Mechanism of Preconditioning

Ionic Alterations

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The mechanism by which preconditioning (brief intermittent periods of ischemia and reflow) improves recovery of function and reduces enzyme release after a subsequent 30-minute period of ischemia was investigated in perfused rat hearts. Specifically, it was hypothesized that ischemia after preconditioning would result in a decreased production of $\text{H}^+$ and therefore a smaller rise in $[\text{Na}^+]$, and $[\text{Ca}^{2+}]$, via $\text{Na}^+\cdot\text{H}^+$ and $\text{Na}^+\cdot\text{Ca}^{2+}$ exchange. To test this hypothesis we measured $\text{pH}$, $[\text{Na}^+]$, $[\text{Ca}^{2+}]$, and cell high-energy phosphates during ischemia and reflow, and we correlated this with recovery of contractile function and release of creatine kinase during reflow. $^{31}P$ nuclear magnetic resonance (NMR) was used to measure $\text{pH}$, and cell phosphates. $[\text{Na}^+]$ was measured by $^{23}\text{Na}$ NMR using the shift reagent thulium 1,4,7,10-tetraazacyclododecane-N,N',N''-tetramethylene phosphonate to distinguish intracellular from extracellular sodium. $[\text{Ca}^{2+}]$ was measured by $^{19}F$ NMR using hearts loaded with 1,2-bis-(2-amino-5-fluorophenoxo)ethane-N,N',N''-tetraacetic acid, termed SF-BAPTA. Basal time-averaged levels of $\text{pH}$, $[\text{Na}^+]$, and $[\text{Ca}^{2+}]$, were 7.07±0.08, 9.4±0.8 mM, and 715±31 mM, respectively. After 30 minutes of ischemia, in preconditioned hearts, $\text{pH}$ was 6.5±0.06, $[\text{Na}^+]$ was 20.9±4.4 mM, $[\text{Ca}^{2+}]$, was 2.1±0.4 µM, and ATP was negligible. In untreated hearts, after 30 minutes of ischemia, $\text{pH}$ was 6.3±0.08, $[\text{Na}^+]$, was 26.7±3.8 mM, $[\text{Ca}^{2+}]$, was 3.2±0.6 µM, and ATP was undetectable. During reperfusion after 30 minutes of ischemia, preconditioned hearts had significantly better recovery of contractile function than untreated hearts (71±9% versus 56±8% initial left ventricular developed pressure), and after 60 minutes of ischemia, preconditioned hearts had significantly less release of the intracellular enzyme creatine kinase (102±12 versus 164±17 IU/g dry wt). We also found that unpreconditioned hearts arrested with 16 mM MgCl$_2$ (to inhibit calcium entry via calcium channels and Na$^+\cdot$Ca$^{2+}$ exchange) before 30 minutes of ischemia recover function on reflow to the same extent as preconditioned hearts with or without magnesium arrest. Thus, preconditioning has no additional benefit in addition to magnesium arrest. In addition, in hearts that received 16 mM MgCl$_2$ just before the 30-minute period of ischemia, preconditioning had no effect on the rise in $[\text{Ca}^{2+}]$, during the 30-minute period of ischemia. These data support the hypothesis that preconditioning attenuates the increase in $[\text{Ca}^{2+}], [\text{Na}^+], \text{and } [\text{H}^+]$, during ischemia, most likely because of reduced stimulation of Na$^+\cdot\text{H}^+$ and Na$^+\cdot\text{Ca}^{2+}$ exchange. The data suggest that interventions that minimize ionic derangements during ischemia are associated with improved recovery of contractility and less enzyme release on reflow. (Circulation Research 1993;72:112-125)

Key Words • myocardial ischemia • preconditioning • nuclear magnetic resonance • cytosolic free calcium concentration • intracellular sodium concentration

An understanding of the mechanisms involved in the transition from reversible to irreversible cell injury has been pursued for many years. Recently, it has been shown that several brief ischemic periods separated by brief periods of reflow (preconditioning) actually reduce necrosis after a subsequent longer (30–60-minute) period of ischemia. The mechanism by which preconditioning delays the transition from reversible to irreversible injury has been the subject of considerable interest, and several theories have been proposed. Thornton et al$^6$ have proposed that adenosine, released during preconditioning, provides protection during subsequent episodes of ischemia. In support of this hypothesis, Downey$^7$ has shown that adenosine antagonists block preconditioning. It has also been suggested that stress proteins may be involved in preconditioning, and although several groups report synthesis of stress proteins or stress protein mRNA during ischemia,$^{8,9}$ preconditioning still occurs if protein synthesis is blocked.$^{10}$ Thus, stress proteins would appear not to be responsible for preconditioning.

A rise in cytosolic free calcium concentration ([Ca$^{2+}$]) has been postulated to activate catabolic enzymes and thereby be an important factor in cell injury.$^{11-13}$ Cardioprotective interventions instituted before ischemia have been shown to delay both the rise in [Ca$^{2+}$]$^{14}$ and lethal injury.$^{15}$ The fundamental protective mechanisms of preconditioning are unknown but could involve a delay in the rise in [Ca$^{2+}$], although there is no direct evidence to support this hypothesis. Murry et al$^7$ reported that, after 40 minutes of ischemia, lactate production in preconditioned hearts was only 60% of untreated hearts. This reduced production of lactate.
would likely result in less intracellular acidification. We hypothesize that reduced [H⁺], in turn, would reduce the rise in [Na⁺] via Na⁺-H⁺ exchange and subsequently the rise in [Ca²⁺] via Na⁺-Ca²⁺ exchange. Furthermore, if catecholamines are released during the preconditioning period, there may be less catecholamine release during the final sustained (30–60-minute) episode of ischemia. Catecholamines are known to increase [Ca²⁺] via increased entry through calcium channels. Indeed, the adenosine effect could be mediated by inhibiting endogenous catecholamine release or attenuating the effects of β-agonists and the rise in cAMP.

In this study, we have examined the effects of preconditioning on ionic alterations during ischemia. Knowledge of ionic alterations during preconditioning and subsequent ischemia is important in defining the mechanisms responsible for preconditioning. Since preconditioning has not been reproduced in isolated cells, it is a phenomenon that must be studied in intact animals or perfused organs. Preconditioning is protective in the isolated perfused heart as well as in vivo, showed in a recent study that found that the reduction in infarct size due to preconditioning was the same in the in situ heart and the isolated heart. The [Ca²⁺] measurements are not presently feasible in intact animals. Therefore, we performed these studies on isolated perfused hearts. ³¹P nuclear magnetic resonance (NMR) provides a noninvasive means of continuously measuring pH and high-energy phosphates. [Na⁺], can be suitably monitored using Na⁺ NMR with infusion of shift reagents to distinguish intracellular from extracellular sodium. Moreover, ⁵²Na has been measured using ¹²-bis(2-amino-5-fluorophenoxo)ethane-N,N,N',N'-tetraacetic acid (5F-BAPTA)–loaded hearts and ¹⁹F NMR. In spite of the reduced contractility ⁵F-BAPTA–loaded hearts, this has been a fruitful method for measuring [Ca²⁺] in perfused organs, especially during ischemia, when contractility ceases. ¹⁹Indo-1 and acouroin have also been loaded into perfused hearts to measure [Ca²⁺]. Using fluorescence and luminescence, and although these indicators do not appreciably alter contractility, there is some question about optical interference and loading into nonmyocytes, as well as the degree to which the surface layers of cells being monitored are representative of the organ as a whole. Since contractility ceases during ischemia and since ⁵F-BAPTA has been useful in measuring changes in [Ca²⁺], during ischemia, we used this method to monitor [Ca²⁺].

In this study, we find that [H⁺], [Na⁺], and [Ca²⁺] all rise to a lesser extent during ischemia in hearts that have been preconditioned.

Materials and Methods

Adult male Sprague-Dawley rats and Hartley guinea pigs (Charles River Suppliers, Wilmington, Mass.) weighing 200–300 g were anesthetized with pentobarbital. The heart was excised, and the aorta was cannulated. Retrograde perfusion was started from a reservoir 90 cm above the aortic cannula. The nonrecirculating perfusate was a Krebs-Henseleit buffer containing (mM) NaCl 120, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.25, NaHCO₃ 25, and glucose 10. The buffer was continuously aerated with humidified 95% O₂–5% CO₂ and was main-
tained at 37°C. After 15 minutes of control perfusion, loading with 300 ml of 5 µM acetoxymethyl ester of 5F-BAPTA was started. With typical flow rates of 10–15 ml/min, loading took 20–30 minutes.

After loading with 5F-BAPTA, hearts were perfused with phosphate-free Krebs-Henseleit buffer (pH adjusted to 7.4 by adding HCl) for approximately 30 minutes, during which time the magnet was shimmed and several preischemic spectra were acquired. Phosphate-free buffer allows us to unambiguously assign the inorganic phosphate peak to the intracellular space as required for measuring pH. The heart was placed in a standard 20-mm NMR tube with the apex of the heart approximately 1 cm from the bottom of the tube. The perfusate was evacuated by a variable speed Masterflex peristaltic pump connected to polyethylene tubing that extended to the bottom of the tube; thus, the heart was not bathed in perfusate.

For most experiments, the hearts were divided into two experimental groups. Both groups received 30 minutes of global ischemia; however, one group was pretreated with four periods of 5 minutes of ischemia each separated by 5 minutes of reflow (preconditioning). After the 30-minute ischemic period, both groups were reperfused with phosphate-free Krebs-Henseleit buffer. In each experimental group, some hearts were evaluated by ¹⁹F NMR, some were evaluated by ³¹P NMR, and some were evaluated by ¹³Na NMR. Measurements of contractility were made in all experiments. In the no-treatment group, four hearts were studied by ¹⁹F NMR, four hearts were studied by ³¹P NMR, and six hearts were studied by ¹³Na NMR. In the preconditioning group, eight hearts were studied by ¹⁹F NMR, seven hearts were studied by ³¹P NMR, and four hearts were studied by ¹³Na NMR. In one set of experiments, hearts were arrested by addition of 16 mM MgCl₂ to the perfusate. [Ca²⁺] during 30 minutes of ischemia and recovery of left ventricular developed pressure during reflow were measured in these hearts with and without preconditioning. [Ca²⁺], was also measured in guinea pig hearts during 30 minutes of ischemia with and without prior preconditioning.

For assessment of contractile function, a latex balloon on the tip of a polyethylene catheter was inserted through the left atrium into the left ventricle. The catheter was connected to a Statham P23d pressure transducer that was outside the magnet at the same height as the heart. The balloon was inflated to give an end-diastolic pressure of 5–10 cm water. Initially, all hearts developed at least 100 cm H₂O peak systolic pressure at an end-diastolic pressure of 10 cm H₂O or less. ⁵F-BAPTA loading has been previously shown to buffer calcium transients and thereby decrease peak systolic pressure to approximately 25 cm H₂O at an end-diastolic pressure of 10 cm H₂O. After loading with ⁵F-BAPTA, hearts were paced at 5 Hz using silver wire and an agar salt bridge connected to a stimulator (Grass Instrument Co., Quincy, Mass.).

These studies were performed on a wide-bore NMR spectrometer (model NT 360, Nicolet Instruments, Fremont, Calif.) with the variable temperature probe at 37°C. For the ¹⁹F studies, we used a 20-mm ³¹P probe tuned to 339.7 MHz (Doty Scientific, Columbia, S.C.). We used a 20-mm broadband probe (Nicolet) tuned to 146.1 MHz for the ³¹P NMR studies and to 95.5 MHz for

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the $^{23}$Na NMR studies. The sample was shimmed on the water signal from the heart, and we routinely obtained a (nonspinning) line width at half height of approximately 0.25 ppm. For the $^{19}$F studies, we used a 40° pulse angle, a ±5-kHz spectral width, a 205-msec recycling time, and 4,000 data points. These pulsing conditions approximately correspond to the Ernst angle for the fluorine resonance of 5F-BAPTA. A 70° pulse angle, a 1-second delay, a ±5-kHz spectral width, and 4,000 data points were used for the $^{31}$P NMR studies. These parameters for $^{31}$P were chosen so that we could measure changes with a 2.5–5-minute time resolution. With these pulsing parameters, we overpulsed the resonances; to obtain a true concentration, we applied correction factors that were dependent on the longitudinal relaxation time ($T_1$) and our pulsing conditions.31–34 For the $^{23}$Na NMR studies, we used 1,000 data points, a 75° pulse angle, ±2,400-Hz spectral width, a 106-msec acquisition time, and a 100-msec delay, giving a 206-msec recycling time.

As shown previously,25,36 calcium binding to 5F-BAPTA exhibits slow exchange kinetics at 8.5 T; thus, [Ca$^{2+}$], is related to the observed resonance intensities by

$$[\text{Ca}^{2+}] = K_0 \times [\text{Ca}^{2+} - 5\text{F-BAPTA}] / [5\text{F-BAPTA}]$$

where the $K_0$ value for 5F-BAPTA is 700 nM at 37°C and [Ca$^{2+}$–5F-BAPTA] and [5F-BAPTA] are proportional to the areas under their respective resonance peaks. Since this measurement requires the comparison of resonance intensities, it is necessary to work under conditions of nonsaturation of the resonances or, alternatively, under conditions of equal saturation. Fortunately, 5F-BAPTA and Ca$^{2+}$–5F-BAPTA have nearly identical spin lattice relaxation times; therefore, the intensity ratio of free 5F-BAPTA to calcium-complexed 5F-BAPTA is essentially independent of the rate of pulsing. Studies by Marban et al37 show that in perfused hearts 5F-BAPTA is not significantly compartmentalized into mitochondria or endothelial cells.

The concentration of intracellular indicator was determined at the end of the experiment by drying the heart to a constant weight and homogenizing in 150 mM KCl, with excess EGTA buffered with Tris-MOPS to pH 7.1. $^{19}$F NMR studies were carried out on the homogenate, to which a known concentration of 6-fluorotryptophan was added. The resonance intensity of 5F-BAPTA was then compared with that of the 6-fluorotryptophan, taking into account the differences in $T_1$ and the number of fluorine nuclei.

pH was measured from the shift between intracellular inorganic phosphate and creatine phosphate, as described previously by Jacobus et al.38 ATP, expressed as percent control, was determined by measuring the area under the $\beta$-phosphate resonance of ATP. In the $^{23}$Na spectra, intracellular sodium was distinguished from extracellular sodium by using an extracellular shift reagent, thulium 1,4,7,10-tetraazacyclododecane-$N,N',N''$-tetramethylbenzenephosphonate (DOTP), which was synthesized as described by Sherry and coworkers.39,40 The sodium-free induction decay was multiplied by a combined exponential and gaussian function of 10 Hz. To correct for any changes in intensity (e.g., due to a change in the tune) during the course of the experiment, a external standard (a round capillary tube, containing 12 mM thulium DOTP plus 400 mM NaCl with no added calcium, placed next to the heart) was included in all experiments, and the area under the intracellular sodium resonance was normalized by comparison with the external standard. The area under the intracellular sodium resonance represents the amount of intracellular sodium in the heart. One means of converting the intracellular sodium signal into [Na$^+$] is by comparison to the intensity of a known concentration standard. Attempts to use our external standard as an absolute intensity standard were not successful, since the signal intensity was found to vary between experiments, depending on the precise placement of the capillary. Thus, our external standard works as a check against changes in intensity during an experiment (which were found to be negligible) but could not be easily used as an absolute concentration standard. The best method we found to quantitate intracellular sodium was to use extracellular sodium as an intensity standard according to the following equation:

$$[\text{Na}^+] = \frac{([A(\text{Na}_0]/[A(\text{Na}_i)]) (V_o/V) (f_o/f_i) [\text{Na}^+]_0}{[\text{Na}^+]_i}$$

where $A(\text{Na}_0)$ is the area under the resonance corresponding to intracellular sodium, $V_i$ is the intracellular volume, $f_i$ is a correction reflecting the NMR visibility of the intracellular sodium resonance, and $A(\text{Na}_i)$, $V_o$, and $f_o$ are the corresponding parameters for extracellular sodium. With radioactive sorbitol used as an extracellular marker in saline-perfused rat hearts, the ratio $V_o/V_i$ was determined to be in the range of 0.9–1.4.41,42

In our calculations, we used a value of 1.0 for control perfusion conditions, and [Na$^+$]$_i$ was 145 mM. There is some controversy regarding the degree of NMR visibility of intracellular sodium. In a study of this parameter, Springer43 reported that intracellular sodium is 40% visible. In our studies, we have set $f_o=0.4$ and $f_i=1.0$ and made the assumption that the visibility of the $^{23}$Na resonance remains constant during the experiment. Changes in [Na$^+$], during ischemia and reflow were calculated as a percentage of the initial [Na$^+$], based on the change in resonance intensity of the intracellular sodium peak.

Creatine kinase (CK) release was measured by collecting the effluent and measuring CK activity spectrophotometrically.44

Values are expressed as mean±SEM. The time-matched data curves during ischemia were evaluated using analysis of variance for repeated measures. When $F$ values indicated that significant differences were present, we then used Tukey's honestly significant difference method, which adjusts for multiple comparisons, to compare samples for statistical significance. Values of $p<0.05$ were considered significant. Statistical evaluations were performed using SYSTAT software (SYSTAT Inc., Evanston, Ill.).

**Results**

Brief intermittent periods of myocardial ischemia (preconditioning) have been shown to provide protection against various manifestations of cell injury produced by a subsequent longer period of ischemia. In intact dogs,1,2 pigs,3 and rabbits,4 preconditioning has been shown to significantly decrease the area of necrosis produced by 30–60 minutes of ischemia. In a recent
study, it was shown that reduction in infarct size due to preconditioning occurs in both the in situ heart and the isolated heart. Hager et al have also shown that preconditioning in rats results in attenuation of reperfusion arrhythmias. Preconditioning has also been shown to reduce stunning. Thus, preconditioning has a variety of beneficial effects. The mechanism(s) responsible for these various effects may or may not be the same. We observe that preconditioning in the rat heart improves recovery of function (Figure 1A) and decreases the release of the intracellular enzyme CK (Figure 1B). In untreated hearts, reperfusion after 30 minutes of total ischemia results in 36±8% recovery of preischemic function. In hearts that have been preconditioned, reperfusion after 30 minutes of ischemia results in recovery of function to 71±9% of the preischemic level, a value significantly different (p<0.05) from that in the untreated heart. As shown previously, there is very little CK release after 30 minutes of ischemia. Therefore, we compared CK release during reperfusion in preconditioned and untreated hearts after 60 minutes of ischemia and found that preconditioned hearts released significantly less CK (164±17 IU/g dry wt for untreated hearts compared with 102±12 IU/g dry wt for preconditioned hearts, p<0.05) (Figure 1B). In addition, preconditioning delays the time to onset of ischemic contracture. Without preconditioning, this occurs at 5.1±0.3 minutes after the onset of ischemia; with preconditioning, ischemic contracture begins at 6.3±0.6 minutes (not significantly different, p=0.13).

Having determined that preconditioning in perfused rat hearts reduces release of intracellular enzymes, delays the onset of ischemic contracture, and improves recovery of function, we were interested in determining if ionic alterations were involved in these effects. NMR spectra obtained from 5F-BAPTA-loaded perfused rat hearts before ischemia and during the last 5 minutes of 30 minutes of global ischemia are shown in Figure 2 for an untreated heart (Figure 2A) and a heart preconditioned with intermittent periods of ischemia.
(preconditioned, Figure 2B). As described, [Ca$^{2+}$], is obtained from the $^{31}$P spectrum by multiplying the $K_d$ by the ratio of the areas under the Ca$^{2+}$–5F-BAPTA and uncomplexed 5F-BAPTA resonances. Each $^{31}$P NMR spectrum is an average of 1,500 accumulations, and since we are not gating to the cardiac cycle, these spectra represent time-averaged values for [Ca$^{2+}$]; in the paced beating heart, this value is 715±31 nM ($n=12$). The preischemic [Ca$^{2+}$] values are taken before preconditioning and, as expected, there is no difference in preischemic [Ca$^{2+}$], values between untreated and preconditioned hearts. In the untreated heart shown in Figure 2A, there was a large increase in the ratio of Ca$^{2+}$–5F-BAPTA to uncomplexed 5F-BAPTA after 25–30 minutes of global total ischemia. In contrast, in the preconditioned heart (Figure 2B), by 25–30 minutes of total ischemia there is still a large uncomplexed 5F-BAPTA resonance, indicating a substantial difference from the untreated ischemic heart.

The $^{31}$P NMR experiments were performed on hearts loaded with 5F-BAPTA. Figure 3 shows a series of $^{31}$P NMR spectra before and during global ischemia (25–30 minutes) in an untreated heart (Figure 3A) and a preconditioned heart (Figure 3B). pH, was determined from the shift difference between creatine phosphate (peak B) and inorganic phosphate (peak A), as described previously. Since the hearts were perfused with a phosphate-free Krebs-Henseleit buffer, the inorganic phosphate signal is derived from intracellular inorganic phosphate. ATP levels (expressed as percent control) were obtained from the $^{31}$P NMR spectra by integrating the area under the $\beta$-phosphate of ATP. Creatine phosphate and inorganic phosphate levels were also obtained by integrating the areas under their respective resonances. Figure 3 shows that, in both an untreated heart and a preconditioned heart, 30 minutes of ischemia results in a large increase in inorganic phosphate and a decrease in creatine phosphate and ATP to undetectable levels (<1.25 mM). Figure 3 also shows that the inorganic phosphate peak is shifted upfield (toward creatine phosphate) by 25–30 minutes of ischemia, consistent with a decrease in pH. In the ischemic spectra, the upfield shift of the inorganic phosphate peak is greater in the untreated heart than in the preconditioned heart, indicating a more acidic pH, in the untreated heart than in the preconditioned heart.

The $^{23}$Na experiments were also performed on hearts loaded with 5F-BAPTA and followed the same protocol, except that to distinguish intracellular sodium from extracellular sodium, 5 mM thulium DOTP was added. Malloy et al showed previously that, if sufficient calcium is present in the perfusate to maintain a physiological free extracellular calcium concentration, thulium DOTP does not alter cardiac function. This was achieved by increasing the total extracellular calcium to 5 mM in the presence of 5 mM thulium DOTP. Figure 4 shows $^{23}$Na NMR spectra before and during global ischemia. There was good separation between the large extracellular peak and the small intracellular peak. By 25–30 minutes of ischemia [Na$^+$], rises in both the untreated and preconditioned hearts.

Creatine phosphate levels determined from $^{31}$P NMR spectra obtained during the last reflow period of the preconditioning sequence were 105±11% of preischemic levels. Thus, at the beginning of the 30-minute period of global ischemia, the creatine phosphate level is essentially the same for both groups of hearts (100% versus 105%). Between 5 and 10 minutes of ischemia, there are measurable amounts of creatine phosphate in preconditioned hearts, whereas untreated hearts have no measurable creatine phosphate. After 10 minutes of ischemia, there is no detectable creatine phosphate in either untreated or preconditioned hearts. Figure 5A also shows that, during reflow, creatine phosphate recovered to 113±37% of preischemic levels in preconditioned hearts compared with 53±17% in untreated hearts.
In contrast to the results for creatine phosphate, the ATP level at the end of the preconditioning period is significantly (p<0.05) below the preischemic value; as shown in Figure 5B, the ATP level in the preconditioned group at the end of the last reflow period before the beginning of the 30-minute period of ischemia is 69±15% of the preischemic mean. However, initially there is a more rapid rate of decline in tissue ATP for the untreated hearts, so that, by 5 minutes of global ischemia, the levels in the two groups do not differ significantly. The recovery of ATP content during reflow is similar in the two groups. Although inorganic phosphate levels appear to be systematically lower in the untreated relative to the preconditioned group (Figure 5C), the differences are not significant.

Figure 6 summarizes the time course of changes in \([\text{Ca}^{2+}]\), (panel A), \([\text{Na}^+]\), (panel B), and \(\text{pH} \) (panel C) before and during 30 minutes of global ischemia in preconditioned and untreated rat hearts. The hypothesis being tested is that preconditioning reduces intracellular H⁺ generation during a subsequent ischemic episode, which results in less \(\text{Na}^+-\text{H}^+\) exchange and therefore attenuates the rise in \([\text{Na}^+]\), and attenuates the subsequent rise in \([\text{Ca}^{2+}]\), via \(\text{Na}^+-\text{Ca}^{2+}\) exchange. Consistent with this hypothesis, Figure 6C shows that, during a 30-minute period of ischemia, preconditioned hearts have a higher \(\text{pH} \) (less H⁺) than untreated hearts. Analysis of variance for repeated measurements shows that the curves are significantly different \((p=0.014)\). The difference in \(\text{pH} \) is significant at 10, 15, 20, and 30 minutes of ischemia. Figure 6B shows that by 30 minutes of ischemia in untreated hearts, \([\text{Na}^+]\), rises to 26.7±3.8 mM, whereas in preconditioned hearts \([\text{Na}^+]\), rises to 20.9±4.4 mM. Although these curves are not significantly different, the direction of the difference is consistent with the hypothesis. This is examined in detail in “Discussion.” A comparison of \([\text{Ca}^{2+}]\) with and without preconditioning (Figure 6A) using analysis of variance for repeated measurements shows that the curves are significantly different \((p=0.033)\). The \([\text{Ca}^{2+}]\),
values are significantly different at 15 and 20 minutes of ischemia (p<0.05). The rise in [Ca$^{2+}$]i is considerably attenuated in the preconditioned heart. Preconditioning delays, but does not prevent, the rise in [Ca$^{2+}$]i. In the untreated hearts, 25–30 minutes of global ischemia caused [Ca$^{2+}$]i to rise to 3.2±0.6 μM; in the preconditioned hearts, 25–30 minutes of ischemia resulted in a [Ca$^{2+}$]i of 2.1±0.4 μM. Figure 6 also shows that, during reflow after the 30-minute ischemic period, pH, [Ca$^{2+}$]i, and [Na$^{+}$]i, return toward preischemic levels.

FIGURE 5. Graphs showing the time course of changes in creatine phosphate (CP, panel A), ATP (panel B), and inorganic phosphate (panel C) during preconditioning (if applicable), 30 minutes of global ischemia, and 10 minutes of reflow in untreated (closed diamonds) and preconditioned hearts (open squares). I and R denote the values during the 5-minute ischemic and reflow periods of preconditioning, respectively. Time-matched curves were tested by analysis of variance for repeated measures. No significant differences were found (for CP, F=1.471, p=0.256; for ATP, F=0.071, p=0.796; and for inorganic phosphate, F=0.099, p=0.761).

FIGURE 6. Graphs showing the time course of changes in intracellular Ca$^{2+}$ concentration (panel A), intracellular Na$^{+}$ concentration (panel B), and intracellular pH (panel C) during preconditioning (if applicable), 30 minutes of global ischemia, and 10 minutes of reflow in untreated (closed diamonds) and preconditioned (open squares) hearts. I and R denote the values during the 5-minute ischemic and reflow periods of preconditioning, respectively. Time-matched curves were tested by analysis of variance for repeated measures. The curves for intracellular Na$^{+}$ concentration in untreated and preconditioned hearts were not significantly different by this test (F=0.0800, p=0.397); therefore, we did not test individual time points. The curves for intracellular Ca$^{2+}$ concentration and intracellular pH did show significant differences between untreated and preconditioned hearts (for intracellular Ca$^{2+}$ concentration, F=6.350, p=0.033; for intracellular pH, F=9.733, p=0.014). After adjustment for multiple comparisons, individual time points were tested for significance. Compared with untreated hearts, intracellular Ca$^{2+}$ concentration was significantly different (p<0.05) in preconditioned hearts at 15 and 20 minutes. For intracellular pH, significant differences (p<0.05) were apparent at 10, 15, 20, and 30 minutes of ischemia.
FIGURE 7. Graph showing the time course of changes in intracellular Ca\(^{2+}\) concentration (Cai) during 30 minutes of ischemia in untreated (open squares), preconditioned (open triangles), and amiloride-pretreated (closed squares) hearts.

The effects of preconditioning on intracellular Ca\(^{2+}\), Na\(^{+}\), and ATP during 30 minutes of ischemia are similar to the effects of pretreatment with inhibitors of Na\(^{+}\)-H\(^{+}\) exchange, such as amiloride. The similarity in the effect of these manipulations on the rise in [Ca\(^{2+}\)], is illustrated in Figure 7.

These data suggest that an important aspect of preconditioning is attenuation of the increase in [Ca\(^{2+}\)]. We reasoned that, if the primary mechanism of preconditioning was due to attenuation of the rise in [Ca\(^{2+}\)] during ischemia, then preconditioning should have no additional beneficial effect when calcium entry via calcium channels and Na\(^{+}\)-Ca\(^{2+}\) exchange is blocked by the addition of 16 mM magnesium. Figure 8 shows the comparison between unpreconditioned hearts arrested with MgCl\(_2\) just before the 30-minute period of ischemia and preconditioned hearts treated with 16 mM MgCl\(_2\) during the fourth reflow just before the 30-minute

FIGURE 8. Bar graph showing left ventricular developed pressure (LVDP) as a percentage of initial LVDP (before preconditioning and ischemia), measured after 20 minutes of reperfusion after 30 minutes of ischemia in hearts treated as indicated.
period of ischemia; after 20 minutes of reperfusion, developed pressure recovers to 68±5% of its preischemic value in preconditioned hearts compared with 72±5% in the absence of preconditioning. Thus, the magnesium-arrested hearts without preconditioning recover function on reflow to the same extent as preconditioned hearts with or without magnesium arrest. Preconditioning has no additional benefit in addition to magnesium arrest. In addition, in hearts that received 16 mM magnesium just before the 30-minute period of ischemia, preconditioning had no effect on the rise in [Ca\(^{2+}\)], during the 30-minute period of ischemia (see Figure 9). Since injury develops much slower in a heart that has been arrested before ischemia, the possibility exists that the lack of difference is due to the time chosen. Thus, in an arrested heart, it may take a longer time for the protective effect of preconditioning to become significant. To evaluate this possibility, we examined 45- and 60-minute periods of ischemia and found preconditioning to have no effect on [Ca\(^{2+}\)], or recovery of function (data not shown).

Our studies of adult rat myocardium show that there is a correlation between the magnitude of the ionic derangements during ischemia and the degree of stunning during reperfusion and between the magnitude of the ionic derangements during ischemia and the amount of enzyme release and that preconditioning can attenuate the ionic derangements, stunning, and enzyme release. We were interested in determining whether similar results would be observed in other species. We chose to study the guinea pig heart and found that, in contrast to the rat heart, preconditioning had no effect on recovery of contractile function after a 20-minute period of ischemia. Similarly, in guinea pigs, precondi-
tioning did not affect the rise in \([\text{Ca}^{2+}]_i\), during the prolonged period of ischemia (see Figure 10). Thus, not all species show the same response to preconditioning. The guinea pig data are consistent with our hypothesis that there is a relation between the magnitude of the rise in \([\text{Ca}^{2+}]_i\) during ischemia and the degree of stunning during reperfusion.

Discussion

Validity of Methods

\([\text{Ca}^{2+}]_i\). This NMR method using 5F-BAPTA to measure \([\text{Ca}^{2+}]_i\) is similar to the fluorescent methods using fura-2.59 The 5F-BAPTA is introduced into the perfused heart as the acetoxymethyl ester, a form that is readily permeable across the plasma membrane. Once in the cell, naturally occurring esterases cleave the ester, leaving the negatively charged 5F-BAPTA trapped in the cytosol. Measurement of \([\text{Ca}^{2+}]_i\), requires the following: 1) minimal background interference, 2) insensitivity of the indicator to other ionic changes in the cell, 3) measurement of the concentration of calcium-complexed and uncomplexed indicator, and 4) preferential loading and retention of the indicator in the cytosol of myocytes. It is possible to carry out \(^{19}\text{F}\) NMR measurements with no observable background because of the absence of naturally occurring fluorine-containing metabolites in cells. This contrasts with optical indicators that must contend with optical background and interferences from other chromophores such as myoglobin, hemoglobin, and NADH, as well as motion artifacts.21-24 Furthermore, the NMR measures the signal of the entire perfused organ and is not restricted to data from the surface layer of cells. Previous studies20,26-28,35,36 have shown that 5F-BAPTA is relatively insensitive to changes in the concentration of other ions that occur under physiological conditions and during ischemia. With this NMR spectral technique, we observe two separate resonances for calcium-complexed and uncomplexed indicator; this allows measurement of \([\text{Ca}^{2+}]_i\), independent of the concentration of the indicator. Several lines of data suggest that 5F-BAPTA is located in the cytosol of myocytes. Experiments in which the measurement of \([\text{Ca}^{2+}]_i\) is gated to the cardiac cycle show diastolic and systolic calcium values consistent with a cytosolic location.52 In addition, Marban and coworkers37 showed that mitochondria isolated from 5F-BAPTA–loaded perfused hearts did not contain 5F-BAPTA. Furthermore, there is no indication that measurable 5F-BAPTA accumulates in endothelial cells or smooth muscle cells. Addition of bradykinin has been shown to increase \([\text{Ca}^{2+}]_i\), in endothelium and smooth muscle cells but not in myocytes.38 It has been shown that addition of bradykinin to 5F-BAPTA–loaded perfused heart does not cause any measurable change in the \(^{19}\text{F}\) NMR spectra, indicating that the 5F-BAPTA resides primarily in the myocytes.37 If ischemia develops to the point at which the cell membrane becomes leaky, thereby allowing the indicator to leak from the cell, this method (and all other methods that rely on an intracellular indicator, such as aequorin and indo-1) is no longer valid. Therefore, we have been careful to limit our calcium measurements to short periods of ischemia, during which there is no significant leakage of intracellular contents. In all cases, we have documented that during the time of the measurements there is no significant enzyme release and no significant leakage of 5F-BAPTA; we only include experiments in which there is no measurable loss of 5F-BAPTA from the heart on reflow. Thus, to correlate ion changes with enzyme release, we measure enzyme release after periods of ischemia that are longer than the duration of ischemia used in experiments for measurement of \([\text{Ca}^{2+}]_i\). In the experiments in which we measured CK release, the hearts were ischemic for 60 minutes; in contrast, in the experiments in which we measured \([\text{Ca}^{2+}]_i\), ischemic periods did not exceed 30 minutes.

\([\text{Na}^+]_i\). \(^{23}\text{Na}\), the naturally occurring sodium isotope, is readily measured using NMR; however, intracellular sodium has the same chemical shift as extracellular sodium. Addition of an extracellular shift reagent allows separation of intracellular from extracellular sodium.43 This method requires that the shift reagent be stable and remain extracellular. Previous studies22,23,39,40 have shown that thulium DOTP meets these requirements. As is the case with 5F-BAPTA, if the plasma membrane becomes damaged, the shift reagent could enter the cell. Thus, we have limited our measurements of \([\text{Na}^+]_i\) to short periods of ischemia, during which there is no plasma membrane damage, as shown by the lack of enzyme release and the lack of 5F-BAPTA release.

CK release. In the perfused-heart model, CK release during reperfusion has often been used as a measure of lethal myocyte injury. CK release has been shown to correlate with the morphological assessment of the proportion of lethally injured myocytes as long as the left ventricle is maintained in a distended state throughout ischemia by inflating an intraventricular balloon that is deflated before reflow.53-55 This was done in the present experiments. Previous work has shown that there is minimal CK release during aerobic perfusion,44 and we collected coronary effluent before the onset of ischemia to confirm that there was no detectable CK release under control conditions. During reflow after the ischemic period, the coronary effluent was collected for 20 minutes to estimate the amount of lethal myocyte injury. Previous work has shown that peak CK release occurs within the first 5 minutes of reflow.53-55 Although CK release is not complete after 20 minutes of reflow, this duration of reflow has been used previously, and a good correlation has been found between the amount of CK released and the morphological determination of the proportion of lethally damaged myocytes. Measurement of CK release is not equivalent to measurement of infarct size in in vivo models of ischemia, which requires 48–72 hours of reperfusion, but CK release provides a qualitative assessment of the amount of contraction band necrosis present during early reflow after a period of ischemia.

Relation Between \([\text{Ca}^{2+}]_i\), and Ischemic Injury

Many investigators have suggested that an increase in \([\text{Ca}^{2+}]_i\), plays a causal role in the generation of ischemic injury.11,14,16,23,26-29,42,56-60 An increase in \([\text{Ca}^{2+}]_i\), has been postulated to activate calcium-dependent degradative processes,57-60 which may lead to irreversible plasma membrane damage. This hypothesis requires that an increase in \([\text{Ca}^{2+}]_i\) precedes lethal cell injury. We14,23,26,59 and other groups16,27,29,58 have shown that
[Ca\(^{2+}\)] increases before lethal ischemic or anoxic myocyte injury. Interventions that delay the rise in [Ca\(^{2+}\)], correspondingly delay the onset of myocyte necrosis.\(^{14,58}\) We have shown that addition of the calcium channel blocker diltiazem and cardioplegic arrest, which have been shown by others to delay the onset of irreversible injury, correspondingly delay the rise in [Ca\(^{2+}\)].\(^{14}\) Reducing extracellular calcium, which has also been shown to delay cell injury, has also been shown to slow the rise in [Ca\(^{2+}\)], both in the perfused heart model\(^{59}\) and in metabolically inhibited cultured heart cells.\(^{58}\) Thus, a strong correlation between an elevated [Ca\(^{2+}\)], and cell injury has been shown. Therefore, we were interested in investigating whether preconditioning, which has been shown to delay the onset of cell injury, also delays the rise in [Ca\(^{2+}\)]. As shown in Figures 2 and 6, preconditioning delays the ischemia-induced rise in [Ca\(^{2+}\)] in the rat heart. These data are consistent with the hypothesis that at least some of the beneficial effects of preconditioning are mediated via a reduced rise in [Ca\(^{2+}\)] during ischemia. Preconditioning also reduced the rise in [Na\(^{+}\)], during ischemia, which could be important during early reperfusion as a stimulus for Ca\(^{2+}\) influx via Na\(^+-\)Ca\(^{2+}\) exchange. Thus, the injurious effects of increased [Ca\(^{2+}\)] may occur during the ischemic period and during early reperfusion. In addition to a possible relation between increased [Ca\(^{2+}\)] and irreversible plasma membrane damage, several groups have suggested a relation between an increase in [Ca\(^{2+}\)] and postischemic contractile dysfunction (stunning).\(^{11,14,23,56}\) It is unclear whether the mechanism(s) involved in stunning is the same as the mechanism(s) of plasma membrane injury; however, the data in this study show that the rapidity and magnitude of the elevation in [Ca\(^{2+}\)], during ischemia correlate with both the degree of postischemic contractile dysfunction after 30 minutes of ischemia, which is not associated with significant lethal injury, and the amount of enzyme release during reflow after 60 minutes of ischemia.

**How Does Preconditioning Result in a Lower Rise in [Ca\(^{2+}\)], During Ischemia?**

The studies described here were designed to evaluate the proposition that the beneficial effect of preconditioning on postischemic functional impairment and on the development of lethal injury in rat hearts may be due to attenuation of ionic derangements during ischemia. Compared with untreated hearts, preconditioned hearts have been shown previously to have decreased production of lactate during subsequent ischemic episodes.\(^{3}\) We hypothesize that the lower H\(^+\) production will decrease Na\(^+-\)H\(^+\), exchange. As discussed in detail previously,\(^{23}\) the lactate (and thus H\(^+\)) generated during 20–30 minutes of ischemia is on the order of 46–66 mM, sufficient to account for the increase in [Na\(^{+}\)] via Na\(^+-\)H\(^+\) exchange. The decrease in [H\(^+\)] in preconditioned hearts will attenuate the rise in [Na\(^{+}\)], during ischemia, and this in turn will reduce the rise in [Ca\(^{2+}\)], via Na\(^+-\)Ca\(^{2+}\) exchange. The data presented here are consistent with this hypothesis. Preconditioning significantly reduced the rise in [H\(^+\)], and [Ca\(^{2+}\)], during a subsequent 30-minute period of ischemia, and there was a slight but statistically insignificant reduction in the rise in [Na\(^{+}\)].

An explanation for the disparity between the effect of preconditioning on the rise in [Ca\(^{2+}\)], and on the rise in [Na\(^{+}\)], during a subsequent ischemic episode can be found by consideration of the thermodynamics of Na\(^+-\)Ca\(^{2+}\) exchange. Assuming that Na\(^+-\)Ca\(^{2+}\) exchange involves three Na\(^{+}\) ions for each Ca\(^{2+}\) ion and that Na\(^+-\)Ca\(^{2+}\) exchange achieves equilibrium conditions during ischemia, we can use the following equation:\(^{61}\)

\[
([\text{Na}^{+}]/[\text{Na}^{+}])_{3}\exp(E_{a}/RT) = ([\text{Ca}^{2+}]/[\text{Ca}^{2+}])_{n}
\]

where \(E_{a}\) is membrane potential, \(F\) is the Faraday constant, \(R\) is the gas constant, and \(T\) is absolute temperature. We assume that [Ca\(^{2+}\)], does not change during ischemia ([Ca\(^{2+}\)] = 1.25 mM) and that membrane potential is \(-40\) mV, according to the data of Kleber\(^{62}\) showing that membrane potential is \(-49.5\) mV after 15 minutes of ischemia at 31–33°C and the data of Hasin et al\(^{63}\) showing that membrane potential is approximately \(-40\) mV within minutes after metabolic blockade with cyanide and 2-deoxyglucose. To simplify the calculations, we also assume that intracellular and extracellular volumes are equal and do not change, so that the net increase in [Na\(^{+}\)], is equal to the net decrease in [Na\(^{+}\)]. Several studies\(^{64-66}\) have suggested that cell swelling occurs during ischemia, but estimates of the magnitude of the increase in intracellular volume are in the range of 10%, which would not substantially affect the calculations. There is a definite contraction of the extracellular space at the onset of total ischemia, which is presumably the result of collapse of the intravascular space, and there is a decrease in the size of the extracellular sodium peak in the 27Na spectra at the onset of ischemia of approximately 30%. As a consequence of the decrease in extracellular volume, when net sodium influx occurs during ischemia, there is a larger absolute change in [Na\(^{+}\)], than in [Na\(^{+}\)], but nevertheless, the percent change in [Na\(^{+}\)], is relatively small. Using our assumptions, including the assumption that there are no volume changes, we calculate that 3.2 \(\mu\)M [Ca\(^{2+}\)], at the end of 30 minutes of ischemia in untreated hearts would be in electrochemical equilibrium with 28.5 mM [Na\(^{+}\)], and 126.5 mM [Na\(^{+}\)]. (If we assume a 30% loss of extracellular volume at the start of ischemia, the calculation would predict an [Na\(^{+}\)], of 27 mM and an [Na\(^{+}\)], of 120.5 mM.) Using the same assumptions, including the assumption of no volume changes, we calculate that an [Na\(^{+}\)], of 25 mM would be in electrochemical equilibrium with the 2.1 \(\mu\)M [Ca\(^{2+}\)], measured in preconditioned hearts after 30 minutes of ischemia. Clearly, given the standard errors of our measurements as well as biological variability, we would not be able to detect a significant difference between 25 and 28.5 mM [Na\(^{+}\)]. Indeed, given all the assumptions involved with calculating sodium concentrations, the values we measure are remarkably close to expectations; we measure 21 mM [Na\(^{+}\)], in preconditioned hearts and 27 mM [Na\(^{+}\)], in untreated hearts. Thus, the sodium data are compatible with the hypothesis that Na\(^+-\)Ca\(^{2+}\) exchange plays a role in setting the cytosolic free calcium concentration during ischemia.

The data presented here are consistent with the report of Neely and Grotlohmann\(^{60}\) that showed that recovery of function correlated inversely with tissue lactate levels at the end of ischemia and that recovery of
function was improved in glycogen-depleted hearts. We have shown that preconditioning, which has been shown to reduce the production of lactate, reduces the fall in cytosolic pH during ischemia and that this attenuates the rise in [Ca$^{2+}$]. The ability of preconditioning to reduce the rise in [Ca$^{2+}$] during ischemia is also very similar to the effect of the Na$^+$-$\text{H}^+$ exchange inhibitors amiloride and dimethylamiloride. Addition of amiloride or dimethylamiloride just before ischemia blocks Na$^+$-$\text{H}^+$ exchange and thereby reduces the rise in [Na$^+$] and [Ca$^{2+}$], during 30 minutes of ischemia. With preconditioning, however, Na$^+$-$\text{H}^+$ exchange is reduced not by blocking the exchanger but by reducing the generation of $\text{H}^+$. Thus, both preconditioning and Na$^+$-$\text{H}^+$ exchange inhibitors delay the rise in [Ca$^{2+}$], during 30 minutes of global ischemia and improve recovery of function during reperfusion.

These data do not exclude the possibility that other factors may be involved in the reduced rise in [Ca$^{2+}$] during ischemia in preconditioned hearts, such as reduced catecholamine release during the subsequent prolonged period of ischemia and antagonism of the catecholamine-induced increase in [Ca$^{2+}$], which are perhaps due to the action of adenosine.

The data in this manuscript and elsewhere show that manipulations slowing the rise in [Ca$^{2+}$], during ischemia decrease the amount of enzyme release and reduce the degree of stunning observed on reflow. We demonstrate here that 16 mM magnesium (which has been shown to block calcium entry via calcium channels and Na$^+$-Ca$^{2+}$ exchange) blocks the increase in [Ca$^{2+}$], during 30 minutes of sustained ischemia to a greater extent than preconditioning does. This suggests that preconditioning does not block calcium entry as effectively as the addition of 16 mM magnesium. Preconditioning is not likely to block calcium entry totally. Calcium channels will still allow calcium entry, and furthermore, although there may be less calcium entry via Na$^+$-Ca$^{2+}$ exchange in preconditioned hearts because of reduced [Na$^+$], calcium entry via Na$^+$-Ca$^{2+}$ exchange is not blocked in a preconditioned heart. Thus, it is not surprising that there is less of a rise in [Ca$^{2+}$]. During ischemia in a magnesium-arrested heart than in a preconditioned heart. Figure 9 also shows that the effects of preconditioning and magnesium arrest are not additive. The rise in [Ca$^{2+}$] is the same in a heart treated with 16 mM magnesium as it is in a preconditioned heart treated with magnesium just before the sustained period of ischemia. In addition, compared with magnesium arrest alone, preconditioning with the addition of magnesium arrest confers no additional improvement in recovery of contractile function upon reflow. Taken together, these data are consistent with the hypothesis that at least some of the beneficial effects of preconditioning are related to the reduced rise in [Ca$^{2+}$]. If we block the rise in [Ca$^{2+}$] using 16 mM magnesium, we retain the beneficial effects of preconditioning, but preconditioning does not further reduce the rise in [Ca$^{2+}$].

It should be noted that a correlation between preconditioning and an attenuated rise in [Ca$^{2+}$], during the sustained period of ischemia has only been demonstrated in rats, and it is uncertain whether this relation will hold in other species. We attempted to confirm whether this correlation would be observed in the guinea pig heart, and we found that preconditioning did not cause an improvement in recovery of function on reflow after the sustained period of ischemia. There are no reports of preconditioning reducing necrosis in guinea pig hearts. Interestingly, the Na$^+$-$\text{H}^+$ exchange inhibitor amiloride, which blocks approximately 50% of the sodium uptake during ischemia in the rat heart, has no effect on sodium uptake in the guinea pig heart; this is attributed to a less active Na$^+$-$\text{H}^+$ exchanger in the guinea pig heart.

If our hypothesis that preconditioning leads to less generation of $\text{H}^+$ and therefore less Na$^+$-$\text{H}^+$ exchange and less increase in [Ca$^{2+}$], via Na$^+$-Ca$^{2+}$ exchange is correct and if the guinea pig heart has a relatively inactive Na$^+$-$\text{H}^+$ exchanger, then one might expect that preconditioning would have little effect on ionic derangements during ischemia in guinea pig myocardium. Furthermore, if Na$^+$-$\text{H}^+$ and Na$^+$-Ca$^{2+}$ exchange are important mechanisms in the rise in [Ca$^{2+}$] during ischemia and if the guinea pig heart has less active Na$^+$-$\text{H}^+$ exchange, then one might expect that [Ca$^{2+}$] would rise more slowly in the guinea pig heart than in the rat heart; this observation is confirmed in Figure 10. In agreement with these data, the study of Galinanes and Hearse reports that, although pretreatment with a cardioplegic solution containing high magnesium significantly improved recovery of cardiac function in the rat and most other animal species studied, cardioplegia did not improve recovery of function in guinea pig hearts. Thus, the guinea pig heart appears to be substantially different from the hearts of most other species. The differences between rat and guinea pig myocardium suggest that significant species variation may be present in the response to preconditioning.

Reduced Metabolic Demand

Although our data suggest that reduced Na$^+$-$\text{H}^+$ and Na$^+$-Ca$^{2+}$ exchange due to decreased H$^+$ production can explain the ionic effects of preconditioning, another hypothesis that must be considered is that the attenuation of ionic changes in ischemic preconditioned hearts is primarily a function of greater high-energy phosphate availability. Previous studies have shown that there is a good correlation between the fall in tissue ATP and the rise in [Ca$^{2+}$] during ischemia under a variety of experimental conditions. However, the ATP levels in preconditioned and untreated hearts are very similar during most of the ischemic period; therefore, it is unlikely that a difference in ATP concentration is responsible for the improved ionic homeostasis. It has been suggested that glycolytically generated ATP is preferentially used to maintain ion pumps, but this is unlikely to be the case in preconditioning, since preconditioned hearts generate less lactate than untreated hearts. However, it is possible that it is not total tissue ATP but ATP in some specific compartment that is important and that preconditioning preferentially preserves this ATP pool.

Although there is no experimental evidence that improved ATP availability is a factor in the attenuation of ionic derangements in ischemic preconditioned hearts, there is good evidence that metabolic demand is reduced in ischemic preconditioned myocardium. One manifestation of the reduced metabolic demand is that the rate of decline in tissue ATP content during the 30-minute period of ischemia is less in the precondi-
tioned heart (see Figure 5 and Reference 1). Furthermore, the rate of decline in tissue ATP content underestimates the actual decrease in metabolic demand because 1) ATP consumption is the sum of ATP produced by anaerobic glycolysis and ATP lost from tissue reserves, and 2) glycolytic ATP production during ischemia is less in preconditioned than in untreated hearts, as estimated by lactate accumulation. Thus, the data indicate that preconditioning both reduces ATP consumption and minimizes early ion derangements. Since [Ca\(^{2+}\)]\(_i\) appears to be an independent regulator of ATP consumption during metabolic inhibition\(^6\)\(^7\) and ischemia,\(^8\) the reduced rate of ATP consumption may be related, at least in part, to a decrease in Na\(^+-\)H\(^+\) and Na\(^+\)-Ca\(^{2+}\) exchange. However, this does not exclude the possibility that other factors are involved as well. Other factors could include adenosine or some other mediator that is produced during the preconditioning period and is not immediately washed out during the final reflow period.\(^6\)\(^7\) Adenosine has been shown to activate a potassium channel,\(^9\)\(^10\) which decreases action potential duration; this could be expected to decrease ATP utilization.

In summary, the present study generally supports the conclusions of our previous studies\(^1\)\(^4\)\(^2\)\(^3\)\(^2\) that cardioprotective interventions instituted before a sustained period of ischemia delay but do not prevent the rise in [Ca\(^{2+}\)]\(_i\) during ischemia and delay but do not prevent lethal myocyte injury. The cardioprotective effects of preconditioning are likely multifactorial and may involve both reduction in metabolic demand and attenuation of ionic derangements. The data are consistent with the hypothesis that increased [Ca\(^{2+}\)]\(_i\) is an important pathogenic factor in the development of stunning and plasma membrane disruption and that one of the beneficial effects of preconditioning is the decrease in anaerobic glycolysis, resulting in less acidosis, less Na\(^+-\)H\(^+\) exchange, and less Na\(^+\)-Ca\(^{2+}\) exchange than in untreated hearts.

Acknowledgment

We thank Scott Gabel for his capable assistance.

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Circ Res. 1993;72:112-125
doi: 10.1161/01.RES.72.1.112

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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