Creatine Kinase of Heart Mitochondria

THE PROGRESSIVE LOSS OF ENZYME ACTIVITY DURING IN VIVO ISCHEMIA AND ITS CORRELATION TO DEPRESSED MYOCARDIAL FUNCTION*

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It is now appreciated that mitochondrial creatine kinase (CKm) may play an important role in heart high-energy phosphate metabolism and that this isozyme is solubilized in vitro by dilute solutions of P2. Since an increase in cellular P2 is known to occur with even brief periods of myocardial ischemia, we investigated the relationship between CKm activity and myocardial performance in rabbit hearts subjected to total global ischemia. CKm activity is expressed as a ratio to mitochondrial malate dehydrogenase (MDHm), a stable marker enzyme. A significant decline in this ratio was observed after only 10 min of ischemia, a time prior to changes in total homogenate creatine kinase activity. After 60 min of ischemia, the CKm/MDHm ratio was depressed by more than 70%. Since there was no restoration of activity following 30 min of reperfusion, we correlected changes in enzyme activity to contractile dysfunction following variable periods of total ischemia. The data showed a close correlation between the decline in the CKm/MDHm ratio and the reduction in performance, measured as left ventricular developed pressure. No correlation was observed between State 3 respiratory rates and performance. Using KCl arrest at 27 °C or hyperthermic ischemia at 40 °C, the CKm/MDHm ratio consistently correlated to the degree of postischemic functional depression, independent of the duration of ischemia. Isoenzyme electrophoresis failed to detect soluble CKm activity in the postischemic supernatant. Therefore, CKm activity appears to be altered rapidly and irreversibly by ischemia. The implications of these observations on the integration of myocardial high-energy phosphate metabolism are discussed.

The depression of cardiac performance after ischemia may be related to persistent alterations in either the production or utilization of ATP. Since heart ATP generation is derived almost exclusively from aerobic metabolism (3), much attention has focused on identifying mitochondrial respiratory defects arising during ischemia that limit the rate of high-energy phosphate synthesis. Many laboratories have demonstrated that mitochondria isolated from ischemic cells have decreased rates of electron transport and oxidative phosphorylation, as well as reduced activities of specific components of the cytochrome chain (4-12). Other studies have illustrated mitochondrial structural abnormalities (13-17). It has been shown in liver that several of these defects induced during ischemia are potentially reversible (18, 19).

It is less well appreciated that postischemic dysfunction might also involve changes in the intracellular integration of the high-energy phosphates. Such integration, or energy transport, involving phosphocreatine has been investigated in a number of laboratories. Early studies correlating the heart’s content of the high-energy phosphates to contractility suggested the existence of two intracellular pools of ATP which were not in direct communication. These pools were thought to be localized within the mitochondrial matrix and near the myofibrils (20, 21). The shuttling of energy by phosphocreatine between these loci presumably involved creatine kinase bound at specific sites (21). Recent 31-phosphorus nuclear magnetic resonance saturation transfer studies (22, 23) have confirmed the nonequilibrium nature of the heart creatine kinase reaction, and have shown that the flux rates of heart creatine kinase can be influenced by changes in cardiac function (24, 25). Together, these NMR results lend in vivo support to the energy transport hypothesis.

A critical enzyme involved in this pathway is the mitochondrial isozyme of creatine kinase (26) which accounts for 30-40% of the creatine kinase activity in heart. It is now established that the enzyme resides on the exterior surface of the inner mitochondrial membrane. At this location, CKm appears to be functionally coupled to the adenine nucleotide translocase both for ATP supply (27-29) and ADP removal (30, 31). Our most recent kinetic studies have underscored the importance of this compartmentation for the effective coupling of phosphocreatine synthesis to oxidative phosphorylation (32), as well as for the general control of heart oxygen consumption (33). Changes in the localization of this enzyme could potentially alter the integration of heart high-energy phosphate metabolism by disrupting this coupling, and thus have a secondary impact on performance.

Mukherjee et al. (34) have shown in vitro that conditions prevailing during ischemia can induce the release of CKm from

* The abbreviations used are: CKm, mitochondrial creatine kinase; MDHm, mitochondrial malate dehydrogenase; LVDP, left ventricular developed pressure; LV dP/dt, the first derivative of the left ventricular pressure curve; EGTA, ethylene glycol bis(β-aminoethly ether)-N,N',N'-tetraacetic acid.

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Ischemic Changes in Heart Mitochondrial Creatine Kinase

intact heart mitochondria. Thus, the goal of this research was to determine the effects of in vitro ischemia on CK\textsubscript{m} activity and evaluate the possible relationships between the activity of CK\textsubscript{m} and the postischemic depression of maximal performance (16). The results show an almost immediate loss of CK\textsubscript{m} activity and a direct correlation to contractile abnormalities. These data are discussed from the view that changes in CK\textsubscript{m} activity, resulting in an alteration of energy transport, may be one of the earliest mitochondrial defects induced by ischemia. They also suggest that a number of important biochemical events transpire progressively, not suddenly, during the presumed "reversible" stages of ischemic cell damage.

**Experimental Procedures**

**Materials**—Phosphocreatine, nucleotides, oxalacetic acid, glucose, malic acid, hexokinase, and glucose-6-phosphate dehydrogenase were purchased from Sigma. Agarose was obtained from Calbiochem Behring. Nagarse was bought from the Enzyme Development Corp., New York, and mannitol was the product of J. T. Baker. All other chemicals were of the highest purity commercially available. The solutions were prepared using deionized water.

**Purification of Rabbit Hearts**—Albino New Zealand rabbits were anesthetized with pentobarbital (50 mg/kg body weight) and injected intraperitoneally. Hearts were perfused within 45 s after removal by a modified Langendorff technique (35). The perfusate was Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 115 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl\textsubscript{2}, 1.2 mM MgSO\textsubscript{4}, 12 mM KH\textsubscript{2}PO\textsubscript{4}, 25 mM NaHC\textsubscript{0}\textsubscript{3}, and 167 mM glucose, oxygenated with 95% O\textsubscript{2} 5% CO\textsubscript{2}. The aorta was cannulated and connected to a column with an overflow apparatus that ensured a constant temperature of 37 °C. At designated intervals, the clamp was released and the hearts were perfused for 30 min, after which time they were reperfused with 5.0 ml of ice-cold solution containing 90 mM NaCl, 37 mM KCl, 24 mM NaHC\textsubscript{0}\textsubscript{3}, and 35 mM glucose at pH 7.4 (36). Immediately following arrest, the balloon was deflated and the hearts were vented. A 16-gauge AngioCath (1.5 cm) was pierced in the aorta and connected to a column with an overflow apparatus.

**Left Ventricular Isovolumic Developed Pressure-End Diastolic Volume**

Immediately following arrest, the balloon was deflated and the hearts were vented. A 16-gauge AngioCath (1.5 cm) was pierced in the aorta and connected to a column with an overflow apparatus.

**Stability of Marker Enzymes**—An aliquot of mitochondria (50 μl) containing 0.25 mg of protein was added to either 0.5 ml of 115 mM KCl + 5 mM KH\textsubscript{2}PO\textsubscript{4}, pH (6.10) for a final pH of 6.12, or to 0.5 ml of 115 mM KCl + 5 mM KHP0\textsubscript{4}, pH (8.10) for a final pH of 8.72. At the start of the 30 °C incubation, samples were removed for the analysis of total activity. After 40 min, the samples were centrifuged at maximum speed for 1 min in a Beckman Microfuge. Supernatant solutions were decanted, and the pellets resuspended for the analysis of total activity and recovery.

**Enzyme Assays**—The assay for creatine kinase was the spectrophotometric method of Eppenberger et al. (38), determined in the reverse direction. The rate of ATP formation was measured by a hexokinase/glucose-6-phosphate dehydrogenase method, and activity calculated from the increase in absorbance accompanying the formation of NADPH, recorded at 340 nm in a Gilford Model 2900 spectrophotometer. The 3.0-ml assay medium contained 100 mM Tris-Cl (pH 7.4), 0.15 mM NADP+, 3.3 mM MgCl\textsubscript{2}, 3.3 mM glucose, 0.5 mM ADP, 8.3 mM phosphoglucose, 5 μg of hexokinase (140 IU/mg), 5 μg of glucose-6-phosphate dehydrogenase (140 IU/mg), and 1.33 mM AMP to inhibit adenylate kinase. The assay was conducted at 30 °C with the reaction initiated by the addition of 12 μg of mitochondrial protein or 15 μg of homogenate protein. Malate dehydrogenase activity was measured by the method of Ochoa (39), while cytochrome oxidase activity was assayed according to Schnaitman and Greenwald (40). Protein was quantitated by a biuret method (41) with crystallized, nitrogen-standardized, bovine serum albumin as the primary standard.

**Isoenzyme Electrophoresis**—Thin-layer agarose gels were prepared 24 h prior to use by spreading 10.0 ml of 1% agarose in 60 mM barbituric buffer (pH 8.4) on 8 × 12-cm glass plates. The gels were electrophoresed in a system at 4 °C (12 mA plate, 60 mA + 40 mA at 8.4). Plates were stained for malate dehydrogenase activity by overlaying with the nitro blue tetrazolium-phenazine methosulfate solution described by Goldberg (42). The plates were incubated at 37 °C for 1 h; the estimated sensitivity was 1.0 mIU. Creatine kinase isozymes (43) were detected by overlaying the plates with 7.5 ml of 100 mM Tris-Cl (pH 7.4), 5.0 mM MgCl\textsubscript{2}, 5.0 mM glucose, 1.0 mM ADP, 15.0 mM phosphocreatine, 3.0 mM AMP, 10.0 mM cytochrome HCl, 10 μg of hexokinase (140 IU/mg), 10 μg of glucose-6-phosphate dehydrogenase (140 IU/mg), 0.3 mM NADP, and incubated for 1 h at 37 °C. Enzyme activity was located by fluorescence and the sensitivity was 1.0 mIU.

**Statistics**—Regression analysis and Student's t test were conducted according to Snedecor and Cochran (44). Comparison of regression analyses were performed according to Duncan (45). Standard statistical programs were used for the one-way analysis of variance. All data reported in the figures, tables, and text are given as the mean ± S.E.

**Results**

**Selection of Mitochondrial Marker Enzymes**—There is some normal variability in the specific activity of creatine kinase in isolated mitochondrial fractions (26, 46). These differences probably result from nonmitochondrial protein contamination. Since postischemic preparations could be more severely contaminated, we sought a stable mitochondrial enzyme for the normalization of CK\textsubscript{m} activity. The activity ratio of CK\textsubscript{m} to other mitochondrial enzymes is relatively constant (46). Table I presents data for the stability of cytochrome oxidase and M DH\textsubscript{m}, classic marker enzymes, under incubation conditions known to solubilize CK\textsubscript{m} (26, 34, 46). The results confirm that MDH\textsubscript{m} and cytochrome oxidase remained associated with the mitochondrial pellet when 50–70% of the CK\textsubscript{m} activity was released. A slight decrease in the recovery of cytochrome oxidase activity was observed at pH 6.1, from 0.90 to 0.78 IU/mg of protein. In contrast, only 2% of the MDH\textsubscript{m} activity was detected in the supernatant and the recovery was...
consistently near 100%. These data suggested that MDH$_m$ was more stable, and thus the preferred enzyme for normalization. In all mitochondrial studies CK$_m$ activity was divided by MDH$_m$ activity, and presented as a CK$_m$/MDH$_m$ ratio.

**Isoenzyme Contaminations**—In control experiments, malate dehydrogenase (42) and creatine kinase (43) isozyme electrophoresis was performed on mitochondria isolated from normal and ischemic hearts. The purpose was to assess the contamination of these preparations by nonmitochondrial isozymes. Mitochondria were diluted in 100 mM K$_2$HPO$_4$ (pH 6.12) and 10 mM cysteine to enzyme activities up to 20 mIU/μl. The samples were applied to plates as 2-μl aliquots, electrophoresed, and stained. Only mitochondrial forms of the isozymes were detected in either normal or ischemic mitochondrial fractions. Since the sensitivity of both methods was 1 mIU, the contamination by other isozymes was apparently less than 2.5%.

**Stability of Total Tissue Homogenate Creatine Kinase Activity during Ischemia**—The release of creatine kinase from the myocardium has been used as an index of irreversible ischemic cell damage. We therefore determined the activity of creatine kinase in heart homogenates during global ischemia (Fig. 1). Fig. 1A shows that the specific activity of creatine kinase has a rather large variance, with average values ranging from 3.0 to 4.5 IU/mg of protein. Even after 120 min of global ischemia, there was no statistically significant decrease in homogenate activity. This time course was similar to that reported by Braasch et al. (47). When homogenate creatine kinase activity was normalized to total malate dehydrogenase activity (Fig. 1B), the variance at each time point was reduced. However, little decrease was noted in the ratio of these enzymes, and only at 120 min was a significant reduction detectable from control (p < 0.02).

**Loss of Mitochondrial Creatine Kinase during Total Global Ischemia**—To determine if the activity of CK$_m$ was altered during ischemia, a group of 24 hearts was subjected to periods of global ischemia ranging from 0 to 60 min followed by 5 min of reperfusion. Mitochondrial fractions were subsequently isolated, and the CK$_m$ and MDH$_m$ activities determined, Fig. 2. CK$_m$ specific activity (Fig. 2A) declined steadily during 1 h of ischemia, with significant differences (p < 0.005) seen at 45 and 60 min. MDH$_m$ activity (Fig. 2B) did not significantly change. When the enzyme data were normalized on a preparation to preparation basis (Fig. 2C), the CK$_m$/MDH$_m$ ratio showed a significant decrease at 10 min of ischemia (p < 0.02). As a result of the normalization process, we now were able to detect very early reductions in CK$_m$ activity.

Brief reperfusion after ischemia could reduce CK$_m$ because of enzyme washout (48). In control experiments, hearts were subjected to 30 min of global ischemia, then reperfused for 30 min. In these hearts, the total homogenerate creatine kinase activity was 3.9 IU/mg of protein, a value similar to control. In 4 other hearts subjected to 30 min of total global ischemia and reflow, we collected the first 5.0 ml of coronary effluent. The samples contained only 40 mIU of creatine kinase activity, which represented less than 1 x 10$^{-4}$% of the heart's total activity. Transmembrane leakage cannot account for the observed changes in CK$_m$ activity.

**Irreversible Loss of CK$_m$ Activity during Ischemia and Reperfusion**—To test the reversibility of CK$_m$ loss in vivo, control hearts were perfused for 75 min at normal flow rates. In mitochondria isolated from these hearts, the CK$_m$/MDH$_m$ ratio was 0.136 ± 0.001, Table II, quite close to the preischemic control value reported in Fig. 2C. Other hearts were made ischemic for 30 min with no reflow. In these, the CK$_m$/MDH$_m$ ratio was significantly depressed (p < 0.005) by 30% to a value of 0.096 ± 0.003. Finally, 4 other hearts were subjected
Ischemic Changes in Heart Mitochondrial Creatine Kinase

Effects of ischemia and reperfusion on the CKm/MDHm ratio
Control hearts were normally perfused for 75 min. Experimental hearts were stabilized for 15 min prior to total global ischemia. Reflow was initiated by releasing the clamp. Hearts were then made totally ischemic at 37°C, and the mitochondrial fractions isolated from each heart. The activities of creatine kinase and malate dehydrogenase were immediately determined as described under "Experimental Procedures."

Table II

<table>
<thead>
<tr>
<th>Conditions</th>
<th>n</th>
<th>CKm/MDHm</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Perfusion (75 min)</td>
<td>4</td>
<td>0.136 + 0.002</td>
<td></td>
</tr>
<tr>
<td>2. Ischemia (30 min) with no reperfusion</td>
<td>4</td>
<td>0.009 + 0.003</td>
<td>0.005 (1 vs 2)</td>
</tr>
<tr>
<td>3. Ischemia (30 min) with reperfusion (30 min)</td>
<td>4</td>
<td>0.091 + 0.007</td>
<td>NS* (2 vs 3)</td>
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</table>

*NS, not significant.

Effects of ischemia and reperfusion on the CKm/MDHm ratio

Contractility Measurements in Isovolumic Rabbit Hearts—The major goal of this study was to determine the relationships between postischemic reductions in energy demand, expressed as contractile performance (16), and alterations in the phosphocreatine energy transport pathway, estimated by the loss of CKm. To achieve this it was necessary to assess maximum contractile performance. Other researchers (49) have shown a correlation between peak developed pressure and maximum O2 consumption in Langendorff paced hearts. Therefore, we monitored contractility by determining performance at the peak of the developed pressure-end diastolic volume curve (Fig. 3). It is well known that ischemia depresses myocardial contractility, which for a limited period of reperfusion appears to be a persistent defect. Fig. 3 shows that peak cardiac performance deteriorated by approximately 14% after only 10 min of total global ischemia. After 60 min of ischemia there was an 88% depression in cardiac performance, with intermediate values at other times. Since ventricular performance was 95 ± 3% after 90 min of perfusion at normal flow rates, the functional alterations observed in Fig. 3 represent damage induced by the ischemic protocol.

Relationships between CKm Activity and Cardiac Performances—Since 30 min of reflow did not restore CKm activity (Table II), the correlations between CKm and performance were evaluated (Fig. 4). In Fig. 4A the CKm/MDHm ratio was plotted versus function, calculated as per cent control peak LVPD. The data show a very close correlation (r = 0.97) between these two parameters. The reduction of maximum dP/dt, expressed as per cent control dP/dt, also closely correlated (r = 0.95) to the loss of CKm activity (Fig. 4B). A more common index of ischemic mitochondrial damage has been the depression of State 3 respiration. In data not shown, there was no relationship between the rates of State 3 and contractile depression. Overall, the data in Figs. 2 and 4 suggest that to 30 min of ischemia, followed by 30 min of reperfusion prior to mitochondrial isolation. Under these latter conditions, CKm activity was not restored to normal, but remained reduced at 0.99 ± 0.007. The data show that the loss of CKm activity was irreversible in nature during brief periods of reflow.

Fig. 2. Mitochondrial enzyme activities during total global ischemia. Twenty-four isolated, perfused rabbit hearts were subjected to global ischemia for 0, 10, 20, 30, 45, and 60 min. Creatine kinase and malate dehydrogenase activities were measured in mitochondrial fractions isolated from each heart. A, mitochondrial creatine kinase activity; IU per mg of mitochondrial protein; B, mitochondrial malate dehydrogenase activity, international units per mg of mitochondrial protein; C, the ratio of mitochondrial creatine kinase activity normalized to the malate dehydrogenase activity from the same mitochondrial preparation. In all panels the lines were drawn by linear regression, and the r values are at the lower left corner.

Fig. 3. Pressure-volume curves of control and postischemic hearts. Left ventricular developed pressure was measured at the peak of the pressure-volume curve. The control curves were generated during the preischemic period as outlined under "Experimental Procedures." Hearts were then made totally ischemic at 37°C followed by reperfusion for 30 min by clamping the perfusion line. Following a 30-min period of reperfusion, postischemic pressure-volume curves were again determined (mean ± S.E., n = 4 at each time).

Table II

<table>
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<th>p value</th>
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<tr>
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<td>0.005 (1 vs 2)</td>
</tr>
<tr>
<td>3. Ischemia (30 min) with reperfusion (30 min)</td>
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<td>0.091 + 0.007</td>
<td>NS* (2 vs 3)</td>
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*NS, not significant.
Ischemic Changes in Heart Mitochondrial Creatine Kinase

Table III

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<tr>
<th>Conditions</th>
<th>CK&lt;sub&gt;m&lt;/sub&gt;/MDH&lt;sub&gt;m&lt;/sub&gt; Ratio</th>
<th>% of control peak LVDP</th>
<th>% peak LVDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0.136 ± 0.002</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2. Ischemia (10 min) at 37 °C</td>
<td>0.119 ± 0.007</td>
<td>88</td>
<td>86 ± 5</td>
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<tr>
<td>3. Ischemia (30 min) at 27 °C</td>
<td>0.115 ± 0.003</td>
<td>87</td>
<td>89 ± 2</td>
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<td>4. Ischemia (10 min) at 40 °C with KCl arrest</td>
<td>0.101 ± 0.003</td>
<td>74</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>5. Ischemia (30 min) at 37 °C</td>
<td>0.091 ± 0.007</td>
<td>67</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>6. Ischemia (120 min) at 27 °C</td>
<td>0.086 ± 0.003</td>
<td>63</td>
<td>59 ± 7</td>
</tr>
</tbody>
</table>

Fig. 4. The relationship between mitochondrial creatine kinase activity and ventricular function in rabbit hearts after 37 °C total global ischemia. Isolated, perfused rabbit hearts were stabilized for 15 min; pressure-volume curves and maximum dP/dt were measured. Total global ischemia was induced at 37 °C for 0, 10, 20, 30, 45, and 60 min. Peak LVDP and maximum dP/dt were again measured after 30 min of reperfusion, and expressed as a percent of control peak developed pressure. Regression for per cent control peak developed pressure (A), or control maximum dP/dt (B) for each heart. After 30 min of reperfusion, the mitochondrial fractions were isolated and assayed for creatine kinase (CK<sub>m</sub>) and malate dehydrogenase (MDH<sub>m</sub>) activities. A, regression for per cent control peak LVDP versus the CK<sub>m</sub>/MDH<sub>m</sub> ratio: per cent peak LVDP = (930 x CK<sub>m</sub>/MDH<sub>m</sub>) - 28; r = 0.97. B, regression for per cent maximum dP/dt versus the CK<sub>m</sub>/MDH<sub>m</sub> ratio: per cent maximum dP/dt = (948 x CK<sub>m</sub>/MDH<sub>m</sub>) - 28; r = 0.95; n = 22.

Other Ischemic Interventions—The data presented in the previous section report enzyme changes as a function of the duration of ischemia. It is also possible to change the "sever-ity" of global ischemia, and thus modify ischemic cell damage. Such approaches could include 40 °C hyperthermic ischemia to increase the rate of damage, or KCl arrest at 27 °C to retard these effects (50). When hearts were subjected to 40 °C hyperthermic ischemia, the magnitude of enzyme loss was greater than that observed for comparable times at 37 °C. Table III. On the other hand, 30 min of KCl arrest at 27 °C preserved CK<sub>m</sub> activity relative to normothermic (37 °C) ischemia. In fact, 120 min of KCl arrest was required to detect enzyme and functional changes similar to those of normothermic 30-min ischemia. Under these conditions, data not shown, the correlation between CK<sub>m</sub> loss and contractile depression was quite similar to that seen in Fig. 4. Therefore, the CK<sub>m</sub>/MDH<sub>m</sub> ratio is more than a measure of ischemic duration since it consistently monitors the extent of damage under variable time condition.

Fig. 5. The fate of mitochondrial creatine kinase in the ischemic rabbit heart. Creatine kinase isoenzyme electrophoresis was performed on soluble fractions of whole heart homogenates from control and 30-min ischemic hearts. All homogenates were prepared in 10 volumes of 210 mM mannitol, 70 mM sucrose, 1.0 mM cysteine, 0.1 mM EGTA, and 10 mM Tris-Cl (pH 7.4). One-half of the control homogenate was incubated in 100 mM KH<sub>2</sub>P<sub>4</sub> (pH 8.0), 0.5% deoxycholate, and 1.0 mM cysteine at 20 °C for 8 min. Soluble fractions of all homogenates were prepared by centrifugation at 37,000 x g for 20 min and diluted in homogenizing medium to final enzyme activities of 1.0 and 1.0 IU/ml. Aliquots, 2 µl, were plated in the agarose gel wells, electrophoresed, and stained as previously described (46). Slot 1: phosphate-deoxycholate control supernatant fraction, 1 IU/ml; slot 2: phosphate-deoxycholate control supernatant fraction, 10 IU/ml; slot 3: control heart supernatant fraction, 1 IU/ml; slot 4: control heart supernatant fraction, 10 IU/ml; slot 5: ischemic (30 min) heart supernatant, 1 IU/ml; slot 6: ischemic (30 min) heart supernatant, 10 IU/ml.
activity, the mechanism of enzyme loss was undefined. Since dilute solutions of inorganic phosphate (5-20 mM) solubilize CK<sub>m</sub> (26, 34, 46), we investigated for CK<sub>m</sub> release into the sarcoplasmic fraction. Isoenzyme electrophoresis was performed on the 37,000 × g supernatant fractions from control and 30-min ischemic hearts. It was assumed that if CK<sub>m</sub> were solubilized and remained active, it would be detected in the supernatant fraction. In control experiments, heart tissue was homogenized in 100 mM potassium phosphate, pH 8.0, containing 0.5% deoxycholate and 1.0 mM cysteine. This resulted in the release of active CK<sub>m</sub>. Fig. 5, slots 1 and 2. Slots 3 and 4 show that no CK<sub>m</sub> activity was detectable in the supernatant fraction from normal homogenates. In postischemic hearts, we were not able to detect enzyme activity in the soluble fraction, slot 5, even when the gel was overloaded, slot 6. The changes in CK<sub>m</sub> activity documented in this article must be explained by mechanisms other than simple release. Such possibilities include enzyme aggregation (51), spontaneous denaturation, or degradation by proteases (52-54).

**DISCUSSION**

To appreciate the integration occurring between high-energy phosphate production and contractile utilization, one must consider the general metabolic restrictions placed on the normal heart. The heart is rather unique in that it utilizes more than 90% of the metabolically generated ATP for a single, well-defined purpose. This is to energize the reactions involved in the excitation-contraction-relaxation cycle (3). While ATP is produced by glycolysis and mitochondrial oxidative phosphorylation, glycolysis usually accounts for less than 10% of the net ATP, with 90% coupled to oxidative phosphorylation. Kobayashi and Neely (55) have more recently fully documented this balance by measuring the rates of ATP production under varying conditions of work stress. At normal work loads, the flux of ATP from glycolysis was 4 μmol/min/g of tissue, while during maximum work this increased to 12 μmol/min/g, the same as observed during anoxia. For oxidative phosphorylation, the rates of ATP synthesis were normally 60 μmol/min/g, which peaked at 120 μmol/min/g.

From the standpoint of our results, it is important to realize that during very high work conditions the rates of ATP production can approach the theoretical metabolic limits. Glycolytic production is restricted by a fixed enzyme content, with hexokinase having the lowest specific activity, 7 IU/g tissue. Since 2 ATPs are synthesized per mol of phosphorylated glucose, the upper limit would be 14 μmol of ATP/min/g, quite close to the measured value of 12. With respect to oxidative phosphorylation, Scarpa and Grazioti (56) reported that the mitochondrial content of the rat heart was 81 mg/g of tissue. Isolated rat heart mitochondria respirole in State 3 at a rate of 500 ng atoms of oxygen/min/mg. Assuming an ADP/O ratio of 3.0, ATP production would be 1.5 μmol/min/mg, or 121.5/g of heart. This value is strikingly close to the observed rate of 120 μmol/min. In other words, during stress the rate of ATP utilization almost reaches the maximum rate of production. For these reasons, investigators recognized that derangements in aerobic energy metabolism could limit ATP production and possibly contractile performance in the postischemic state. This is also why we assessed myocardial function under maximal conditions. Under normal conditions, modest derangements in mitochondrial metabolism might not be detectable since there is an apparent 2-fold metabolic reserve.

The data presented in the report are consistent with the idea that changes in localization of a critical integrative enzyme may affect the fidelity of phosphocreatine energy transport, and be expressed as decreased performance. However, such an interpretation must be taken with some caution. For example, we have not ruled out the possibility that ischemic induced changes in the regulation of the actomyosin ATPase may occur, although there are reports that such is not the case (57). Likewise, we now appreciate that there are persistent changes in the ATP content of the myocardium that correlate with regional contractile depression when hearts are subjected to short-term ischemia (58, 59). It is clear that a number of potentially damaging events transpire during ischemia to summate as contractile depression. It would therefore be inappropriate to overinterpret our results and consider any single event "responsible" for posts ischemic contractile failure.

As a final point, for over two decades investigators have successfully defined a sequence of events occurring during the progression to irreversible myocardial damage. Cell death and necrosis are the end result. Because the morphological changes can quickly disappear upon reperfusion, pathological studies suggested that up to some transition point, ischemic-induced damage may be rapidly and completely reversible (16). The extension of this work has led to the concept of a "point of no return" between reversible and irreversible damage (7), implying that such a change is abrupt. However, in spite of the fact that cell morphology may be normal, myocardial performance deteriorates in a way directly related to the duration of ischemia (compare Fig. 3 versus Figs. 1 and 2 in Ref. 16). More recently it has been recognized that there are regions in a previously ischemic zone that have normal morphology but depressed function. This has led to the concept of an ischemic induced "stunned" myocardium (60). Since both our biochemical (Fig. 2) and functional (Fig. 3) data progressively degenerate, the results presented in this article suggest that "reversible ischemia" may be characterized by a definable sequence of events occurring in a time-dependent manner. In other words, the transition to cell death is gradual, and not an all or none event. This important recognition now opens the door for more detailed biochemical studies of the subtle changes induced during the early, and potentially correctable reversible phase. The sensitivity of the CK<sub>m</sub>/MDH<sub>m</sub> ratio to the duration and severity of ischemia suggests that this approach may provide useful information about cell damage in normally appearing tissue.

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Ischemic Changes in Heart Mitochondrial Creatine Kinase

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