovery after reperfusion.

In the present study, isovolumic left-heart preparations perfused at low flow were used as a model of acute myocardial ischemia. In this model, major determinants of myocardial performance including cardiac frequency, ventricular size (muscle length), coronary flow, and composition of the perfusate are controlled, and changes in peak systolic dP/dt and in end-diastolic pressure may be utilized as indexes of cardiac contractility and diastolic elastic stiffness ("compliance"), respectively (18). Although the isolated perfused heart is useful for assessing intrinsic changes in cardiac performance, this preparation has certain limitations. Reduced perfusion with hemoglobin-free media mimicks ischemia only in terms of flow, but undoubtedly delivers less oxygen to the muscle compared to true ischemia and eliminates potentially important blood-mediated effects. Furthermore, control of coronary flow and ventricular volume produces nonphysiological coronary and ventricular dynamics. The perfused heart preparation is however particularly valuable for the evaluation of interventions that, in the intact organism, may act directly and/or indirectly on the heart. Thus, nifedipine, a potent vasodilator with direct negative inotropic effects (6, 9, 29), may act in vivo on the ischemic heart by decreasing left ventricular afterload, or by augmenting coronary flow, or by reducing cardiac contractility. Results of the present study demonstrate that nifedipine exerts potent direct effects on the ischemic myocardium and, in this respect, probably differs from other vasodilators such as nitroglycerin and nitroprusside.

Calcium plays an important role in the regulation of the energy metabolism of cardiac muscle (7, 14, 16), and disturbances in the amount and distribution of intracellular calcium may be expected to affect the energetics of myocardial cells. In a number of studies, myocardial injury has been found to be associated with myocardial accumulations of calcium (6, 69, 11, 25). However, the relationship between accumulation of calcium and myocardial injury is still poorly understood. In the present study, changes in ventricular mechanics induced by decreased perfusion were related to an index of cellular calcium. The measurement of intracellular calcium in the ischemic myocardium poses methodological problems. Theoretically, intracellular calcium can be calculated as the difference between total tissue calcium and calcium contained in the extracellular space. Estimates of extracellular calcium, a substantial fraction of total tissue calcium (5, 25), depend upon the measurements of the mean extracellular calcium concentration and of the extracellular water...
with appropriate markers under steady-state conditions (21). The distribution of commonly used extracellular fluid markers in ischemic myocardium is, however, difficult to ascertain. Cell membranes that permit macromolecules to leak out of ischemic cells may not be impermeable to markers, and it may be difficult to achieve a steady state in a condition in which total tissue water and its distribution may not be constant (5, 26). It would be desirable to measure the free calcium concentration (calcium ion activity) surrounding the major organelles of the cells, but applicable methods for making such measurements in intact hearts are not available. Because of these considerations, we have utilized the calcium concentration in the mitochondrial fraction as an indicator of intracellular calcium, using copper as a mitochondrial marker. This approach is based on the observations that much of the calcium accumulating in ischemic myocardium can be recovered in the mitochondrial fraction (26), and that copper in blood-free myocardium constitutes an index of mitochondria and mitochondrial membranes in the tissue (19). In this approach, it is assumed that accumulation of mitochondrial calcium is a reflection of an increased calcium concentration in the myoplasm, since a rise in extramitochondrial calcium is the major factor determining energy-linked calcium uptake by mitochondria (23). To minimize spurious intrapreparative changes in mitochondrial calcium, hearts were perfused with isolation medium to wash out extracellular calcium before cell fractionation and ruthenium red, an inhibitor of mitochondrial calcium uptake (28), was added to all preparative solutions. To evaluate whether the mitochondrial fraction was accumulating or releasing calcium during its isolation, uptake and discharge of calcium by the mitochondrial fraction during exposure to isolation medium at 0°C were studied. Results demonstrated that 0.5 mM ruthenium red largely inhibited uptake, but did not influence release of calcium from calcium-loaded mitochondria. Retention of calcium was not enhanced by adding substrate and phosphate. In this respect, the mitochondrial preparations did not appear to behave like those obtained from guinea pig skeletal muscle (28). Thus, under our experimental conditions, ruthenium red provided only an unidirectional calcium seal, and the possibility that mitochondria lost some calcium during their preparation cannot be ruled out. However, preparation of mitochondria without ruthenium red did not yield significantly higher values. Our calcium values for mitochondria from fresh myocardium ranged between 6.2 and 6.8 ng-atom/mg protein, in agreement with previously reported values (25) but substantially lower than those obtained by Carafoli and Lehninger (5). Copper has been found to be a useful marker of mitochondria and mitochondrial membrane in blood-free myocardium. The concentration of copper measured in the mitochondrial fraction averaged 1.38 ± 0.04 ng-atom/mg protein, in good agreement with the values of 1.47 and 1.41 obtained by Griffiths and Wharton (8) and Page et al. (19) in mitochondria from beef and rat hearts, respectively. The yields of protein in the mitochondrial pellets of 4.01% (ca. 5–6 mg/g fresh myocardium) were low in the present study, but characteristic of preparative procedures using no Nagarse (12). Myocardial ischemia and hypoxia are known to produce marked changes in the functional properties of the mitochondrial cell fraction (10, 13). The advantage of using copper as a mitochondrial marker is that it may be less susceptible to alterations by ischemia compared to markers depending upon complex enzymic properties of the mitochondrion. This is supported by the observation that the copper-to-protein ratio in the mitochondrial pellets did not change appreciably in hearts subjected to perfusion at low flow.

As cell membranes are not impermeable to calcium and as the ratio of extracellular to intracellular free calcium is of the order of 10,000, the low intracellular calcium ion activity (≈10^{-7} M) in myocardial cells must be maintained by active processes (20). If ischemia interferes with these processes, calcium may be expected to accumulate inside of the cell. As nifedipine appears to act by blocking the slow, inward calcium current (6), inhibition of the accumulation of calcium by this drug in myocardial ischemia suggests that influx of calcium through the slow channels contributes to the calcium overload. However, the mechanism of action of nifedipine will require further evaluation and other mechanisms, such as decreased sequestration of calcium by the sarcoplasmic reticulum and diminished trans-sarcolemmal extrusion of calcium, may be operative in promoting accumulation of intracellular calcium. It should be emphasized that the increases in mitochondrial calcium observed in this and other studies (25) imply a net increase in myocardial calcium and do not merely represent a redistribution of tissue calcium.

The salient finding of this study is the correlation between cardiomechanical changes typical of ischemia and accumulation of calcium in the mitochondrial fraction. Incomplete or delayed myocardial relaxation and decreased myocardial compliance during episodes of myocardial ischemia have been demonstrated in patients suffering from coronary arterial disease (1, 17) and in patients undergoing cardiopulmonary bypass (4). Relaxed muscle represents a high-energy state of the contractile apparatus as ATP is required to dissociate the actin-ADP-myosin complexes (crossbridges) (7). It appears likely that undissociated crossbridges are involved in producing ischemic myocardial stiffness, although early muscle mechanical changes of ischemia are known to occur with nearly intact ATP stores (30). Depletion of ATP stores in ischemic myocardium are probably primarily determined by a lack of oxygen limiting mitochondrial synthesis of ATP. However, by inappropriately activating myofibrillar ATPase activity (7) and inhibiting oxidative phosphorylation in mitochondria (3, 14), an excess intracellular free calcium could enhance ATP utilization and simultaneously diminish its production. Thus, calcium could play an important role in depleting the ischemic cell of its energy stores and limiting the availability of ATP for crossbridge relaxation.
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