Low Ca^{2+} Reperfusion and Enhanced Susceptibility of the Postischemic Heart to the Calcium Paradox

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This study was designed to define the effect of postischemic low Ca^{2+} perfusion on recovery of high-energy phosphates, intracellular pH, and contractile function in isolated rat hearts. Phosphorus-31 nuclear magnetic resonance spectroscopy was used to follow creatine phosphate, adenosine triphosphate, intracellular inorganic phosphate, and intracellular pH during control perfusion (15 minutes), total ischemia (30 minutes), and reperfusion (30 minutes). In Group I the perfusate [Ca^{2+}] was 1.3 mmol/l throughout the experiment, whereas in Group II the perfusate [Ca^{2+}] was reduced to 0.05 mmol/l during the first 10 minutes of reperfusion. Hearts from Group III were not made ischemic but were subjected to 10 minutes of low Ca^{2+} perfusion followed by 20 minutes of normal Ca^{2+} perfusion. During low Ca^{2+} reperfusion (Group II) recovery of high-energy phosphates and pH was significantly better than in controls (Group I). However, after reexposure to normal Ca^{2+}, metabolic recovery was largely abolished, coronary flow was suddenly impaired, and contracture developed without any rhythmic contractions. These observations indicated the occurrence of a calcium paradox rather than postponed ischemia-reperfusion damage. On the other hand, normoxic hearts (Group III) tolerated temporary perfusion with 0.05 mmol/l Ca^{2+} very well with respect to left ventricular developed pressure, coronary flow, and metabolic parameters. In conclusion, postischemic low Ca^{2+} (0.05 mmol/l) perfusion may reduce reperfusion damage, but at the same time ischemia appears to enhance the susceptibility of the heart to the calcium paradox. (*Circulation Research* 1989;64:1158–1164)

Myocardial ischemia has numerous consequences, which can be explained by a reduced availability of oxygen and substrate and accumulation of metabolic waste products.[1] Reperfusion after a limited period of ischemia may either initiate a direct or delayed restoration of metabolism and function or lead to a rapid deterioration, loss of cellular constituents, and accelerated cell death.[1,2] The severity and duration of the ischemic event are generally believed to determine the eventual outcome.

Several interventions preceding a temporary ischemic event have been found to limit ischemic damage and thereby enhance recovery of metabolism and function on reperfusion.[3–5] Preservation of high-energy phosphates and maintenance of ionic homeostasis during ischemia appear to be the key determinants of the protective action.[3,6,7] More recently, interest has been diverted to protective interventions at the time of reperfusion, and several studies indicate that reversibility of ischemic or hypoxic damage may be affected by reperfusion conditions.[3,8–12]

Since Ca^{2+} accumulation in myocardial cells has been shown to play a major role in reperfusion injury,[2,13] limitation of uncontrolled Ca^{2+} influx might enhance survival of cells after ischemia. Calcium antagonists, however effective when given before ischemia,[3,5,14] generally fail to protect the heart when given only during reperfusion[3,6,9] or offer only limited protection[8]; this suggests a pathway for Ca^{2+} entry during reperfusion other than the slow channels.[6] On the other hand, a large reduction of the extracellular [Ca^{2+}] during reperfusion may well afford protection, although at the same time this may set the stage for the calcium paradox.[8,15]

The present study was performed to investigate whether a temporary reduction of the perfusate [Ca^{2+}] during the initial phase of reperfusion, before reexposure to normal Ca^{2+}, can modify reperfusion

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injury. Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy offers the unique possibility of following the time course in high-energy phosphates, inorganic phosphate, and pH during ischemia and the two-step reperfusion protocol in isolated rat hearts, with a simultaneous assessment of myocardial function.

Materials and Methods

Animal Preparations

Male Wistar rats weighing 325–375 g were anesthetized with diethyl ether and heparinized (250 IU i.v.). The heart was rapidly excised and cooled in ice-cold perfusate. After cannulation of the aorta, retrograde perfusion was started at a constant pressure of 100 cm H_2O (10.0 kPa). The standard perfusate contained (mmol/l) NaCl 124.0, KCl 4.7, $MgCl_2$ 1.0, $CaCl_2$ 1.3, $NaHCO_3$ 24.0, Na_2HPO_4 0.5, and glucose 11.0. The perfusate was filtered (0.8-μm filters, Millipore, Bedford, Massachusetts) before use and saturated with 95% O_2-5% CO_2, resulting in a pH of 7.35±0.05 at 37° C. For assessment of contractile function, a perfusate-filled catheter[16,17] was inserted through the apex and connected to a Statham P23dB pressure transducer (Gould, Cleveland, Ohio). The pressure signal was recorded on a Gould Brush recorder, and the difference between end-systolic and end-diastolic pressure was taken to be the left ventricular developed pressure. Heart rate was maintained at 300 beats/min throughout the experiment by right ventricular pacing with two sodium chloride wick electrodes connected to a Grass S88 stimulator (Grass Instrument, Quincy, Massachusetts). Hearts were placed in a 20-mm NMR tube together with a capillary containing methylene diphosphonate as a spectral reference. The glass tube with the heart was then lowered into the NMR coil. The effluent was removed from a level above the heart, leaving the heart submerged in a fixed volume of perfusate. The effluent was collected in 5-minute fractions for determination of coronary flow. Myocardial temperature was maintained at 37° C by water-jacketed perfusion lines to the heart and a continuous stream of air (37° C) around the sample tube.

NMR Measurements

^{31}P NMR spectra were obtained at 81.0 MHz on an MSL 200 spectrometer (Bruker, Karlsruhe, FRG) equipped with a 4.7 Tesla vertical bore magnet. For each spectrum, 128 free-induction decays were accumulated after 90° pulses by use of 2K data points and a 5-kHz spectral width at a repetition time of 2.3 seconds. Accumulated free-induction decays were exponentially filtered, resulting in 10-Hz line broadening. After an automatic polynomial baseline correction of the spectra, quantitation of metabolites was achieved by integration of the areas under the individual peaks of interest in each spectrum. Values for creatine phosphate (CP) and ATP (β-ATP) were expressed as a percentage of their respective preischemic values; intracellular inorganic phosphate (P_i) was expressed as a percentage of the sum of phosphate from CP, ATP, and P_i during preischemic control perfusion by the equation

$$(P_i/\{CP+3\ ATP+P_i\}_{preischemic})\times 100\%$$

CP and P_i were corrected for partial saturation; the saturation factors, 1.5 and 1.1, respectively, were determined using a 10-second recycle time. Intracellular and extracellular pH values were calculated from the chemical shift of the respective P_i peaks relative to methylene diphosphonate. A value of 0 ppm was assigned to CP.

Experimental Protocol

After a stabilization period of about 20 minutes, three control spectra were obtained in all hearts. The hearts were then randomly subjected to one of three protocols. In Groups I and II the hearts were made totally ischemic for 30 minutes, followed by 30 minutes of reperfusion. In control hearts (Group I) the $[Ca^{2+}]$ in the perfusate was 1.3 mmol/l throughout the experiment, whereas in Group II the $[Ca^{2+}]$ during the initial 10 minutes of reperfusion was lowered to 0.05 mmol/l. For this purpose a second perfusion line was used, which allowed immediate start of reperfusion at a low $[Ca^{2+}]$. During the last 20 minutes of reperfusion the hearts from Group II were perfused with the standard perfusate. The hearts from Group III were not made ischemic but were exposed to 10 minutes of low Ca^{2+} perfusion, followed by 20 minutes of normal Ca^{2+} perfusion.

Statistical Analysis

Results are presented as mean±SD of nine of 11 experiments. Analysis of variance with repeated measurements was used for determination of the effect of temporary reperfusion with low Ca^{2+} containing perfusate on metabolic parameters. Results obtained during ischemia, during the first 10 minutes of reperfusion, and during the remainder of the reperfusion period were analyzed separately. In normoxic hearts exposed to 10 minutes of low Ca^{2+} (Group III), analysis of variance with repeated measurements was carried out for comparison of normal Ca^{2+} reperfusion with the initial control perfusion. A test result of $p<0.05$ was considered significant.

Results

Ischemia

Figure 1 shows the effect of total interruption of coronary perfusion on CP, ATP, and intracellular P_i. The rapid degradation of CP and ATP was balanced by an increase in intracellular P_i. Intracellular pH dropped rapidly from 7.06±0.02 during control perfusion to 5.84±0.06 after 15 minutes of ischemia. Contractile activity was no longer observed after 2.5–3 minutes of ischemia.

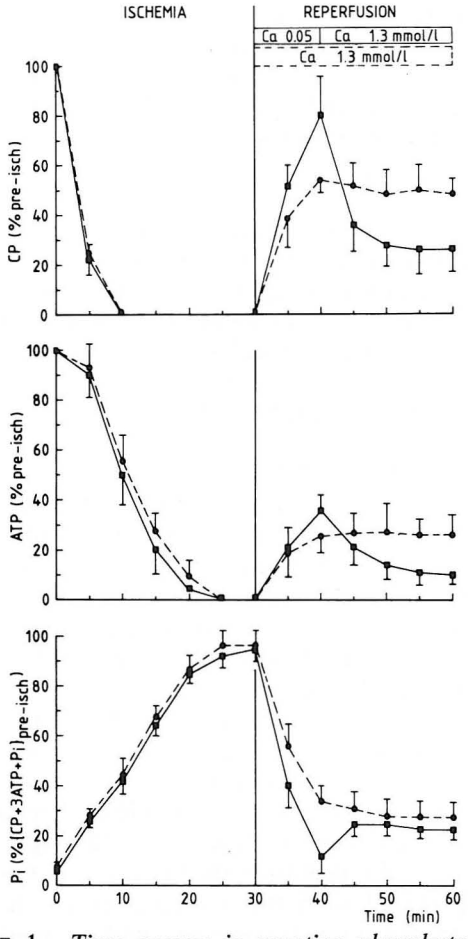

FIGURE 1. *Time course in creatine phosphate (CP), ATP, and intracellular inorganic phosphate (P_i) in isolated rat hearts during ischemia (30 minutes) and reperfusion (30 minutes) as measured by ^{31}P nuclear magnetic resonance. During ischemia no significant differences were observed between control Group I (●), in which [Ca^{2+}] was 1.3 mmol/l throughout the experiment* (n=9), *and Group II (■), in which [Ca^{2+}] was lowered to 0.05 mmol/l during the first 10 minutes of reperfusion* (n=11). *Low Ca^{2+} reperfusion temporarily improved recovery of CP (* $p<0.001$) *and ATP (* $p<0.02$) *and restored low levels of intracellular P_i (* $p<0.001$) *as compared with controls. Normalization of perfusate Ca^{2+} largely abolished this recovery. For further explanation, see text. Data are presented as mean±SD.*

Normal Ca^{2+} Reperfusion

Reperfusion with the standard perfusate (Group I) resulted in a partial recovery of CP and ATP (Figure 1). Intracellular P_i decreased, but remained elevated in comparison with preischemic levels. Statistical analysis indicated that after 10 minutes of reperfusion, no further changes occurred. In addition, the ^{31}P NMR spectra were characterized by the presence of multiple P_i peaks (Figure 2), most likely due to differences of intracellular pH among myocardial cells.[18] Despite this incomplete or nonhomogeneous recovery, the main P_i peak corresponded with a pH of 7.00–7.10. Figure 3 shows that coronary flow

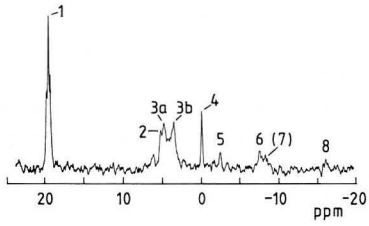

FIGURE 2. *^{31}P nuclear magnetic resonance spectrum obtained from an isolated rat heart between 25 and 30 minutes of normal Ca^{2+} reperfusion after 30 minutes of ischemia (Group I). Numbered peaks are as follows: 1, methylene diphosphonate; 2, perfusate inorganic phosphate (P_i) at pH 7.40; 3a, intracellular P_i at a normalized pH of 7.05; 3b, extracellular or intracellular P_i at pH between 7.00 and 5.85; 4, creatine phosphate; 5, 6, and 8, γ-, α-, and β-phosphate group of ATP, respectively; and (7), nicotinamide adenine dinucleotide.*

upon reperfusion was impaired, which, in combination with the different pH values, indicated a partial no-reflow phenomenon. By the end of 30 minutes of reperfusion, left ventricular function had recovered to 40±18% of preischemic values.

Low Ca^{2+} Reperfusion

Initial reperfusion with 0.05 mmol/l Ca^{2+} (Group II) resulted in a significantly better recovery of CP ($p<0.001$) and ATP ($p<0.02$) than in control hearts (Figure 1). During low Ca^{2+} reperfusion, intracellular P_i levels returned to preischemic levels and were significantly lower than intracellular P_i levels in control hearts during the corresponding period ($p<0.001$). In eight of 11 hearts intracellular pH recovered homogeneously to preischemic values, although in four hearts accurate pH determination was hampered due to low levels of intracellular P_i.

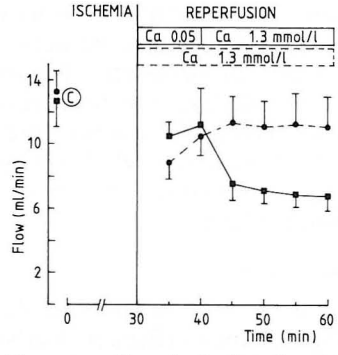

FIGURE 3. *Coronary flow in isolated rat hearts during control perfusion (C) and during reperfusion after 30 minutes of ischemia. Coronary flow during control perfusion did not differ between control Group I (●), in which [Ca^{2+}] was 1.3 mmol/l throughout the experiment* (n=9), *and Group II (■), in which [Ca^{2+}] was lowered to 0.05 mmol/l during the first 10 minutes of reperfusion* (n=11). *During normalization of perfusate [Ca^{2+}] after 10 minutes of low Ca^{2+} reperfusion, coronary flow was significantly less than in controls (* $p<0.001$). *Data are presented as mean±SD.*

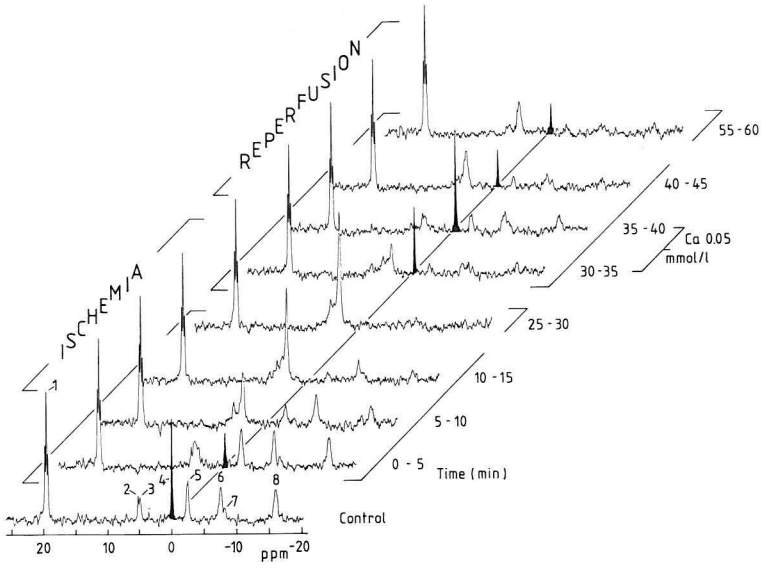

FIGURE 4. *^{31}P nuclear magnetic resonance spectra obtained from an isolated perfused rat heart during control perfusion, ischemia, and reperfusion. During the first 10 minutes of reperfusion, perfusate [Ca^{2+}] was lowered to 0.05 mmol/l (Group II). Numbered peaks are as follows: 1, methylene diphosphonate; 2, extracellular inorganic phosphate (P_i); 3, intracellular P_i; 4, creatine phosphate; 5, 6, and 8, γ-, α-, and β-phosphate group of ATP, respectively; 7, nicotinamide adenine dinucleotide.*

In three hearts the intracellular P_i peak was split into a peak corresponding with normalized pH and a peak corresponding with low intracellular pH. In all respects metabolic recovery at the end of the 10-minute reperfusion period was significantly better in Group II hearts than in Group I (control) hearts.

However, on returning to the standard perfusate the initial metabolic recovery was largely abolished, as indicated by a second decrease in CP and ATP (Figure 1). The simultaneous increase in intracellular P_i did not parallel the fall in high-energy phosphates, indicating a decrease of total phosphate. The metabolic state at the end of the reperfusion period was significantly worse than in control hearts ($p<0.0001$). Figure 4 shows a set of ^{31}P NMR spectra obtained from a heart from Group II during control perfusion, ischemia, and reperfusion.

Coronary flow during the first 10 minutes of reperfusion (Figure 3) was better (although not significantly) than in control hearts, but reexposure to normal Ca^{2+} caused a considerable drop in flow, indicating a sudden rise in coronary resistance.

In Group II, despite metabolic recovery, no contractile activity was observed during the initial period of reperfusion due to the low extracellular [Ca^{2+}]. Immediately after reexposure to normal Ca^{2+}, most hearts showed severe contracture without any rhythmic contractile activity.

Normoxic Low Ca^{2+} Perfusion

For assessment of whether temporary reduction of the extracellular [Ca^{2+}] to 0.05 mmol/l would initiate similar adverse effects in nonischemic hearts, a separate series of experiments was performed (Group III). Figure 5 shows that in these nonischemic hearts metabolic parameters were almost unaffected by the switch to 0.05 mmol/l Ca^{2+} for 10 minutes and back to 1.3 mmol/l Ca^{2+} for another 20 minutes. The large changes in high-energy phos-

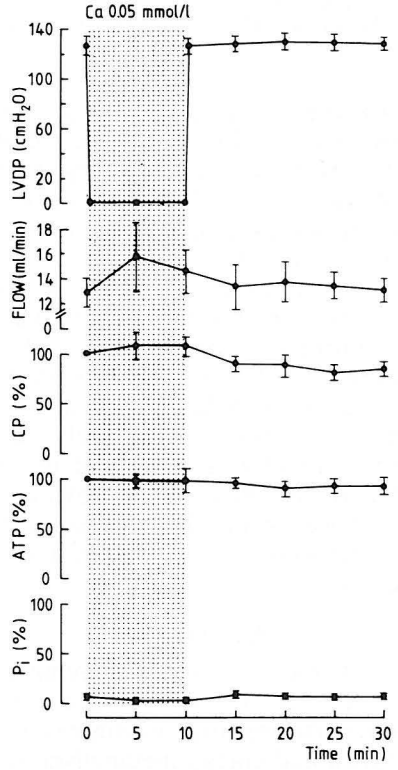

FIGURE 5. *Functional and metabolic parameters in normoxic hearts subjected to 10 minutes of perfusion with 0.05 mmol/l Ca^{2+} followed by 20 minutes with 1.3 mmol/l Ca^{2+} (Group III). Creatine phosphate (CP) and ATP are given as a percentage of their respective control values obtained before low Ca^{2+} perfusion. Intracellular inorganic phosphate (P_i) is expressed as a percentage of the sum of phosphate from CP, ATP, and P_i during control perfusion preceding low Ca^{2+} perfusion. Each point represents mean±SD of nine experiments. LVDP, left ventricular developed pressure.*

TABLE 1. Summary of Literature on Temporary Low Ca^{2+} Reperfusion After Myocardial Ischemia

Authors	Species	Model	$[Ca^{2+}]_{low}$ (mmol/l)	Protection
Allen et al[8]	Dog	Regional ischemia in vivo	0.25* 0.15*	+ +
Chappell et al[9]	Rabbit, cat	Hypoxic/reoxygenated isolated papillary muscle	0.125	+
Ferrari et al[3]	Rabbit	Langendorff perfused heart	0.75 0.15 0.05	– +/–† +
Follette et al[10]	Dog	Hypothermic cardiac arrest in vivo	0.5 <0.05	+ –
Koomen et al[27]	Rat	Langendorff perfused heart	0.1	–
Kuroda et al[11]	Rat	Working heart	0.5‡	+
Nayler[28]	Rabbit	Isolated mitochondria from Langendorff perfused heart	0.05	+
Shine and Douglas[12]	Rabbit	Perfused interventricular septum	0.75	+
Watts et al[29]	Rat	Langendorff perfused heart	0.75	–

*In presence of diltiazem.
†Protection did not last after normalization of extracellular $[Ca^{2+}]$.
‡Optimal $[Ca^{2+}]$.

phates and P_i that occurred during Ca^{2+} repletion in postischemic hearts (Group II) were not observed. Intracellular pH did not change during the protocol, although during the low Ca^{2+} period pH could not always be accurately determined due to very low levels of intracellular P_i.

Left ventricular function recovered completely immediately after the return to normal Ca^{2+}. Coronary flow returned to control values after a slight increase during the low Ca^{2+} perfusion. Concomitantly, CP increased slightly and P_i decreased during low Ca^{2+} perfusion, most likely due to the absence of contractile activity and the observed increased coronary flow. It is obvious that, in contrast to postischemic hearts, normoxic hearts tolerated a temporary reduction of Ca^{2+} to 0.05 mmol/l very well.

Discussion

The present study showed that a reduction of the perfusate $[Ca^{2+}]$ to 0.05 mmol/l during the first 10 minutes of reperfusion after 30 minutes of ischemia enabled myocardial energy metabolism to recover. However, upon reintroduction of 1.3 mmol/l Ca^{2+}, this beneficial effect was not only abolished but even became a detrimental effect.

By now it is accepted that reperfusion injury is associated with uncontrolled Ca^{2+} influx during the first minutes of reperfusion.[13,19] Therefore, it seems appropriate that the extracellular $[Ca^{2+}]$ be lowered during this vulnerable period. On the other hand, complete omission of Ca^{2+} from the perfusion fluid followed by readmission of Ca^{2+} inevitably leads to the calcium paradox, characterized by contracture of the myofibrils, rapid depletion of high-energy phosphates, accumulation of Ca^{2+} and P_i by mitochondria, and uncoupling of oxidative phosphorylation, sarcolemmal disruption, and loss of intracellular constituents.[15,20–24] It is generally assumed that a $[Ca^{2+}]$ of 0.05 mmol/l is safe in that the calcium paradox will not occur, although this is based mainly on electron microscopic observations in rabbit myocardium at 28° C that separation of the glycocalyx could be prevented by an extracellular $[Ca^{2+}]$ of 0.05 mmol/l.[25] However, in normothermic rabbit hearts 0.05 mmol/l did not completely prevent the calcium paradox when leakage of intracellular enzymes was used to define myocardial cell damage.[26]

The question of the optimal $[Ca^{2+}]$ early during reperfusion after ischemia is yet to be solved, but will certainly depend on the experimental conditions and the extent of ischemic damage and possibly also on the species used. This may explain why the results of previous studies, in which reperfusate $[Ca^{2+}]$ ranging from 0.05 to 0.75 mmol/l has been used, are not uniform (Table 1).

Our results showed that reduction of the $[Ca^{2+}]$ to 0.05 mmol/l on reperfusion resulted in a significantly better recovery of myocardial energy metabolism in comparison with hearts reperfused with 1.3 mmol/l Ca^{2+}. The explanation for this may be threefold. 1) Resumption of mitochondrial respiration on reperfusion with a normal $[Ca^{2+}]$ leads to the accumulation of Ca^{2+} and P_i in mitochondria at the expense of ATP production.[12,28] This is prevented by a large reduction of the $[Ca^{2+}]$, leaving all newly produced energy available for repair processes and

restoration of ionic homeostasis. 2) The absence of contractile activity may also contribute to metabolic recovery by diminishing energy expenditure. 3) Furthermore, Ca^{2+}-induced activation of (phospho)lipases and proteases[28,30] will not occur, thereby preventing a further aggravation of tissue damage.

When, after 10 minutes of low Ca^{2+} reperfusion, the standard perfusate was used again, a sharp drop in CP and ATP levels occurred, which was not accompanied by an equal increase of intracellular P_i, as was observed during ischemia. Together with the concomitant contracture of the heart and the sudden increase in coronary resistance, these observations suggested the occurrence of a calcium paradox.[23] The decreased total phosphate content may be explained by washout of P_i or by deposition of calcium phosphate in mitochondria, which is NMR invisible.[23] In addition, small amounts of CP and ATP may have left the cells before breakdown.[31]

Since in our experiments the normoxic hearts tolerated temporary perfusion with 0.05 mmol/l Ca^{2+} very well, it must be concluded that postischemic hearts are more susceptible to the calcium paradox. A possible additive effect of Ca^{2+} depletion and ischemia in the isolated rat heart has been discussed in one other study.[32] It was demonstrated that the volume of Ca^{2+}-free perfusate needed to evoke a calcium paradox was largely reduced when the heart was made ischemic between Ca^{2+} depletion and Ca^{2+} repletion. However, one may also argue that the exposure to the Ca^{2+}-free perfusate lasted longer when the hearts were made ischemic and, therefore, Ca^{2+} depletion was more complete, without any specific additive effect of the intervening ischemic period. At the same time, ischemic damage may have been less, attributable to the Ca^{2+} depletion before ischemia.[5,27] In our experiments ischemia preceded low Ca^{2+} perfusion, and, therefore, a true additive effect occurred, since these hearts (Group II) finally recovered significantly less than after ischemia (Group I) or low Ca^{2+} perfusion alone (Group III).

It is unclear why ischemia enhances the susceptibility of the heart to the calcium paradox. It is possible that the effects of Ca^{2+} depletion[24,25] and ischemia[33,34] on sarcolemmal integrity are additive, as suggested by Jynge.[32] Alternatively, this enhanced sensitivity may also be associated with intracellular Na^+, which increases during both ischemia[35–37] and Ca^{2+} depletion.[38,39] Increased levels of intracellular Na^+ during ischemia are supposed to be caused by failure of the sarcolemmal Na^+/K^+ pump due to lack of ATP[35–37] or high levels of P_i.[35] The cause of Na^+ influx during Ca^{2+}-free perfusion is not completely understood, but a reduced activity of the Na^+/K^+ pump has been reported[40] as well as transport of Na^+ through the Ca^{2+} channels.[38] High levels of intracellular Na^+ may eventually lead to a massive Ca^{2+} influx through the Na^+/Ca^{2+} exchange mechanism[20,35–38,40] after normalization of the perfusate $[Ca^{2+}]$ in the hearts of Group II rats. This in turn may lead to an addition of the postponed reperfusion injury and the newly introduced mild calcium paradox damage. It may be expected that Na^+/Ca^{2+} exchange will be reactivated during resynthesis of ATP after an inhibition during ischemia due to dephosphorylation[41] and acidosis,[35] but that massive exchange cannot occur unless the extracellular $[Ca^{2+}]$ is normalized. It is conceivable that in normoxic hearts, in contrast with postischemic hearts, during 10 minutes of perfusion with 0.05 mmol/l Ca^{2+}, weakening of the sarcolemma and the increase in intracellular Na^+ are not sufficient to predispose the heart to the calcium paradox.[38]

In conclusion, this study demonstrates that recovery of myocardial energy metabolism upon reperfusion is not solely determined by the extent of ischemic damage but can be modulated by temporary lowering of the perfusate $[Ca^{2+}]$ to 0.05 mmol/l. However, a previous period of ischemia may enhance the susceptibility of the heart to the calcium paradox. This may be of importance for future investigations, both experimental and clinical, dealing with protective interventions at the time of reperfusion.

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