Relation Between Glycolysis and Calcium Homeostasis in Postischemic Myocardium

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This study examined the hypothesis that glycolysis is required for functional recovery of the myocardium during reperfusion by facilitating restoration of calcium homeostasis. $[Ca^{2+}]_i$ was measured in isolated perfused rabbit hearts by using the Ca^{2+} indicator 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (5F-BAPTA) and ^{19}F nuclear magnetic resonance spectroscopy. In nonischemic control hearts, inhibition of glycolysis with iodoacetate did not alter $[Ca^{2+}]_i$. In hearts subjected to 20 minutes of global zero-flow ischemia, $[Ca^{2+}]_i$ increased from 260±80 nM before ischemia to 556±44 nM after 15 minutes of ischemia ($p<0.05$). After reperfusion with 5 mM pyruvate as a carbon substrate, $[Ca^{2+}]_i$ increased further in hearts with intact glycolysis to 851±134 nM ($p<0.05$ versus ischemia) during the first 10 minutes of reperfusion, before returning to preischemic levels. In contrast, inhibition of glycolysis during the reperfusion period resulted in persistent severe calcium overload ($[Ca^{2+}]_i$, 1,380±260 nM after 15 minutes of reperfusion, $p<0.02$ versus intact glycolysis group). Furthermore, despite the presence of pyruvate and oxygen, inhibition of glycolysis during early reperfusion resulted in greater impairment of functional recovery (rate/pressure product, 3,722±738 mm Hg/min) than did reperfusion with pyruvate and intact glycolysis (rate/pressure product, 9,851±590 mm Hg/min, $p<0.01$). Inhibition of glycolysis during early reperfusion was also associated with a marked increase in left ventricular end-diastolic pressure during reperfusion (41±5 mm Hg) compared with hearts with intact glycolysis (16±2 mm Hg, $p<0.01$). The detrimental effects of glycolytic inhibition during early reperfusion were, however, prevented by initial reperfusion with a low calcium solution ($[Ca]_o$, 0.63 mM for 30 minutes, then 2.50 mM for 30 minutes). In these hearts, the rate/pressure product after 60 minutes of reperfusion was 12,492±1,561 mm Hg/min ($p<0.01$ versus initial reflow with $[Ca]_o$ of 2.50 mM). These findings indicate that the functional impairment observed in postischemic myocardium is related to cellular Ca^{2+} overload. Glycolysis appears to play an important role in restoration of Ca^{2+} homeostasis and recovery of function of postischemic myocardium. (*Circulation Research* 1992;70:1180–1190)

KEY WORDS • myocardial ischemia • calcium • glycolysis • reperfusion

Under most circumstances, oxidative metabolism of free fatty acids is the predominant energy source for the myocardium,[1] but myocardial viability is maintained by anaerobic glycolysis during periods of ischemia or hypoxia.[2,3] After reperfusion, oxidative phosphorylation resumes in postischemic myocardium and glycolysis is believed to resume a secondary role. Some recent findings, however, suggest that glycolysis may continue to be important in the postischemic myocardium. Although fatty acids are the primary fuel source after reperfusion,[4–6] oxidative metabolism of fatty acids appears to be reduced compared with normal myocardium.[2,4,7] In contrast, experimental[8] and clinical[9] positron emission tomography studies indicate that glucose utilization is increased in postischemic myocardium, probably via nonoxidative metabolism.[10] In addition, the functional recovery of isolated reperfused hearts is improved when glucose is available as a metabolic substrate,[11] while recent studies in our laboratory have shown that recovery of postischemic myocardium is impaired when glycolysis is inhibited during early reperfusion.[12]

The reason for this apparent need for glycolysis in postischemic myocardium is uncertain but may reflect a need for restoration of ion homeostasis during early reperfusion. Several studies have documented a rise in $[Ca^{2+}]_i$ during ischemia and early reperfusion.[13–17] Such calcium overload can result in sustained myocardial contractile dysfunction and may be an important factor in the pathogenesis of stunned myocardium.[13,18]

There is evidence to support a connection between glycolysis and ion transport in the myocardium. Glycogenolytic enzymes are associated with the sarcoplasmic reticulum,[19] and enzymes of the glycolytic pathway have also been found to be associated with the sarcolemma.[20] Studies of isolated membrane vesicles from smooth muscle have shown that membrane Ca^{2+} transport appears to be preferentially supported by glycolysis.[21] Although glycolytic ATP may be directed toward ion transport processes, ATP generated via oxidative phos-

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phorylation appears to be preferentially used by the contractile apparatus.[22–25] In addition, calcium overload of the myocyte may also result in excessive mitochondrial calcium accumulation, with subsequent uncoupling of oxidative phosphorylation,[26–28] which would further increase the dependency of the postischemic myocardium on glycolysis.

The relation between glycolysis and intracellular calcium levels during ischemia and reperfusion was studied in isolated, perfused rabbit hearts by ^{19}F nuclear magnetic resonance (NMR) spectroscopy with the calcium indicator 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (5F-BAPTA).[16,29,30] The functional consequences of glycolytic inhibition during reperfusion were further examined by comparing the effects of reperfusion with a normocalcemic ($[Ca]_o$, 2.50 mM) or hypocalcemic ($[Ca]_o$, 0.63 mM) solution on recovery of contractile function of postischemic myocardium.

Materials and Methods

Experimental Preparation

Female New Zealand White rabbits (1–2 kg) were heparinized (1,000 units i.v.) and anesthetized with pentobarbital sodium (30 mg i.v.). The hearts were rapidly excised and the aortas cannulated for retrograde perfusion of the coronary circulation. A small latex balloon was inserted via the left atrium into the left ventricle and connected by thin polyethylene tubing to a pressure transducer (Statham P23Db, Gould Instruments, Indianapolis, Ind.). The balloon was inflated with saline solution, so that left ventricular end-diastolic pressure was 10–12 mm Hg. The hearts then beat isovolumically throughout the experiment. Heart rate and left ventricular pressure were continuously recorded on chart paper (RS 3400, Gould). The rate/pressure product was calculated as the product of heart rate and left ventricular developed pressure.

Measurement of Intracellular Calcium

Measurements of $[Ca^{2+}]_i$ were made using ^{19}F NMR spectroscopy and the Ca^{2+} indicator 5F-BAPTA in 14 hearts.[16,29,30] These hearts were perfused with modified Tyrode's solution containing (millimolar) NaCl 117, KCl 5.9, $MgCl_2$ 1, HEPES 5, and $CaCl_2$ 2.5 with pyruvate 5. The pH was adjusted to 7.40 at a constant temperature of 30°C, and the perfusate was bubbled continuously with 100% oxygen. A constant coronary flow rate of 25 ml/min was maintained via a peristaltic pump. In these hearts, the intraventricular latex balloon contained an aqueous solution of 1 mM 6-fluorotryptophan (6-FT) as an NMR reference standard. Inclusion of the 6-FT standard allowed estimation of the intracellular concentration of 5F-BAPTA, as previously described.[29]

After initial stabilization, the hearts were loaded with 5F-BAPTA by addition of the tetraacetoxymethyl ester (5 μM, 5F-BAPTA-AM, Molecular Probes, Inc., Eugene, Ore.) to the perfusate. Perfusion with 5F-BAPTA-AM was continued until adequate myocardial loading was achieved, manifest as the appearance of Ca^{2+}-bound and free 5F-BAPTA peaks on the NMR spectra. The usual duration of loading required was 20–25 minutes. On completion of the 5F-BAPTA loading, perfusion with the Tyrode's solution was resumed but $[Ca]_o$ was increased to 8 mM to counter the intracellular Ca^{2+} buffering effect of 5F-BAPTA.[16,29] The organic anion transport inhibitor probenecid (1 mM) was included in the perfusate to inhibit active extrusion of 5F-BAPTA from the cells. In previous experiments, addition of probenecid has not altered myocardial contractility or $[Ca^{2+}]_i$.[16,30]

The hearts were placed in a temperature-controlled jacket inside a wide-bore 8.5-T superconducting magnet. The ^{19}F NMR spectra were obtained with a model AM-360 FT NMR spectrometer (Bruker Instruments, Billerica, Mass.) operating in pulsed Fourier transform mode. A 25-mm broadband probe was tuned to the ^{19}F resonance frequency of 338.86 MHz. After shimming the magnetic field on the proton signal, spectra were acquired from 750 consecutive free induction decays by using a spectral width of 5 kHz and 45° pulses at a repetition interval of 400 msec. The spectra were processed with a line broadening of 75 Hz, and chemical shifts were referenced to the 6-FT standard, assigned to 0 ppm.

The concentrations of Ca^{2+}-bound and free 5F-BAPTA in individual spectra were calculated by integrating the areas under the Ca^{2+}-bound and free peaks, with calibration from the 6-FT standard, and applying the relation

$$[Ca^{2+}]_i = K_d \cdot ([B]/[F])$$

where K_d is the dissociation constant of Ca^{2+}-5F-BAPTA (285 nM at 30°C), [B] is the concentration of Ca^{2+}-bound 5F-BAPTA, and [F] is the concentration of free 5F-BAPTA.[29] The spin-lattice relaxation times of the Ca^{2+}-bound and free peaks of 5F-BAPTA are similar, and thus the ratio [B]/[F] is independent of pulse repetition frequency.[16]

Measurements of $[Ca^{2+}]_i$ were made for three groups of hearts (Table 1). Control hearts (group 1, $n=5$) were perfused with pyruvate for 60 minutes, without an ischemic interval. Consecutive time-averaged ^{19}F spectra were acquired in 5-minute blocks throughout the experiment. After 30 minutes of perfusion, glycolysis was blocked by the addition of iodoacetate to the perfusate (150 μM for 15 minutes, then 50 μM for 15 minutes). Iodoacetate is a potent, irreversible inhibitor of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in the glycolytic pathway.[31,32] This dose of iodoacetate is similar to that used by other investigators[31,33] and is efficacious in inhibiting glycolysis. In previous experiments in our laboratory, hearts perfused with glucose demonstrated a decrease in the rate/pressure product to 30% of baseline within 10 minutes of introduction of iodoacetate and to 10% of baseline after 20 minutes. In contrast, hearts perfused with pyruvate maintained a rate/pressure product at 86% of baseline and myocardial oxygen consumption at 81% of baseline after 45 minutes of perfusion with iodoacetate. Function in these hearts was similar to that in hearts perfused with pyruvate and no iodoacetate. These observations are consistent with other studies showing that this dose range of iodoacetate does not interfere with oxidative metabolism.[32,34,35]

In group 2 ($n=5$), hearts were perfused with pyruvate for 20 minutes, followed by 20 minutes of global zero-flow ischemia, and then 20 minutes of reperfusion with

TABLE 1. Study Groups and Perfusion Protocols

Group	n	Preischemic substrate	Reperfusion substrate	Iodoacetate	$[Ca]_o$ (mM) Early	$[Ca]_o$ (mM) Late
Measurement of $[Ca^{2+}]$						
1	5	Pyruvate	No ischemia	Yes	8	8
2	5	Pyruvate	Pyruvate	No	8	8
3	4	Pyruvate	Pyruvate	Yes	8	8
Measurement of left ventricular function						
4	7	Glucose	Glucose	No	2.5	2.5
5	8	Pyruvate	Pyruvate	Yes	2.5	2.5
6	8	...	Pyruvate	No	2.5	2.5
7	8	...	Pyruvate	Yes	2.5	2.5
8	4	Pyruvate	Pyruvate	No	0.63	2.5
9	7	Pyruvate	Pyruvate	Yes	0.63	2.5
10	4	...	Pyruvate	Yes	0.63	2.5

The early $[Ca]_o$ refers to the first 30 minutes of reperfusion, and the late $[Ca]_o$ refers to the last 30 minutes of reperfusion.

pyruvate at the same flow rate as during preischemia. Hearts in group 3 ($n=4$) were studied according to the same protocol of ischemia and reperfusion, but this group also received iodoacetate (150 μM for the first 15 minutes of reperfusion, then 50 μM for 15 minutes) in the reperfusion solution. In both groups 2 and 3, time-averaged ^{19}F spectra were acquired in 5-minute blocks throughout the control, ischemia, and reperfusion periods.

Functional Effects of Glycolytic Inhibition

Because loading of myocytes with 5F-BAPTA results in buffering of $[Ca^{2+}]_i$ and a marked negative inotropic effect,[16] the contractile function of postischemic myocardium, in the presence or absence of glycolysis, was studied in an additional four groups of hearts, which were not loaded with 5F-BAPTA (Table 1). These hearts were perfused at a constant pressure (80 mm Hg) with a bicarbonate-buffered Krebs-Henseleit solution (pH 7.40) containing (millimolar) NaCl 117, $NaHCO_3$ 24, KCl 5.9, $MgSO_4$ 0.9, $CaCl_2$ 2.5, EDTA 0.5, and NaH_2PO_4 1. The perfusate was continuously bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide. Either glucose (16 mM) or sodium pyruvate (5 mM) was supplied as a carbon substrate. When pyruvate was used, the concentration of NaCl in the perfusate was reduced accordingly.

The first group served as controls (Table 1, group 4 [$n=7$]). These hearts were perfused with glucose for 20 minutes, made globally ischemic for 20 minutes by complete occlusion of the perfusion line, and then reperfused with glucose for 60 minutes. In the other three groups, the effect of glycolytic inhibition on recovery of postischemic function was examined. Glycolysis was inhibited by 1) addition of iodoacetate, 2) depletion of glycogen, or 3) both interventions. In group 5 ($n=8$), hearts were perfused with pyruvate before and after 20 minutes of global ischemia. During reperfusion, glycolysis was inhibited by the addition of iodoacetate to the perfusate (150 μM for the first 15 minutes, followed by 50 μM for the next 45 minutes). In group 6 ($n=8$) and group 7 ($n=8$), partial glycogen depletion was induced by an initial 60 minutes of substrate-free perfusion before 20 minutes of global ischemia was imposed. Pilot studies in our laboratory have shown that this perfusion protocol reduces myocardial glycogen stores to approximately 20% of normal levels. After 60 minutes of substrate-free perfusion and 20 minutes of ischemia, residual myocardial glycogen stores were 2.5±0.4 μmol/g wet wt compared with 13.1±1.8 μmol/g in nonischemic hearts perfused for 60 minutes with 5 mM pyruvate. Both groups were reperfused with pyruvate, but while the glycolytic pathway was intact in group 6, it was blocked in group 7 by iodoacetate given according to the previous dose schedule. In all of these groups, the standard $[Ca]_o$ was 2.50 mM.

The effects of initial reperfusion with a low calcium solution on recovery of myocardial function were studied in another three groups of hearts, to evaluate whether the effects of glycolytic blockade in postischemic myocardium could be antagonized. In each of these groups, hearts were subjected to 20 minutes of global ischemia, but $[Ca]_o$ in the reperfusion solution was reduced to 0.63 mM for the first 30 minutes of reperfusion, before being increased to 2.50 mM for the final 30 minutes of the study. In group 8 ($n=4$), hearts were reperfused with pyruvate without iodoacetate. In group 9 ($n=7$), hearts were reperfused with pyruvate and iodoacetate, and in group 10 ($n=4$), hearts were depleted of glycogen by 60 minutes of substrate-free perfusion before the ischemic period and then reperfused with pyruvate and iodoacetate.

Statistical Analysis

Data within treatment groups at different time intervals were compared by repeated-measures analysis of variance.[36] Comparisons of ventricular function between groups were made with Student's unpaired t test with Bonferroni correction for repeated comparisons. Measurements of $[Ca^{2+}]_i$ were compared between the normal and glycolytic inhibition groups by the Mann-Whitney test. A two-tailed value of $p<0.05$ was regarded as significant, and data are reported as mean±SEM.

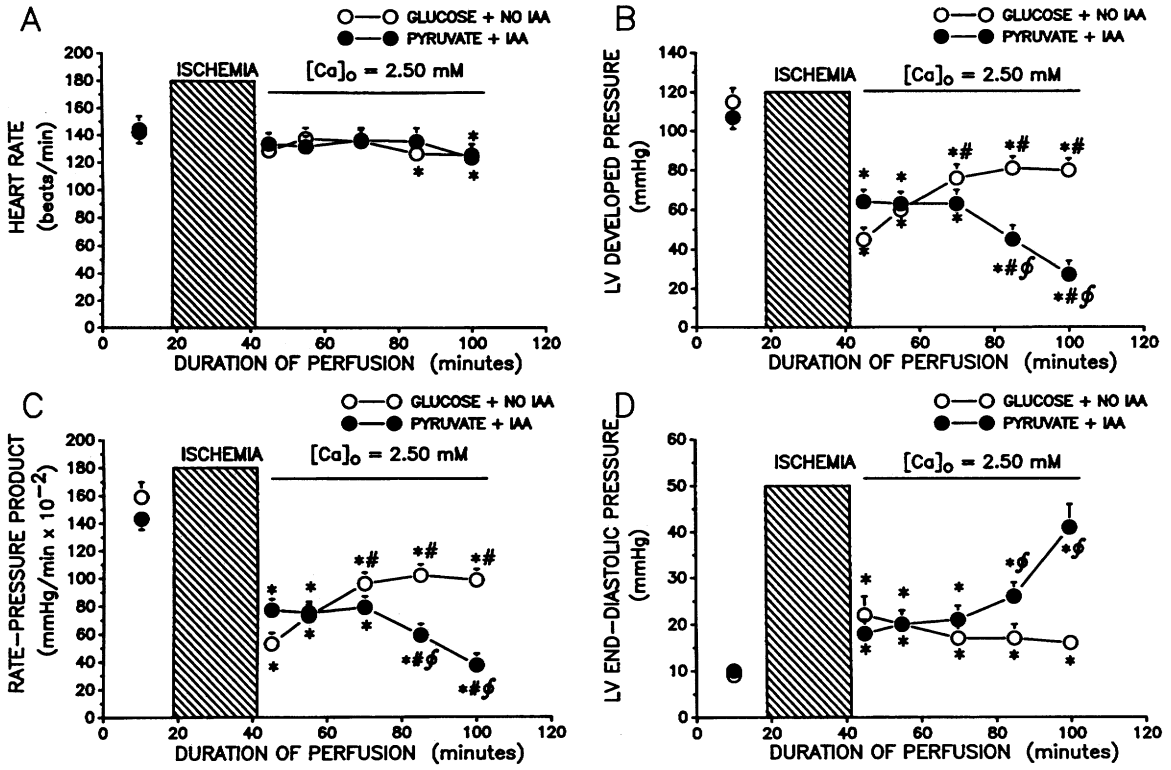

FIGURE 1. *Graphs showing comparison of myocardial function after 20 minutes of ischemia in hearts reperfused with glucose (n=7) or with pyruvate (n=8). Glycolysis was inhibited in the pyruvate group by administration of iodoacetate (IAA) at the onset of reperfusion. The [Ca]$_o$ was 2.50 mM in both groups during reperfusion. Changes in heart rate* (panel A), *left ventricular (LV) developed pressure* (panel B), *rate-pressure product* (panel C), *and LV end-diastolic pressure* (panel D) *are shown for both groups.* *p<*0.01 vs. preischemia,* #p<*0.01 vs. 5 minutes of reperfusion,* ϕp<*0.01 vs. glucose perfusion.*

Results

Glycolysis and Postischemic Function

Blockade of glycolysis with iodoacetate severely impaired the functional recovery of postischemic myocardium during reperfusion with normal calcium solutions (Figure 1). When hearts were reperfused with glucose, there was a progressive recovery of the rate/pressure product to 9,851±590 mm Hg/min after 1 hour. The left ventricular end-diastolic pressure was initially elevated on reperfusion (22±4 mm Hg) but decreased to 16±2 mm Hg ($p<0.05$) after 1 hour. In contrast, glycolytic blockade resulted in poor recovery of function, despite the presence of pyruvate as an alternate carbon substrate. The rate/pressure product after 1 hour of reperfusion was only 3,722±738 mm Hg/min ($p<0.001$ versus glucose). In addition, these hearts exhibited a progressive rise in left ventricular end-diastolic pressure from 18±3 mm Hg after 5 minutes of reperfusion to 41±5 mm Hg after 1 hour ($p<0.01$ versus glucose).

Glycogen depletion alone did not impair recovery of postischemic hearts (Figure 2). After 60 minutes of substrate-free perfusion and 20 minutes of ischemia, reperfusion with 5 mM pyruvate resulted in progressive recovery in contractile function, similar to that observed in hearts reperfused with glucose. Heart rate was mildly reduced after ischemia, but left ventricular developed pressure recovered toward baseline levels during reperfusion. The rate/pressure product increased from 5,022±771 mm Hg/min after 5 minutes of reflow to 11,017±735 mm Hg/min ($p=0.002$) after 1 hour. The left ventricular end-diastolic pressure was elevated at the time of reperfusion (21±4 mm Hg) but decreased to 11±3 mm Hg after 1 hour ($p<0.01$). Thus, residual glycogen stores after substrate-free perfusion and ischemia (approximately 20% of normal levels) were still sufficient to support recovery of the reperfused myocardium. Addition of iodoacetate blocked usage of this residual glycogen and severely impaired recovery of postischemic myocardium, which exhibited low developed pressures. After 1 hour the rate/pressure product in this group was only 2,143±882 mm Hg/min ($p<0.001$ versus reperfusion with pyruvate alone). The end-diastolic pressure increased progressively during reperfusion in this group, to 57±11 mm Hg after 1 hour ($p<0.01$ versus reperfusion with pyruvate alone).

Glycolysis and Intracellular Calcium

Individual ^{19}F NMR spectra obtained during ischemia and reperfusion are illustrated in Figure 3 from a heart with intact glycolysis reperfused with pyruvate (panel A) and from another heart in which glycolysis was blocked during reperfusion (panel B). In preischemic (control) myocardium, the Ca^{2+}-bound (B) and free (F) 5F-BAPTA resonances are of approximately equal intensity ([B]/[F]≈1). After 15 minutes of ischemia, there is an increase in $[Ca^{2+}]_i$, which is evident as an increase in the [B]/[F] ratio. The $[Ca^{2+}]_i$ increased further during the first few minutes of reperfusion. After 15–20 min-

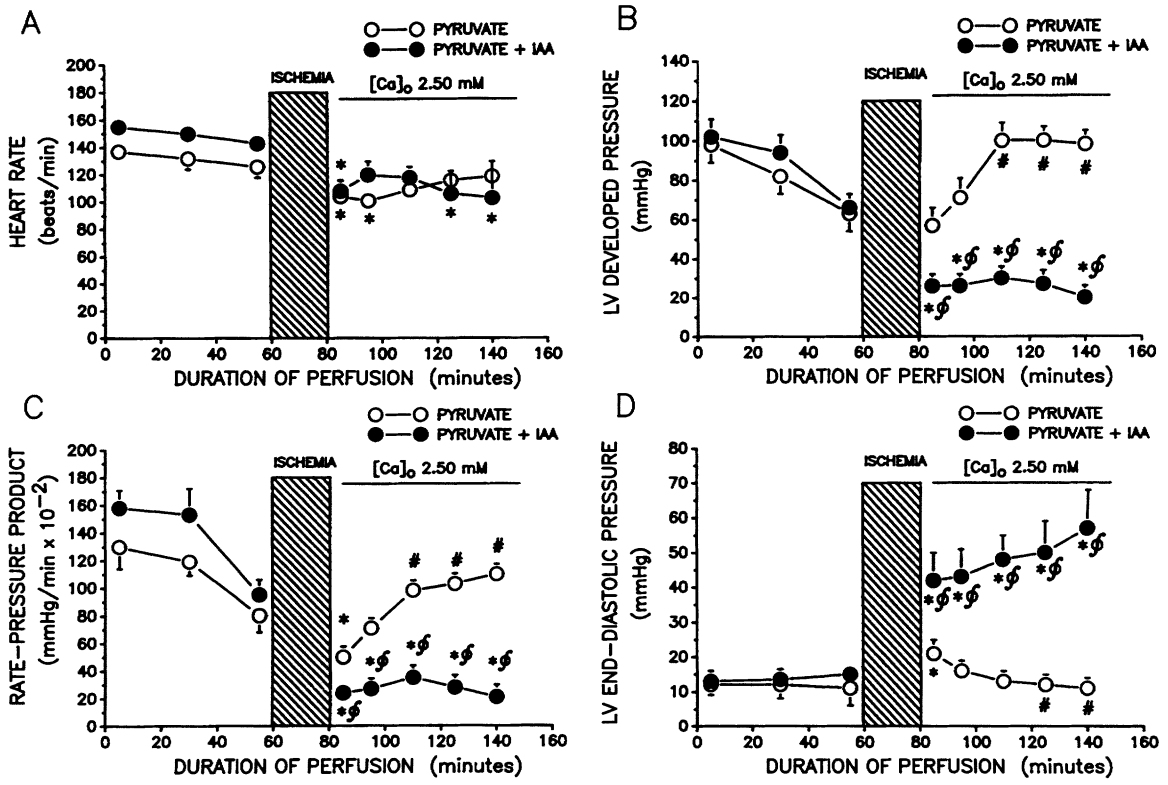

FIGURE 2. *Graphs showing comparison of the effects of glycolysis on recovery of function of postischemic myocardium in hearts depleted of glycogen by substrate-free perfusion during the first 60 minutes of the experiment. Hearts were reperfused with pyruvate, in the absence* (n=*8) or presence* (n=*8) of iodoacetate (IAA), from the onset of reperfusion. The [Ca]$_o$ was 2.50 mM in both groups during reperfusion. Changes in heart rate* (panel A), *left ventricular (LV) developed pressure* (panel B), *rate-pressure product* (panel C), *and LV end-diastolic pressure* (panel D) *are shown for both groups.* *p<*0.05 vs. presichemia,* #p<*0.01 vs. 5 minutes of reperfusion,* $p<*0.01 vs. pyruvate alone group.*

utes of reperfusion with intact glycolysis, the Ca^{2+}-bound and free 5F-BAPTA resonances recover toward their control values (panel A, reperfusion), indicating at least partial restoration of intracellular calcium homeostasis. In contrast, when glycolysis is inhibited by iodoacetate, the Ca^{2+}-bound 5F-BAPTA resonance remains very large in the postischemic myocardium (panel B, reperfusion), indicating persistence of intracellular Ca^{2+} overload.

The effect of glycolytic inhibition on $[Ca^{2+}]_i$ in reperfused myocardium is illustrated for the two groups in Figure 4. The myocardial concentration of 5F-BAPTA was stable throughout the experiments and was not affected by iodoacetate treatment (panels C and D). In the five control hearts perfused with pyruvate for 1 hour, without an ischemic period, $[Ca^{2+}]_i$ was stable throughout the experiment and was similar before (450±160 nM) and after (570±180 nM) iodoacetate treatment (data not shown). After 15 minutes of ischemia, before reperfusion, $[Ca^{2+}]_i$ increased approximately twofold from the preischemic value (260±80 to 560±50 nM, $p=0.035$ in the normal group, and 330±70 to 780±90 nM, $p=0.03$ in the group receiving iodoacetate during reperfusion). A further increase in $[Ca^{2+}]_i$ was observed during the first 5 minutes of reperfusion (normal reperfusion group, 820±150 nM, $p=0.02$ versus end ischemia; iodoacetate group, 1,100±180 nM, $p=0.07$ versus end ischemia). Subsequently, in the normal reperfusion group, $[Ca^{2+}]_i$ returned to preischemic levels. In the group with glycolytic inhibition, however, persistent elevation of $[Ca^{2+}]_i$ (1,290±120 nM, $p=0.016$ versus normal reperfusion) was observed throughout the 20-minute reperfusion period.

Low Calcium Reperfusion and Postischemic Function

In view of the associations between blockade of glycolysis, persistent elevation of $[Ca^{2+}]_i$, and poor functional recovery of postischemic myocardium, the effect of low calcium reperfusion on functional recovery was examined in the presence or absence of glycolytic blockade (Figure 5). Postischemic hearts with intact glycolysis reperfused with pyruvate and $[Ca]_o$ of 0.63 mM for the first 30 minutes, followed by $[Ca]_o$ of 2.50 mM for the next 30 minutes, exhibited a recovery in function to approximately 90% of preischemic levels at 1 hour (rate/pressure product, 12,492±1,561 mm Hg/min). Both heart rate and left ventricular developed pressure progressively recovered during the reperfusion period. This recovery was better than that observed in hearts reperfused with glucose and $[Ca]_o$ of 2.50 mM (rate/pressure product, 9,851±590 mm Hg/min, $p=0.01$). Initial reperfusion with a low calcium solution antagonized the effects of glycolytic inhibition with iodoacetate. In hearts reperfused with pyruvate and iodoacetate and exposed to 0.63 mM $[Ca]_o$ for the first

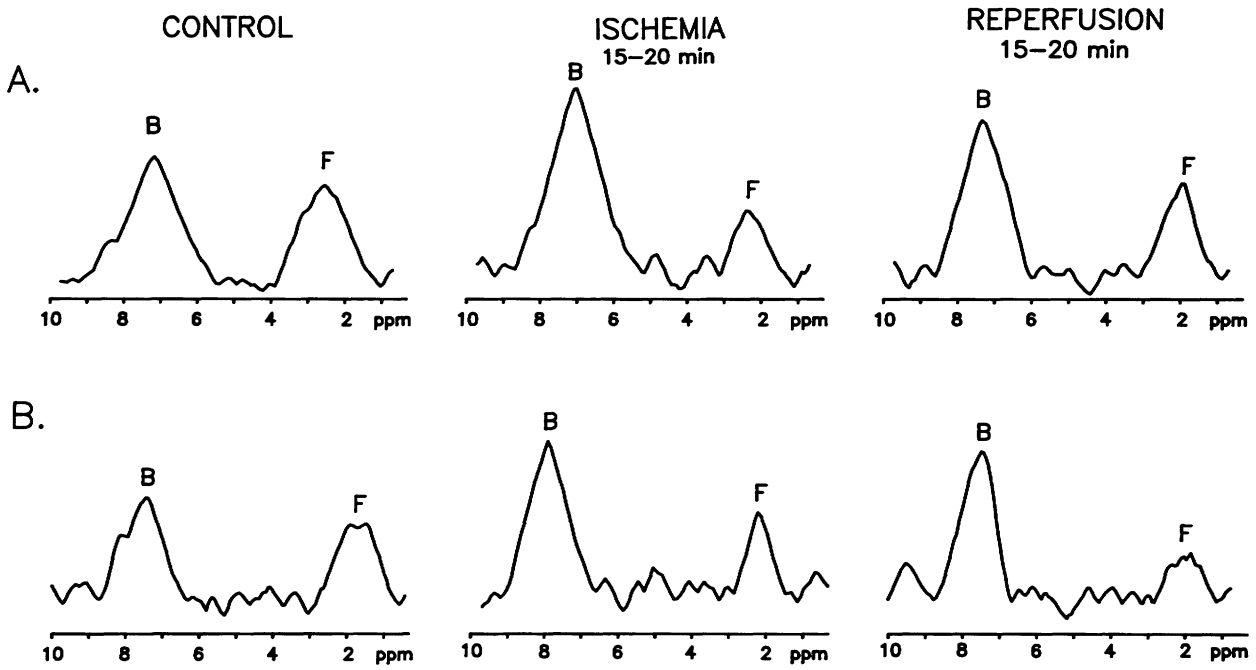

FIGURE 3. *Examples of ^{19}F nuclear magnetic resonance spectra of the calcium indicator 5F-BAPTA before and after ischemia and reperfusion from two hearts, one with intact glycolysis* (panel A) *and one with glycolytic inhibition* (panel B). *The resonance peak B represents Ca^{2+}-bound 5F-BAPTA, and peak F represents free 5F-BAPTA. In the heart with intact glycolysis (panel A), the increase in $[Ca^{2+}]_i$ during ischemia is evident, with resolution toward normal levels after 15 minutes of reperfusion. In contrast, in a heart reperfused with iodoacetate (panel B), the Ca^{2+} overload observed during ischemia persists throughout reperfusion.*

30 minutes, recovery of function at 1 hour (rate/pressure product, 13,858±2,073 mm Hg/min) was equivalent to hearts reperfused without iodoacetate. The left ventricular developed pressure in these hearts remained similar to that in hearts with intact glycolysis. This is in marked contrast to the findings in hearts reperfused with 2.50 mM $[Ca]_o$ and iodoacetate, which had severely impaired function after 1 hour of reperfusion (rate/pressure product, 3,722±732 mm Hg/min, $p<0.001$ versus low calcium reflow group). Initial reperfusion with $[Ca]_o$ of 0.63 mM also prevented the rise in left ventricular end-diastolic pressure that occurred with glycolytic blockade when $[Ca]_o$ was 2.50 mM. After 30 minutes of perfusion with $[Ca]_o$ of 0.63 mM, return to perfusion with $[Ca]_o$ of 2.50 mM was associated with a further improvement in left ventricular function in both groups, suggesting that the critical period for glycolytic activity is within the first 30 minutes of reperfusion.

Initial reperfusion with $[Ca]_o$ at 0.63 mM also ameliorated the functional impairment observed with iodoacetate in glycogen-depleted hearts (Figure 6). Glycogen-depleted hearts receiving iodoacetate after ischemia exhibited little recovery of function when $[Ca]_o$ was 2.50 mM throughout reperfusion (rate/pressure product, 2,143±882 mm Hg/min, after 1 hour). These hearts had a low left ventricular developed pressure and a progressive rise in left ventricular end-diastolic pressure during the 1-hour reperfusion period. In contrast, reperfusion with $[Ca]_o$ of 0.63 mM for the first 30 minutes resulted in a significant recovery of contractile function over the 1-hour reperfusion period (rate/pressure product, 7,071±1,400 mm Hg/min, $p=0.01$ versus $[Ca]_o$ of 2.50 mM). Left ventricular developed pressure was higher and end-diastolic pressure significantly lower than in hearts reperfused with $[Ca]_o$ of 2.50 mM.

Discussion

The present findings support the hypothesis that glycolysis continues to play an important role during the early reperfusion period in postischemic myocardium. Inhibition of glycolysis with iodoacetate during reperfusion results in persistent myocardial calcium overload, indicating that glycolytic ATP is required for restoration of calcium homeostasis after ischemia. Furthermore, the functional recovery of postischemic myocardium is impaired in the presence of glycolytic inhibition, most probably as a result of the persistent myocyte calcium overload. This relation between glycolysis, calcium homeostasis, and myocardial function is further supported by the present observations that the functional impairment associated with inhibition of glycolysis in postischemic myocardium is significantly ameliorated by reperfusion with a hypocalcemic solution.

Methods of Inhibition of Glycolysis

A principal requirement for this study was the rapid and irreversible inhibition of glycolysis during early reperfusion. Several techniques of glycolytic inhibition have been described, including use of iodoacetate, 2-deoxyglucose, and glycogen depletion.[31] Because the competitive substrate antagonist 2-deoxyglucose is an incomplete inhibitor of glycolysis and is slow to effect inhibition,[31] it was not used in the present study. Iodoacetate is a potent and irreversible inhibitor of glycolysis.[32] At the concentrations used in this study, iodoace-

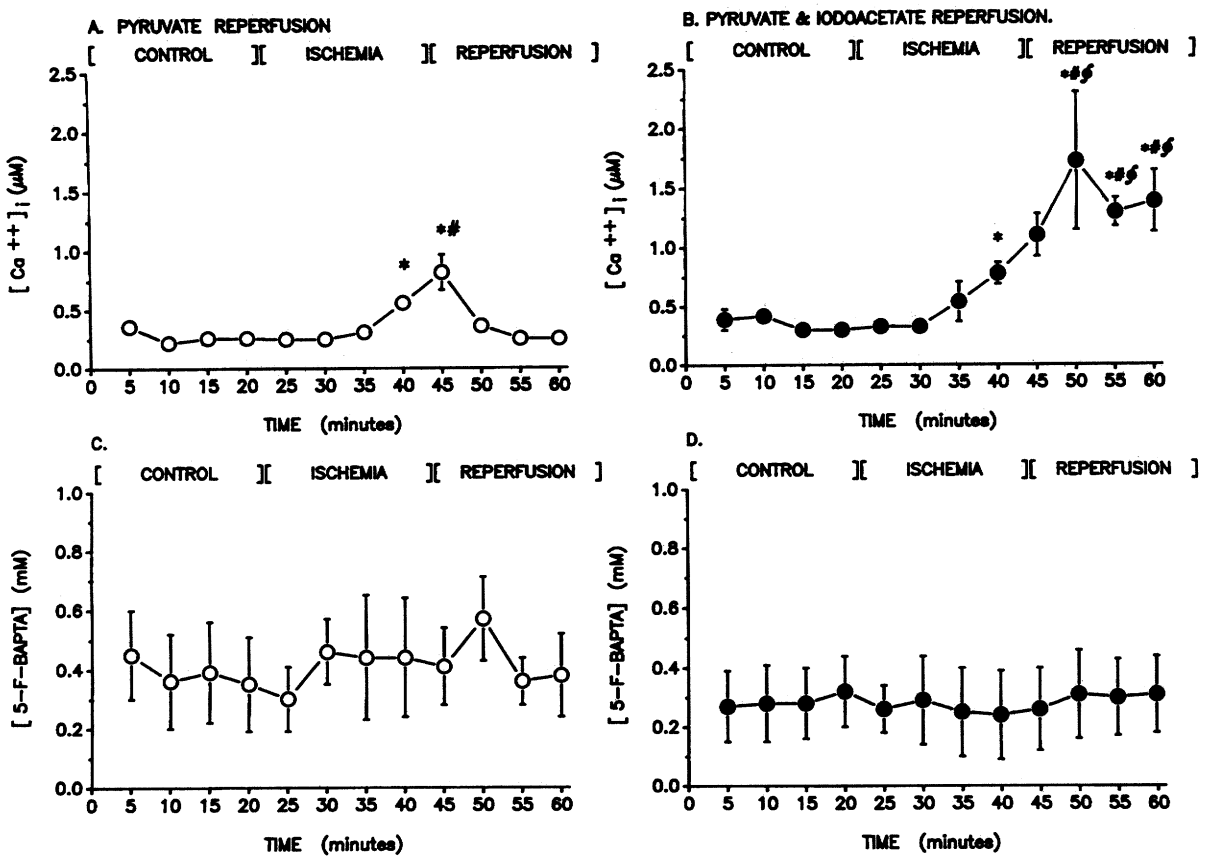

FIGURE 4. *Graphs showing changes in myocyte $[Ca^{2+}]_i$* (panels A *and* B) *during ischemia and reperfusion in hearts with intact glycolysis (pyruvate reperfusion,* n=*5) and in hearts with glycolytic inhibition by iodoacetate* (n=*4). Persistence of Ca^{2+} overload is evident in hearts reperfused with iodoacetate. In both groups, myocardial concentrations of 5F-BAPTA remained stable throughout the experiment* (panels C *and* D). *p<*0.05 vs. preischemic control period,* #p<*0.05 vs. end ischemia,* $p<*0.02 vs. pyruvate alone group.*

tate does not inhibit other enzymes, such as hexokinase, phosphofructokinase, pyruvate kinase,[33] or succinic dehydrogenase.[34] In addition, oxidative phosphorylation in isolated myocardial mitochondria is unaffected by these concentrations of iodoacetate.[32,35] The specificity of iodoacetate as an inhibitor of glycolysis is supported by observations that iodoacetate does not impair the function of normal myocardium when isolated hearts are perfused with pyruvate[37] or butyrate[33] as substrates for oxidative phosphorylation. A nonspecific effect of iodoacetate on $[Ca^{2+}]_i$ is excluded by the observation that $[Ca^{2+}]_i$ in normal myocardium is unchanged by the addition of iodoacetate. The rate of inhibition of glycolysis by iodoacetate is relatively rapid, but not instantaneous, with approximately 95% inhibition of glyceraldehyde-3-phosphate dehydrogenase after 20 minutes of perfusion with iodoacetate.[33]

The glycogen depletion induced by the present protocol of 1 hour of substrate-free perfusion, before the ischemic period, was incomplete. Other investigators have suggested that glycogen depletion may be accelerated by β-adrenergic stimulation of the heart during substrate-free perfusion,[31] but complete depletion of myocardial glycogen stores would be incompatible with the heart surviving the subsequent ischemic period. The residual glycogen stores appeared to be sufficient to support necessary glycolysis during the early reperfusion period, because no functional impairment was observed on reperfusion with pyruvate in this study. However, glycogen depletion was synergistic with iodoacetate in the impairment of function of postischemic myocardium. This observation is consistent with the initial time dependency of achieving complete glycolytic blockade with iodoacetate.

Measurement of Intracellular Calcium

In this study, we have used ^{19}F NMR spectroscopy and the intracellular calcium chelator 5F-BAPTA. This technique has several advantages, including the fact that 5F-BAPTA does not interfere with myocardial high energy phosphate metabolism and does not alter myocardial metabolic changes occurring during ischemia and reperfusion.[16] There are, however, several caveats to the use of 5F-BAPTA. First, the intracellular calcium buffering induced by 5F-BAPTA results in a marked negative inotropic effect. Although this can be partially countered by increasing $[Ca]_o$ to 8 mM,[29] as was done in the present study, evaluation of functional changes during reperfusion requires parallel experiments in hearts not exposed to 5F-BAPTA. The pattern of changes in $[Ca^{2+}]_i$ observed during ischemia and reperfusion is similar with $[Ca]_o$ of 8 mM and $[Ca]_o$ of 2 mM.[16]

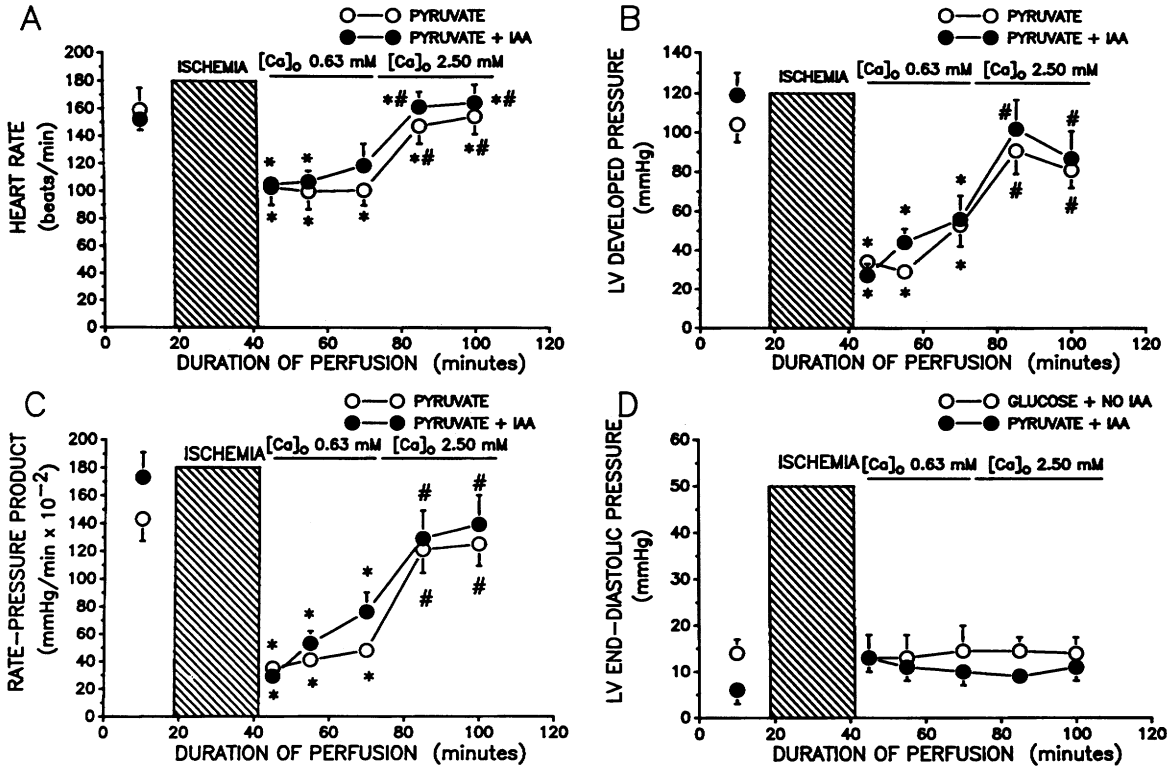

FIGURE 5. *Graphs showing the effects of 30 minutes of initial reperfusion with a low calcium ($[Ca]_o$, 0.63 mM) solution on the recovery of contractile function in hearts reperfused with pyruvate* (n=4) *or pyruvate and iodoacetate (IAA,* n=7*). Changes in heart rate* (panel A), *left ventricular (LV) developed pressure* (panel B), *rate-pressure product* (panel C), *and LV end-diastolic pressure* (panel D) *are shown for both groups. In both groups the $[Ca]_o$ was 0.63 mM for the first 30 minutes of reperfusion, before being increased to 2.50 mM. Despite glycolytic inhibition with IAA, reperfusion with a low calcium solution was associated with good functional recovery, similar to that of hearts reperfused without IAA.* *p<0.01 *vs. preischemia,* #p<0.01 *vs. 30 minutes of reperfusion.*

At high levels of Ca^{2+}_i, the 5F-BAPTA indicator becomes saturated and will then tend to underestimate the actual degree of myocardial Ca^{2+} loading. As a result, the present measurements of the absolute degree of $[Ca^{2+}]_i$ overload occurring during late ischemia and early reperfusion are probably a lower limit estimate of the true $[Ca^{2+}]_i$.[16] The temporal resolution of the 5F-BAPTA method is also limited,[16] and in this study data for measurements of $[Ca^{2+}]_i$ were acquired in 5-minute blocks. As a result of this temporal averaging, it is possible that some brief changes in $[Ca^{2+}]_i$ may not be detected by the present technique. A rapid onset and short-lived peak in $[Ca^{2+}]_i$ may occur immediately on reperfusion and not be evident in the spectra in this study. The principal finding of this study, that $[Ca^{2+}]_i$ overload is prolonged in the presence of glycolytic inhibition, is not, however, invalidated by the temporal constraints of $[Ca^{2+}]_i$ measurement.

Intracellular Calcium During Ischemia and Reperfusion

In these perfused hearts intracellular calcium levels increased after 15 minutes of ischemia, and this time course is similar to observations of other investigators.[13,14] The further increase in $[Ca^{2+}]_i$ during early reperfusion is also consistent with studies in a ferret model of ischemia and reperfusion.[16,29] The potential sources of this $[Ca^{2+}]_i$ overload during ischemia and reperfusion include direct entry via voltage-dependent channels,[38] intracellular acidosis and Na^+ accumulation with subsequent Ca^{2+} entry via Na^+-Ca^{2+} exchange,[15,39,40] or redistribution of Ca^{2+} from intracellular stores such as the mitochondria or the sarcoplasmic reticulum.[41] There is some evidence to support each of these mechanisms. Treatment with calcium channel blockers can reduce $[Ca^{2+}]_i$ accumulation during ischemia.[38] Alteration of the transsarcolemmal H^+-Na^+ balance, resulting from treatment with amiloride[39] or reperfusion with an acidotic medium,[42] also reduces $[Ca^{2+}]_i$ overload. Finally, calcium transport by the Ca^{2+},Mg^{2+}-ATPase of the sarcoplasmic reticulum appears to be reduced in postischemic myocardium,[41] possibly as a result of membrane damage by oxygen free radicals.[43] The present experiments do not allow definition of the specific pathogenesis of $[Ca^{2+}]_i$ overload during late ischemia and early reperfusion, but recent studies suggest that oxygen free radicals may be directly involved.[44]

In previous studies, myocardial $[Ca^{2+}]_i$ overload has been found to resolve after the first 5 minutes of reperfusion.[13,16] The present findings are the first evidence that postischemic myocardial $[Ca^{2+}]_i$ overload can be prolonged. Furthermore, this prolongation of $[Ca^{2+}]_i$ overload appears to result specifically from inhibition of glycolysis during early reperfusion.

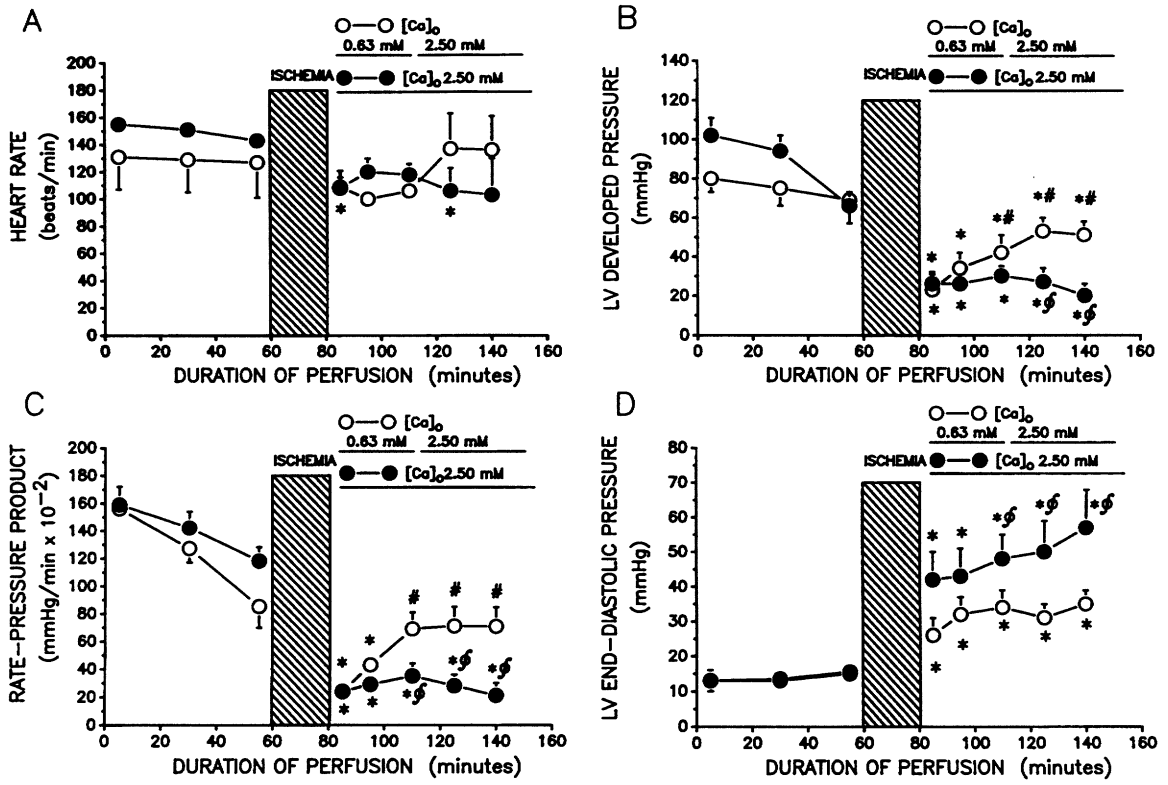

FIGURE 6. *Graphs showing the effects of different $[Ca]_o$ on function of hearts partially depleted of glycogen by substrate-free perfusion during the first 60 minutes of the experiment, with inhibition of glycolysis by iodoacetate during reperfusion. Changes in heart rate* (panel A), *left ventricular (LV) developed pressure* (panel B), *rate-pressure product* (panel C), *and LV end-diastolic pressure* (panel D) *are shown for both groups. Hearts reperfused with $[Ca]_o$ of 2.50 mM (filled circles) exhibited poor recovery of LV developed pressure and high end-diastolic pressure. Hearts reperfused with $[Ca]_o$ of 0.63 mM for the first 30 minutes after ischemia (open circles) showed better recovery of LV developed pressure and lower end-diastolic pressure, which was maintained after increasing $[Ca]_o$ to 2.50 mM.* *p<*0.01 vs. preischemia,* #p<*0.01 vs. 5 minutes of reperfusion,* ϕp<*0.01 vs. low calcium reperfusion.*

Relation Between Glycolysis and Calcium Homeostasis

The exact mechanisms of resolution of $[Ca^{2+}]_i$ overload are still undefined, but presumably involve pumping of $Ca^{2+}{}_i$ into the sarcoplasmic reticulum and/or transport of $Ca^{2+}{}_i$ out of the myocyte into the interstitium, both of which are energy-requiring processes.[45,46] The active nature of resolution of $[Ca^{2+}]_i$ overload is supported by the present findings, which further indicate that glycolysis is the required energy source.

There are three possible reasons for the observed relation between glycolytic inhibition, failure of Ca^{2+} homeostasis, and impaired functional recovery after ischemia. In the first case, the $[Ca^{2+}]_i$ overload may reflect absolute depletion of myocardial ATP.[47] In the second case, oxidative phosphorylation may be impaired during early reperfusion, so that myocardial ATP synthesis remains dependent on glycolysis. Finally, there may be a specific relation between ATP synthesis via glycolysis and Ca^{2+} transport processes.

During ischemia, myocardial phosphocreatine is depleted, but after reperfusion phosphocreatine levels are rapidly restored and ATP levels are typically 70–80% of preischemic values.[16,48] It is unlikely, however, that $[Ca^{2+}]_i$ overload in the present study was primarily caused by ATP depletion resulting from glycolytic inhibition, as the rise in $[Ca^{2+}]_i$ preceded the introduction of iodoacetate. The levels of myocardial ATP that have been observed after 20 minutes of ischemia[48] still considerably exceed the levels that have been associated with failure of $[Ca^{2+}]_i$ homeostasis and rigor during ischemia.[49] After reperfusion, recovery of contractile function is largely independent of myocardial ATP levels[48] and normoxic perfused hearts exhibit preserved contractile function, even in the presence of marked ATP depletion.[50]

A characteristic feature of postischemic myocardium is depressed contractile function in the presence of near-normal levels of myocardial oxygen consumption.[51,52] This may reflect either uncoupling of oxidative phosphorylation or inefficient use of ATP by the contractile apparatus. Although myocardial high energy phosphate metabolism was not measured in this study, current evidence indicates that oxidative phosphorylation is not uncoupled in the myocardium after brief periods of ischemia.[53] If oxidative phosphorylation was uncoupled after ischemia, then oxygen consumption in the arrested, postischemic heart should be increased, but this was not found to be the case in recent studies by Laster et al.[51] It is more likely that the disparity between oxygen consumption and myocardial contractile function reflects inefficient use of ATP by the myofilaments,

perhaps as a result of altered Ca^{2+} sensitivity of the contractile apparatus or impaired activity of the myofibrillar ATPase. Such a defect at the level of the myofilaments would be consistent with observations that the Ca^{2+} transient is paradoxically increased in postischemic myocardium.[54]

The probable explanation of the present findings is a specific relation between glycolytic ATP and Ca^{2+} transport processes. A functional compartmentation of glycolytic and oxidative ATP use has been suggested by several investigators.[22–25] Both glycogenolytic and glycolytic enzymes appear to be associated with the sarcoplasmic reticulum and the sarcolemma.[19–21] A compartmentation of ATP use would impart to glycolytic ATP a qualitative, rather than quantitative, significance. Even maximal stimulation of glycolysis appears capable of yielding <10% of the ATP available from normal basal rates of oxidative phosphorylation.[55]

The functional recovery of postischemic myocardium is impaired when glycolysis is inhibited. The findings of Sako et al,[11] that functional recovery of postischemic hearts is improved when glucose is added to the perfusion medium, are complementary to our findings. The question then arises of why the postischemic myocardium should be particularly dependent on glycolysis. One reason may be the magnitude of the intracellular Ca^{2+} overload, with increases in $[Ca^{2+}]_i$ of up to 10 times normal during early reperfusion. The rapid resolution of such an intracellular Ca^{2+} load would greatly increase demand for glycolytic ATP compared with normal myocardium. In addition, the Ca^{2+} membrane transport pumps may be impaired during ischemia and reperfusion, most probably as a result of membrane damage mediated by oxygen free radicals.[43] Indeed, there is evidence to indicate that the calcium pumping capacity of the sarcoplasmic reticulum is impaired in postischemic myocardium.[41] Reduced efficiency of the Ca^{2+} pump of the sarcoplasmic reticulum may then also be expected to increase ATP requirements during early reperfusion. The early reperfusion period may thus be viewed as a "stress" period for the heart, which is faced with Ca^{2+} overload and impaired mechanisms for effecting its resolution.

Conclusion

Glycolysis continues to play an important role during the early reperfusion period. Inhibition of glycolysis is associated with persistent Ca^{2+} overload and impaired functional recovery of postischemic myocardium. There appears to be a specific relation between glycolytic activity and restoration of Ca^{2+} homeostasis in postischemic myocardium.

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Relation between glycolysis and calcium homeostasis in postischemic myocardium.

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