Preconditioning limits mitochondrial Ca\(^{2+}\) during ischemia in rat hearts: role of K\(_{\text{ATP}}\) channels

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1Division of Cardiovascular Medicine, University of California, Davis 95616; Department of Veterans Affairs, Northern California Health Care System, Mather 95655; and 2Division of Cardiology, San Francisco General Hospital, San Francisco, California 94110

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Wang, Lianguo, Gennady Cherednichenko, Lisa Hernandez, Jessica Halow, S. Albert Camacho, Vincent Figueroed, and Saul Scaefher. Preconditioning limits mitochondrial Ca\(^{2+}\) during ischemia in rat hearts: role of K\(_{\text{ATP}}\) channels. Am J Physiol Heart Circ Physiol 280: H2321–H2328, 2001.—Prolonged myocardial ischemia results in an increase in intracellular calcium concentration ([Ca\(^{2+}\)]\(_{\text{i}}\)), which is thought to play a critical role in ischemia-reperfusion injury. Ischemic preconditioning (PC) improves myocardial function during ischemia-reperfusion, a process that may involve opening mitochondrial ATP-sensitive potassium (K\(_{\text{ATP}}\)) channels. Because pharmacological limitation of 

Opening of mitochondrial K\(_{\text{ATP}}\) channels has been shown to protect the heart during ischemia-reperfusion (11, 14), although the mechanism of this protection is largely unknown. One hypothesis proposed is that opening the mitochondrial K\(_{\text{ATP}}\) channel dissipates the inner mitochondrial membrane potential established by the proton pump (26). This dissipation is expected to reduce the driving force for Ca\(^{2+}\) influx through the Ca\(^{2+}\) uniporter, thus attenuating mitochondrial calcium concentration ([Ca\(^{2+}\)]\(_{\text{m}}\)) overload (8–10). This postulate has been supported in a recent study using isolated rat mitochondria and intact rat cardiomyocytes in which K\(_{\text{ATP}}\) channel openers prevented [Ca\(^{2+}\)]\(_{\text{m}}\) overload by both reducing the driving force for Ca\(^{2+}\) uptake via the uniporter and by activating cyclosporin-sensitive Ca\(^{2+}\) efflux (20).

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Thus the purpose of this study was to test the hypotheses that 1) PC limits the accumulation of [Ca\(^{2+}\)]\(_{\text{im}}\) during ischemia and reperfusion, and 2) this effect is mediated by opening mitochondrial \(K_{\text{ATP}}\) channels. These experiments used the fluorescence indicator indo 1 to measure [Ca\(^{2+}\)] in isolated, perfused hearts with parallel measurement of hemodynamics. The role of mitochondrial \(K_{\text{ATP}}\) was elucidated using the mitochondrial \(K_{\text{ATP}}\) channel opener diazoxide and the mitochondrial \(K_{\text{ATP}}\) channel blocker 5-hydroxydecanoic acid (5-HD).

**MATERIALS AND METHODS**

**Heart Perfusion and Measurements of Function**

Male Sprague-Dawley rats (300–350 g) were administered heparin (100 units ip) and anesthetized with pentobarbital sodium (65 mg/kg ip). As soon as deep anesthesia was achieved, evidenced by lack of eye-blink reflex and foot withdrawal reflex, hearts were rapidly isolated and retrograde perfused with a modified Krebs-Henseleit buffer containing the following (in mM): 118 NaCl, 4.7 KCl, 1.2 MgSO\(_4\), 1.2 CaCl\(_2\), 25 NaHCO\(_3\), and 11 glucose. The hearts were not paced, and perfusion pressure was set at 100 cmH\(_2\)O. The perfusion apparatus was temperature controlled with heated baths used for the perfusate and a water jacketing around the perfusion tubing to maintain heart temperature at 37 ± 0.05°C under all conditions. A gas mixture of 95% O\(_2\)-5% CO\(_2\) was directly bubbled in the perfusate containers. Left ventricular end-diastolic pressure (LVEDP) and systolic pressure were measured throughout the protocols via a H\(_2\)O-filled latex balloon inserted into the left ventricle. Hemodynamic data were obtained using a computer-based physiological recording system (Biopac Systems; Santa Barbara, CA).

**Fluorescence Measurements**

**Fluorescence instrumentation.** Fluorescence measurements were performed as previously described in detail (3, 4) using a modified spectrofluorometer (model SLM8100, SLM Instruments; Rochester, NY). Excitation light from a 450-W xenon arc lamp (Ushio) was filtered through a 350-nm interference filter and focused onto the ingoing leg of a quartz bifurcated fiber bundle (Dolan-Jenner Industries; Lawrence, MA). The common leg of this 1.57-mm diameter fiber bundle was girdled against the epicardial surface of the left ventricle, avoiding visible vessels. A shutter in front of the excitation light was opened for only seconds at a time during data acquisition to prevent bleaching of the indo 1 fluorescence. The fluorescence signal was transferred via the outgoing leg of the bundle and separated by 385- and 456-nm interference filters before detection by photomultiplier tubes.

**Indo 1 acetoxymethyl ester loading.** After a 15-min equilibration period, baseline background fluorescence [primarily NADH (3, 4)] was measured and subtracted from all subsequent fluorescence measurements. Hearts were then loaded for 25 min by retrograde perfusion with Krebs-Henseleit buffer containing indo 1, acetoxymethyl ester (indo 1-AM; 6 μM, dissolved in dimethyl sulfoxide and Pluronic F-127, 20% wt/vol, Molecular Probes; Johnston City, OR) and fetal bovine serum (1%). Probenvecid (0.1 mM, Sigma; St. Louis, MO) was added to all perfusates to slow the extrusion of indo 1 from the myocytes (1). Residual indo 1-AM was washed out by perfusing with standard buffer for 25 min. An experiment was discarded if the fluorescent intensity at either 385 or 456 nm was less than twice the background fluorescence during any phase of the experimental protocol.

**Experimental Protocols**

Hearts were randomly assigned to one of five groups described below (\(n = 5\) in each group). All hearts had a 15-min equilibration period with baseline measurements of LVEDP, systolic pressure, and background fluorescence. Loading of indo 1-AM was followed by a 25-min washout period. Control hearts then had a 50-min period of normal perfusion before 25 min of no-flow ischemia and 30 min of reperfusion. PC hearts had four PC episodes (5-min ischemia and 5-min reperfusion) followed by 10 min of normal perfusion before ischemia. The effects of mitochondrial \(K_{\text{ATP}}\) channel blockade on PC were examined by perfusing with 5-HD (100 μM) for 10 min before PC (PC + 5-HD). The effects of pharmacological mitochondrial \(K_{\text{ATP}}\) channel opening by adding diazoxide (100 μM) to the perfusate for 10 min before ischemia with or without 5-HD (100 μM) (diazoxide and diazoxide + 5-HD). Drug concentrations used in this study are active in the perfused rat heart model (12). [Ca\(^{2+}\)]\(_{\text{im}}\) and [Ca\(^{2+}\)]\(_{\text{m}}\) were measured separately in parallel experiments. Hemodynamic and indo 1 fluorescence intensity measurements were repeated every 5 min.

**Mn\(^{2+}\) quenching of cytosolic fluorescence.** To determine mitochondrial fluorescence, cytosolic fluorescence was quenched by adding MnCl\(_2\) at a final concentration of 17.5 μM to the perfusate 10 min before the perfusate 10 min before the 25-min ischemic period. Adequate quenching was verified by the loss of calcium transients after manganese loading. The addition of MnCl\(_2\) did not alter cardiac function (heart rate or developed pressure).

**Calculations**

**Calculation of [Ca\(^{2+}\)]\(_{\text{im}}\), [Ca\(^{2+}\)]\(_{\text{m}}\), was calculated using the standard equation for fluorescent calcium indicators (15)**

\[
[\text{Ca}^{2+}] = K_d \cdot \frac{((R - R_{\text{min}})/(R_{\text{max}} - R)) \cdot S_{456}}{R_{\text{max}}} - R_{\text{min}}
\]

where \(R\) is the ratio of fluorescence at 385 nm and 456 nm, \(R_{\text{min}}\) and \(R_{\text{max}}\) are the fluorescence ratios at zero and saturating [Ca\(^{2+}\)], respectively, as determined in a previous study (3). A dissociation constant (\(K_d\)) of 594 nM was used to calculate [Ca\(^{2+}\)], (2, 34). \(S_{456}\) is the ratio of fluorescence intensities during saturating and zero [Ca\(^{2+}\)], at 456-nm emission wavelength (2).

**Calculation of [Ca\(^{2+}\)]\(_{\text{m}}\), [Ca\(^{2+}\)]\(_{\text{im}}\) was calculated using the same equation employed to calculate total [Ca\(^{2+}\)] from the fluorescent intensity after manganese quenching. On the basis of prior studies (28, 29, 35), the calibration parameters were assumed to be the same in the cytosolic and mitochondrial compartments.**

The above calculations, the results of which are presented in Tables 1 and 2, do not take into account the confounding issues of NADH autofluorescence, leak of indo 1 into the extracellular space, or the effect of manganese quenching on extracellular calcium or mitochondrial calcium during myocardial ischemia. Experiments were undertaken to semi-quantitatively assess these effects and determine whether these issues would alter the findings of the study.

**NADH autofluorescence.** NADH is known to increase during ischemia, thus increasing the background fluorescence signal. Although it was not possible to simultaneously measure NADH and indo 1 fluorescence in each individual heart, NADH fluorescence was measured in parallel experiments in all groups. The change in NADH fluorescence was similar between groups, increasing minimally at 385 nm but dou-
Statistical Analysis

Data presented are means ± SE. Differences in data between groups were analyzed using ANOVA. Student-Newman-Keuls post-test was used if the ANOVA was significant. A value of P < 0.05 was considered statistically significant.

RESULTS

Hemodynamics

Diazoxide and 5-HD had little effect on left ventricular developed pressure (LVDP) and LVEDP before ischemia. After ischemia and 30 min of reperfusion, LVDP was significantly greater in diazoxide-treated and PC hearts than in control hearts (Fig. 2A). During the reperfusion period, LVEDP was elevated in control hearts, whereas both diazoxide and PC significantly reduced LVEDP during reperfusion (Fig. 2B). The effects of diazoxide and PC on LVDP and LVEDP were partially or completely abolished by 5-HD (Fig. 2).

\[
\frac{[Ca^{2+}]_i}{[Ca^{2+}]_m},
\]

versus time for all groups are shown in Table 1 and Fig. 3. \([Ca^{2+}]_i\), in control hearts increased steadily during 25 min of ischemia and reached an average concentration of 650 nM at the end of ischemia. The increase in \([Ca^{2+}]_i\), was similar in other groups with the exception of hearts treated with diazoxide, which had higher \([Ca^{2+}]_i\); after 25 min of ischemia. \([Ca^{2+}]_i\), in control hearts increased further during the first 20 min of reperfusion and then fell, but did not return to preischemic levels after 30 min of reperfusion. PC abolished the increase in \([Ca^{2+}]_i\), during reperfusion, resulting in significantly lower \([Ca^{2+}]_i\); than in control hearts. In contrast, \([Ca^{2+}]_m\), increased during reperfusion in the diazoxide-treated hearts (Fig. 3A). 5-HD reversed the effects of both diazoxide and PC on \([Ca^{2+}]_i\) (Fig. 3B).

\[
\frac{[Ca^{2+}]_m}{[Ca^{2+}]_i}
\]

was identical in all groups before the initiation of ischemia (Table 2). However, the increase in \([Ca^{2+}]_m\) was significantly lower in both diazoxide-treated and PC hearts than in control hearts during 25 min of ischemia and 30 min of reperfusion (Table 2 and

Table 1. Calculated \([Ca^{2+}]_i\), during ischemia and reperfusion

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control</th>
<th>Diazoxide</th>
<th>Diazoxide + 5-HD</th>
<th>PC</th>
<th>PC + 5-HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>236 ± 7</td>
<td>257 ± 20</td>
<td>226 ± 11</td>
<td>262 ± 16</td>
<td>233 ± 11</td>
</tr>
<tr>
<td>5</td>
<td>259 ± 10</td>
<td>280 ± 9</td>
<td>242 ± 12</td>
<td>292 ± 19</td>
<td>235 ± 8</td>
</tr>
<tr>
<td>10</td>
<td>333 ± 24</td>
<td>391 ± 30</td>
<td>303 ± 20</td>
<td>388 ± 25</td>
<td>284 ± 26</td>
</tr>
<tr>
<td>15</td>
<td>448 ± 36</td>
<td>477 ± 44</td>
<td>376 ± 20</td>
<td>468 ± 64</td>
<td>376 ± 30</td>
</tr>
<tr>
<td>20</td>
<td>555 ± 45</td>
<td>613 ± 28</td>
<td>413 ± 13</td>
<td>608 ± 68</td>
<td>453 ± 32</td>
</tr>
<tr>
<td>25</td>
<td>648 ± 37</td>
<td>878 ± 30*</td>
<td>542 ± 39</td>
<td>675 ± 62</td>
<td>588 ± 36</td>
</tr>
<tr>
<td>30</td>
<td>941 ± 75</td>
<td>1,349 ± 95</td>
<td>847 ± 113</td>
<td>679 ± 48*</td>
<td>761 ± 57</td>
</tr>
<tr>
<td>35</td>
<td>1,025 ± 70</td>
<td>1,461 ± 91</td>
<td>912 ± 41</td>
<td>734 ± 65*</td>
<td>921 ± 105</td>
</tr>
<tr>
<td>40</td>
<td>999 ± 120</td>
<td>1,441 ± 85</td>
<td>910 ± 46</td>
<td>655 ± 52*</td>
<td>795 ± 113</td>
</tr>
<tr>
<td>45</td>
<td>932 ± 119</td>
<td>1,508 ± 113</td>
<td>875 ± 55</td>
<td>608 ± 53*</td>
<td>605 ± 59*</td>
</tr>
<tr>
<td>50</td>
<td>795 ± 127</td>
<td>1,249 ± 82*</td>
<td>702 ± 41</td>
<td>567 ± 50*</td>
<td>533 ± 50*</td>
</tr>
<tr>
<td>55</td>
<td>722 ± 147</td>
<td>1,010 ± 44</td>
<td>681 ± 38</td>
<td>435 ± 30*</td>
<td>507 ± 55*</td>
</tr>
</tbody>
</table>

Values are means ± SE. \([Ca^{2+}]_i\), intracellular calcium concentration (in nM). 5-HD, 5-hydroxydecanoic acid; PC, preconditioning. The ischemic time period is 5–25 min. *P < 0.05 vs. control.

Table 2. Calculated \([Ca^{2+}]_m\) during ischemia and reperfusion

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Control</th>
<th>Diazoxide</th>
<th>Diazoxide + 5-HD</th>
<th>PC</th>
<th>PC + 5-HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>252 ± 20</td>
<td>271 ± 11</td>
<td>236 ± 21</td>
<td>277 ± 7</td>
<td>231 ± 15</td>
</tr>
<tr>
<td>5</td>
<td>347 ± 29</td>
<td>291 ± 21</td>
<td>267 ± 8</td>
<td>273 ± 20</td>
<td>269 ± 17</td>
</tr>
<tr>
<td>10</td>
<td>385 ± 45</td>
<td>328 ± 28*</td>
<td>291 ± 9</td>
<td>277 ± 7*</td>
<td>341 ± 32</td>
</tr>
<tr>
<td>15</td>
<td>471 ± 59</td>
<td>372 ± 29*</td>
<td>397 ± 22</td>
<td>316 ± 25*</td>
<td>426 ± 39</td>
</tr>
<tr>
<td>20</td>
<td>720 ± 63</td>
<td>477 ± 33*</td>
<td>503 ± 47*</td>
<td>394 ± 34*</td>
<td>606 ± 36</td>
</tr>
<tr>
<td>25</td>
<td>895 ± 60</td>
<td>607 ± 41*</td>
<td>753 ± 66*</td>
<td>488 ± 41*</td>
<td>880 ± 22</td>
</tr>
<tr>
<td>30</td>
<td>1,242 ± 86</td>
<td>734 ± 33*</td>
<td>1,365 ± 51</td>
<td>787 ± 105*</td>
<td>1,454 ± 141</td>
</tr>
<tr>
<td>35</td>
<td>1,498 ± 118</td>
<td>630 ± 22*</td>
<td>1,451 ± 160</td>
<td>695 ± 82*</td>
<td>1,347 ± 83</td>
</tr>
<tr>
<td>40</td>
<td>2,026 ± 125</td>
<td>692 ± 37*</td>
<td>1,484 ± 138</td>
<td>734 ± 70*</td>
<td>1,357 ± 62*</td>
</tr>
<tr>
<td>45</td>
<td>2,293 ± 97</td>
<td>736 ± 40*</td>
<td>1,525 ± 128*</td>
<td>716 ± 78*</td>
<td>1,418 ± 100*</td>
</tr>
<tr>
<td>50</td>
<td>2,392 ± 130</td>
<td>729 ± 41*</td>
<td>1,573 ± 125*</td>
<td>645 ± 71*</td>
<td>1,227 ± 136*</td>
</tr>
<tr>
<td>55</td>
<td>2,580 ± 195</td>
<td>697 ± 37*</td>
<td>1,691 ± 130*</td>
<td>622 ± 33*</td>
<td>1,268 ± 134*</td>
</tr>
</tbody>
</table>

Values are means ± SE. \([Ca^{2+}]_m\), mitochondrial calcium concentration (in nM). The ischemic time period is 5–25 min. *P < 0.05 versus control.
These differences occurred early in the ischemic period (10 min). 5-HD abolished the effects of both diazoxide and PC in limiting mitochondrial Ca\(^{2+}\) overload during ischemia and in the early reperfusion period. However, control hearts still had higher \([\text{Ca}^{2+}]_m\) in the last 15 min of reperfusion compared with the 5-HD-treated hearts (Table 2 and Fig. 4B).

**Relationship of [Ca\(^{2+}\)] to Functional Recovery**

The absence of functional recovery in most of the control and diazoxide + 5-HD-treated hearts precluded quantitative assessment of the relationship of \([\text{Ca}^{2+}]\) to functional recovery in these groups. Functional recovery defined by the LVDP after 30 min of reperfusion was inversely related to \([\text{Ca}^{2+}]_m\). As seen in Fig. 5, there was a linear relationship across the PC, PC + 5-HD, and diazoxide groups, with >50% recovery only seen in those hearts (PC and diazoxide) with \([\text{Ca}^{2+}]_m\) below 750 nM. These results parallel those of Miyamae et al. (27).

**DISCUSSION**

Previous studies using isolated rat hearts have shown that the cardioprotective effect of PC is due in part to opening of mitochondrial K\(_{\text{ATP}}\) channels and that diazoxide, a mitochondrial K\(_{\text{ATP}}\) channel opener, mimics PC (11, 12, 26). However, the mechanism of this effect is unknown. This study shows that opening mitochondrial K\(_{\text{ATP}}\) channels by either PC or a K\(_{\text{ATP}}\) channel opener can reduce mitochondrial calcium overload during ischemia and reperfusion in isolated rat hearts. Although the reduction in \([\text{Ca}^{2+}]_m\) was associated with greater recovery of LVDP, the protective effect was independent of total \([\text{Ca}^{2+}]_i\). These data are consistent with the hypothesis that opening mitochondrial K\(_{\text{ATP}}\) channels, either pharmacologically or by PC, limits mitochondrial Ca\(^{2+}\) overload during ischemia and reperfusion.

**[Ca\(^{2+}\)]_m Overload and Ischemia-Reperfusion Injury**

These experiments validate that mitochondrial Ca\(^{2+}\) accumulates during and after ischemia. Mitochondria have distinct pathways for Ca\(^{2+}\) influx and efflux (17). Under normoxic conditions, uptake of Ca\(^{2+}\) occurs predominantly by a uniporter and is driven by the mitochondrial membrane potential (17). Ca\(^{2+}\) efflux occurs principally via the Na\(^+\)/Ca\(^{2+}\) antiporter, the maximum capacity of which is roughly one-tenth that of influx via the uniporter. Another regulatory pathway is referred to as the mitochondrial permeability transition pore (16). Opening of this pore can result from massive mitochondrial calcium overload (24), causing mitochondria to become uncoupled and the proton-translocating ATPase to turn from synthesizing ATP to actively hydrolyzing ATP, eventually resulting in cell death.

Prior studies have indicated that reperfusion/reoxygenation injury correlates well with \([\text{Ca}^{2+}]_m\) uptake, although there is no correlation with \([\text{Ca}^{2+}]_i\) (27, 28). In Miyamae et al. (27), \([\text{Ca}^{2+}]_m\) was measured by Mn\(^{2+}\) quenching 20 min into reperfusion with and without exposure to ruthenium red to block entry of Ca\(^{2+}\) into the mitochondria. This study found that functional recovery was greater in hearts exposed to ruthenium red to block entry of Ca\(^{2+}\) into the mitochondria. This study found that functional recovery was greater in hearts exposed to ruthenium red, was inversely correlated with \([\text{Ca}^{2+}]_m\), but was independent of \([\text{Ca}^{2+}]_i\) (which was unaffected by blockade of mitochondrial calcium entry). These findings were consistent with a critical role of pharmacological limitation of \([\text{Ca}^{2+}]_m\) during reperfusion after ische-
current findings indicate that both PC and K\textsubscript{ATP} channel opening with diazoxide limit [Ca\textsuperscript{2+}]\textsubscript{m} during ischemia as well as reperfusion in parallel with greater functional recovery. As in the prior study (27), the degree of functional recovery was linearly and inversely related to [Ca\textsuperscript{2+}]\textsubscript{m} below a threshold level (Fig. 5). This may represent a threshold value of [Ca\textsuperscript{2+}]\textsubscript{m} above which mitochondria cannot function or, possibly, varying proportions of [Ca\textsuperscript{2+}]\textsubscript{m} values from populations of mitochondria that have normal or lethal calcium concentrations.

### The Role of Mitochondrial K\textsubscript{ATP} Channels

Recent studies suggest that the cardioprotective effects induced by the mitochondrial K\textsubscript{ATP} channel opener diazoxide and PC are due to preservation of mitochondrial function (11, 23). It has been proposed that mitochondrial K\textsubscript{ATP} channel openers, by virtue of their ability to dissipate the mitochondrial membrane potential (6, 19), may reduce the driving force for Ca\textsuperscript{2+} accumulation (25). Recently, Holmuhamedov et al. (20) showed, at the level of isolated mitochondria as well as in intact cardiomyocytes, that K\textsubscript{ATP} channel openers...
not only impede mitochondrial Ca$^{2+}$ uptake but also promote mitochondrial Ca$^{2+}$ release, thereby diminishing the amount of accumulated Ca$^{2+}$ within the mitochondrial matrix. In the current study, diazoxide, a selective mitochondrial KATP channel opener, reduced [Ca$^{2+}$]$_{m}$ overload during ischemia and reperfusion, which was abolished by the mitochondrial KATP channel-selective blocker 5-HD.

Previous studies suggest that PC-induced cardioprotection is mediated via activation of mitochondrial KATP channels (12, 25). Recently, Fryer et al. (11) examined the role of mitochondrial KATP channels in a rat model of PC and demonstrated that inhibition of the mitochondrial KATP channel attenuated cardioprotection induced by PC. The present study provides further evidence that PC-induced cardioprotection is indeed mediated by opening of the mitochondrial KATP channel in the isolated rat heart because cardioprotection was abolished by the mitochondrial KATP channel-selective blocker 5-HD. More importantly, PC-induced opening of mitochondrial KATP channels limited [Ca$^{2+}$]$_{m}$ overload. These data are consistent with the previous hypothesis that PC-induced opening of the mitochondrial KATP channel would reduce [Ca$^{2+}$]$_{m}$ overload (25). As has been proposed, the mitochondrial membrane potential may play an important role in this process. Recently, Ylitalo et al. (38) showed that the decrease in the mitochondrial membrane potential during prolonged ischemia was more rapid in preconditioned hearts. On the basis of these data, we can speculate that lower [Ca$^{2+}$]$_{m}$ in the preconditioned hearts resulted, at least in part, from a decrease in the driving force for Ca$^{2+}$ entering mitochondria through the Ca$^{2+}$ uniporter.

Although the current results do not indicate whether lower [Ca$^{2+}$]$_{m}$ during ischemia and reperfusion was directly responsible for the cardioprotective effect of the mitochondrial KATP channel opener and PC, previous studies (27) with Ca$^{2+}$-uniporter blockers suggest this is likely an important component of cardioprotection. By assessing mitochondrial Ca$^{2+}$ and the functional recovery in isolated rat hearts, this study provides a link between ischemia-reperfusion injury, [Ca$^{2+}$]$_{m}$ overload, and mitochondrial KATP channel activation.

**Mitochondrial Versus Cellular Calcium Overload**

It is interesting to note that, although diazoxide and PC had a profound effect on mitochondrial Ca$^{2+}$, PC did not limit the accumulation of [Ca$^{2+}$]$_{i}$ during ischemia and diazoxide-treated hearts had even higher end-ischemic [Ca$^{2+}$]$_{i}$ than control hearts. Even more noteworthy, [Ca$^{2+}$]$_{i}$ in PC hearts was significantly lower than in control hearts after 30 min of reperfusion, whereas [Ca$^{2+}$]$_{i}$ in the diazoxide-treated group was higher than control. Thus there was a dissociation between functional recovery (seen in both groups) and lower [Ca$^{2+}$]$_{i}$, (seen only in the PC hearts). These data parallel the findings of Miyamae et al. (27), who found that recovery of ischemic-reperfused hearts was dependent on lower [Ca$^{2+}$]$_{m}$ but was independent of [Ca$^{2+}$]$_{i}$.

The role of lower [Ca$^{2+}$]$_{i}$ in the PC hearts and the mechanism of this observation is only speculative. It is...
unclear whether the limitation of $[Ca^{2+}]_{i}$ was a primary effect of PC or, more likely, a secondary effect of improved mitochondrial function resulting in greater ATP production and, hence, sarcolemmal Ca-ATPase function (10). Alternatively, other mechanisms of PC, such as preservation of sarcoplasmic reticulum (SR) function, may play a role in regulating $[Ca^{2+}]_{i}$. Investigators (31, 36) have reported that SR Ca$^{2+}$ release and Ca$^{2+}$ uptake activity was relatively preserved in PC hearts after reperfusion, an effect that may result in greater SR accumulation of calcium and lower cytosolic calcium. Another factor that may affect intracellular calcium in PC hearts is the rapid reduction in intracellular sodium observed during reperfusion (33), an event that would alter the kinetics of Na$^{+}$/Ca$^{2+}$ exchange across the sarcolemma and limit $[Ca^{2+}]_{i}$. In contrast, the pharmacological opening of mitochondrial K$_{ATP}$ channels with diazoxide would not be expected to produce these ancillary effects of PC and, by limiting mitochondrial Ca$^{2+}$ accumulation, result in higher $[Ca^{2+}]_{i}$.

These data suggest that elevation of the intracellular level of $[Ca^{2+}]_{i}$ may not be necessarily pathological when the mitochondrial level of $[Ca^{2+}]_{m}$ is limited. This may occur because a critical element in determining cellular recovery and the return of ion homeostasis on reperfusion is the ability of the cell to synthesize ATP and maintain the free energy of hydrolysis ($\Delta G$) above a critical level.

**Limitations**

In contrast to other techniques, the use of indo 1 calcium fluorescence has distinct advantages with minimal effects on contractility, excellent time resolution, and the ability to identify $[Ca^{2+}]_{m}$ (3, 4, 27–29, 34). However, certain limitations to this technique must be considered, as noted in MATERIALS AND METHODS. We have considered these potential confounding factors and provided evidence that the effects of NADH autofluorescence, indo 1 leakage, and manganese quenching are valid. Inves.

**REFERENCES**


