Mechanisms of ischemic preconditioning effects on Ca\textsuperscript{2+} paradox-induced changes in heart

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Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, and Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R2H 2A6; and Second Department of Internal Medicine Yamanashi Medical University, Yamanashi 409-3898, Japan

Kawabata, Ken-Ichi, Thomas Netticadan, Mitsuru Osada, Kohji Tamura, and Naranjan S. Dhalla. Mechanisms of ischemic preconditioning effects on Ca\textsuperscript{2+} paradox-induced changes in heart. Am. J. Physiol. Heart Circ. Physiol. 278: H1008–H1015, 2000.—The effects of ischemic preconditioning (IP) on changes in cardiac performance and sarcoplasmic reticulum (SR) function due to Ca\textsuperscript{2+} paradox were investigated. Isolated perfused hearts were subjected to IP (three cycles of 3-min ischemia and 3-min reperfusion) followed by Ca\textsuperscript{2+}-free perfusion and reperfusion (Ca\textsuperscript{2+} paradox). Perfusion of hearts with Ca\textsuperscript{2+}-free medium for 5 min followed by reperfusion with Ca\textsuperscript{2+}-containing medium for 30 min resulted in a dramatic decrease in the left ventricular (LV) developed pressure and a marked increase in LV end-diastolic pressure. Alterations in cardiac contractile activity due to Ca\textsuperscript{2+} paradox were associated with depressed SR Ca\textsuperscript{2+}-uptake, Ca\textsuperscript{2+}-pump ATPase, and Ca\textsuperscript{2+}-release activities as well as decreased SR protein contents for Ca\textsuperscript{2+}-pump and Ca\textsuperscript{2+} channels. All these changes due to Ca\textsuperscript{2+} paradox were significantly prevented in hearts subjected to IP. The protective effects of IP on Ca\textsuperscript{2+} paradox changes in cardiac contractile activity as well as SR Ca\textsuperscript{2+}-pump and Ca\textsuperscript{2+}-release activities were lost when the hearts were treated with 8-([p-sulphophenyl]-theophylline, an adenosine receptor antagonist; KN-93, a specific Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMK II) inhibitor; or chelerythrine chloride, a protein kinase C (PKC) inhibitor. These results indicate that IP rendered cardioprotection by preventing a depression in SR function in Ca\textsuperscript{2+} paradox hearts. Furthermore, these beneficial effects of IP may partly be mediated by adenosine receptors, PKC, and CaMK II.

ischemic preconditioning; calcium paradox; cardiac function; cardiac sarcoplasmic reticulum

BRIEF PERIODS OF MYOCARDIAL ischemia and reperfusion have been shown to render cardioprotection against a prolonged period of ischemia-reperfusion; this phenomenon has been termed as ischemic preconditioning (IP) (19). It has been previously reported that IP has beneficial effects with respect to myocardial infarct size, arrhythmias, and cellular injury due to ischemia-reperfusion (14, 19, 21, 26). Several endogenous agonists such as adenosine (14), norepinephrine (27), and bradykinin (7), as well as free radicals (5), have been implicated in mediating the beneficial effects of IP. Protein kinase C (PKC), a major signal transduction mechanism, has been shown to play an important role in protecting the ischemic-preconditioned heart (15). Other targets such as tyrosine kinase (6), ATP-sensitive K (K\textsubscript{ATP}) channels (23), and phosphorylation by Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMK II) (20) have also been considered as mechanisms underlying the cardioprotective effects of IP. In a recent study, we have shown that IP exerted beneficial effects in hearts subjected to Ca\textsuperscript{2+} paradox (11), a phenomenon where a brief period of Ca\textsuperscript{2+} depletion followed by Ca\textsuperscript{2+} repletion results in marked myocardial cell damage and contractile dysfunction (31). Because both Ca\textsuperscript{2+} paradox and ischemia-reperfusion injury are known to be associated with the occurrence of intracellular Ca\textsuperscript{2+} overload (3, 24), it is possible that IP may elicit beneficial effects on the myocardium by preventing the development of intracellular Ca\textsuperscript{2+} overload. Whereas a great deal of work has been carried out to understand the mechanisms of the cardioprotective effects of IP with respect to alterations due to ischemia-reperfusion, the mechanisms underlying the IP-mediated cardioprotection of changes due to Ca\textsuperscript{2+} paradox are not known. Thus a study concerning the nature of IP effects on the Ca\textsuperscript{2+} paradox-induced alterations in the heart can be seen to provide further information in the area of cardioprotection.

Under physiological conditions, a small amount of Ca\textsuperscript{2+} entering through the L-type Ca\textsuperscript{2+} channels in the sarcolemmal (SL) membrane induces a release of Ca\textsuperscript{2+} from the sarcoplasmic reticulum (SR) stores and thus...
results in cardiac contraction (8). Cardiac relaxation occurs when the major part of the cytosolic Ca\(^{2+}\) is pumped back into the SR via the Ca\(^{2+}\)-pump ATPase and the rest is extruded by the SL Na\(^+/\)Ca\(^{2+}\) exchanger and the SL Ca\(^{2+}\)-pump ATPase. Because of its remarkable ability to regulate the intracellular concentration of Ca\(^{2+}\), SR is known to play a central role in the contraction-relaxation cycle of the cardiac muscle (8). Earlier studies have demonstrated that IP may exert beneficial effects on the ischemia-reperfusion induced alterations in the SR Ca\(^{2+}\)-uptake and Ca\(^{2+}\)-release activities in the heart (2, 20, 32) and these may partly explain the cardioprotective effects of IP. Although an impairment in the SR function has been reported to occur in the Ca\(^{2+}\) paradox hearts (2), the effects of IP on Ca\(^{2+}\) paradox-induced changes in SR have not been investigated. The present study was therefore undertaken to examine the effects of IP on cardiac SR function by employing an isolated rat heart model of Ca\(^{2+}\) paradox. Because adenosine, PKC, and CaMK II (14, 15, 20) are considered to be important in mediating the effects of IP on ischemia-reperfusion-induced changes in the heart, we examined the role of these mediators in hearts subjected to Ca\(^{2+}\) paradox.

**MATERIALS AND METHODS**

Heart perfusion and experimental protocol. Male Sprague-Dawley rats (300–350 g) were anesthetized by an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) mixture. The hearts were rapidly excised and perfused by the Langendorff procedure. The perfusion medium containing (in mmol/l) 120 NaCl, 25 NaHCO\(_3\), 4.7 KCl, 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 1.25 CaCl\(_2\), and 11 glucose was gassed with 95% O\(_2\)-5% CO\(_2\) and maintained at pH 7.4 at 37°C. The hearts were paced at 300 beats/min by an electrical stimulator (Phipp and Bird, Richmond, VA), and the coronary flow rate was maintained at 10 ml/min. For measuring the left ventricular pressure, the left atrium was removed and a latex balloon connected to a pressure transducer was inserted through the mitral valve into the left ventricle. The balloon was filled with the perfusion medium and adjusted to the left ventricular end-diastolic pressure (LVEDP) of 5–7 mmHg. Values for the left ventricular developed pressure (LVDP), rate of pressure development (+dP/dt), and rate of pressure decay (−dP/dt) were obtained through the program AcqKnowledge for Windows 3.0 (Biopac System, Goleta, CA). The balloon technique employed here allowed stable recording of the hemodynamic parameters over a period of more than 60 min. All hearts were stabilized for 30 min by perfusion with the oxygenated medium. The experimental protocols for inducing IP and Ca\(^{2+}\) paradox as well as for drug treatments are shown in Fig. 1. For the Ca\(^{2+}\) paradox group, the hearts were perfused for 18 min with oxygenated medium followed by 3 or 5 min of Ca\(^{2+}\)-free perfusion and 30 min of repletion. The durations of Ca\(^{2+}\) depletion and repletion were adapted from our previous study (11). For the preconditioning group, the hearts were subjected to three cycles of 3-min global ischemia and 3-min reperfusion, followed by 3 or 5 min of Ca\(^{2+}\)-free perfusion and 30 min of repletion. For the control group, the hearts were perfused for a comparable period with the oxygenated medium. To test whether the inhibitors of adenosine receptors,
CaMK II, or PKC attenuate the protective effect of IP against Ca\textsuperscript{2+} paradox, we used 8-(p-sulfophenyl)-theophylline (8-SPT) (Research Biochemicals International, Natick, MA), KN-93 (Sigma Chemical, St. Louis, MO), or chelerythrine chloride (Sigma Chemical), respectively. The vehicle (perfusion medium) or each inhibitor was delivered by an infusion pump into the perfusion stream directly above the aortic cannula at 1 ml/min for 10 min before starting the preconditioning protocol as well as during the three cycles of intermittent reflow. The final concentrations of 8-SPT, KN-93, and chelerythrine were 10 µM, 1 µM, and 5 µM, respectively; these concentrations were selected on the basis of previous studies (11, 12, 16) showing inhibitory constant (K\textsubscript{i}) value for 8-SPT for adenosine receptors (2.63 µM), KN-93 for CaMK II (0.37 µM), and chelerythrine for PKC (0.67 µM).

Preparation of SR vesicles. The SR preparation was isolated by the method of Osada et al. (20) with slight modifications. Briefly, the LV tissue was pulverized and homogenized twice for 20 s each at a one-half maximal setting using a Polytron homogenizer (Brinkmann, Westbury, NY). The homogenization buffer contained (in mmol/l) 10 NaHCO\textsubscript{3}, 5 Na\textsubscript{2}HPO\textsubscript{4}, 15 Tris-HCl, pH 6.8, and protease inhibitors (in µmol/l): 1 leupeptin, 1 pepstatin, and 100 phenylmethylsulfonyl fluoride. The homogenate was then centrifuged for 20 min at 9,500 rpm (Beckman, JA 20.0), and the supernatant was further centrifuged for 45 min at 19,000 rpm (Beckman, JA 20.0). The pellet obtained was suspended in a buffer containing 0.6 M KCl and 20 mM Tris-HCl, pH 6.8, and recentrifuged at the same speed and duration as indicated in the previous step. The resulting pellet obtained was suspended in a mixture of 250 mM sucrose and 10 mM histidine, pH 7.0. All steps were performed at 0–4°C, and the resulting SR preparation was used for various assays. The purity of the SR preparations was determined by measuring the activities of marker enzymes such as ouabain-sensitive Na\textsuperscript{+}–K\textsuperscript{+} ATPase, cytochrome-c oxidase, glucose-6-phosphatase, and rotenone-insensitive NADPH cytochrome-c reductase according to methods described earlier (1). The SR preparations from all groups were found to contain minimal (3–5%) but equal cross-contamination by other subcellular organelles.

Measurement of Ca\textsuperscript{2+}-uptake activity. Ca\textsuperscript{2+}-uptake activity of SR was determined by the procedure described earlier (20) with slight modifications. The standard reaction mixture (total volume 250 µl) contained (in mmol/l) 50 Tris-maleate (pH 6.8), 5 Na\textsubscript{2}HPO\textsubscript{4}, 5 ATP, 5 MgCl\textsubscript{2}, 120 KCl, 5 K-oxalate, 0.1 EGTA, 0.1 \textsuperscript{45}CaCl\textsubscript{2} (12,000 counts·min\textsuperscript{-1}·mmol\textsuperscript{-1}), and 0.025 ruthenium red. The initial free Ca\textsuperscript{2+} in this medium determined by the program of Fabiato (9) was 8.2 µmol/l. Ruthenium red was added to inhibit Ca\textsuperscript{2+}-release channel activity under these conditions. The reaction was initiated by the addition of SR membranes to the Ca\textsuperscript{2+}-uptake reaction mixture and terminated after 1 min by filtering a 200-µl aliquot of the reaction mixture. The filters were washed, dried, and counted in a beta scintillation counter.

Determination of Ca\textsuperscript{2+}-stimulated ATPase activities. The status of the Ca\textsuperscript{2+} pump in the SR membranes was determined by measuring the Ca\textsuperscript{2+}-stimulated ATPase activities according to the method used previously (10). Total (Mg\textsuperscript{2+}- and Ca\textsuperscript{2+}-) and basal (Mg\textsuperscript{2+}) ATPase activities were determined in the presence or absence of Ca\textsuperscript{2+} in a reaction medium containing (in mmol/l) 100 KCl, 20 Tris-HCl, 5 MgCl\textsubscript{2}, and 5 Na\textsubscript{2}HPO\textsubscript{4}, respectively. The reaction was started by the addition of 5 mM Tris-ATP in the presence of 0.05 mg/ml of SR protein and was terminated with 12% cold TCA. Inorganic phosphate liberated during the reaction was estimated in the protein-free filtrate by a spectrophotometric method (10). The Ca\textsuperscript{2+}-stimulated, Mg\textsuperscript{2+}-dependent ATPase (Ca\textsuperscript{2+}-pump ATPase) activity was calculated as the difference between the total and basal ATPase activities.

Measurement of ryanodine-sensitive, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. The Ca\textsuperscript{2+}-release activity of SR vesicles was measured by a procedure described previously (20). In brief, the SR fraction (62 µg protein) was suspended in a total volume of 625 µl of loading buffer containing (in mmol/l) 100 KCl, 5 MgCl\textsubscript{2}, 5 K-oxalate, 5 Na\textsubscript{2}HPO\textsubscript{4}, and 20 Tris-HCl (pH 6.8). After incubation with 10 µM \textsuperscript{45}CaCl\textsubscript{2} (20 mCi/ml) and 5 mM ATP for 45 min at room temperature, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release was carried out by adding 1 mM EGTA plus 1 mM CaCl\textsubscript{2} to the reaction mixture. The reaction was terminated at 15 s by filtering a 100-µl aliquot of the reaction mixture. The filters were washed, dried, and counted in a beta scintillation counter. The Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release was completely prevented (90–97%) by the treatment of SR with 20 µM ryanodine.

Western blot analysis. The protein contents of Ca\textsuperscript{2+}-release channel [ryanodine receptor (RyR)], Ca\textsuperscript{2+} pump [sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2)], and phospholamban (PLB) in the SR membrane were determined by Western immunoblotting techniques. For immunoassay of RyR, SERCA2a, and PLB, SR (20 µg protein/ lane) samples were subjected to SDS-PAGE in 6%, 10%, and 15% gels, respectively. The quantity of protein loaded was within the linear range (14). The protein bands from these gels were then transferred electrophoretically to nitrocellulose membrane (for RyR) or polyvinylidene difluoride membrane (for SERCA2a and PLB). The membranes were used for incubation with anti-RyR receptor (1:1400), anti-SERCA2a (1:1400), or anti-PLB (1:3500) antibodies. A peroxidase-linked, anti-mouse IgG was used for SERCA2a or PLB as the secondary antibody (1:5000), whereas anti-mouse IgG was used for Ca\textsuperscript{2+}-release channel as the secondary antibody (1:2500) and then incubated with streptavidin-conjugated horseradish peroxidase solution (1:5000). Protein bands reactive with antibodies were visualized using the ECL detection system from Amersham (Buckinghamshire, UK). The intensity of each band was scanned by Imaging Densitometer with the aid of Molecular Analyst Software version 1.3 (Bio-Rad, Hercules, CA). After the detection of SR proteins by Western blot analysis, the membranes were stained with Coomassie Blue, and each lane was scanned to normalize the well-to-well variability in protein loading. The total intensity of the bands in each lane was calculated, but no significant changes in total intensity were observed among different lanes.

Statistical analysis. Data are expressed as means ± SE.

The statistical difference among different groups was determined by factorial ANOVA (Statview 4.02, Abacus Concepts). All groups were analyzed with post hoc testing by the Scheffé’s procedure. P < 0.05 was considered to be significant.

RESULTS

Left ventricular function and Ca\textsuperscript{2+} uptake. Changes in the left ventricular function and SR Ca\textsuperscript{2+} uptake due to Ca\textsuperscript{2+} paradox with or without IP are shown in Fig. 2. During predepletion phase, IP did not affect the LVDP, LVEDP, and SR Ca\textsuperscript{2+} uptake; there was no change in LVEDP in hearts that did not undergo IP; Ca\textsuperscript{2+}-free perfusion for 5 min (depletion phase) produced a marked depression of LVDP and SR Ca\textsuperscript{2+}-uptake activity in hearts with or without IP; however, the extent of changes in both conditions was similar. A marked increase in LVEDP and a depression in SR Ca\textsuperscript{2+}-uptake activity were seen on perfusing the Ca\textsuperscript{2+}-depleted...
hearts with normal medium for 10 or 30 min without any recovery in LVDP. At the 10-min repletion phase, IP significantly improved the recovery of LVDP and decreased the LVEDP of the Ca\textsuperscript{2+} paradox hearts; however, there was no significant difference in SR Ca\textsuperscript{2+}-uptake activities between groups with or without IP. At the 30-min repletion phase, IP significantly improved LVDP as well as SR Ca\textsuperscript{2+} uptake and markedly decreased the LVEDP of the Ca\textsuperscript{2+} paradox hearts (Fig. 2).

SR protein content in IP and Ca\textsuperscript{2+} paradox. The SR content of RyR, SERCA2a, and PLB in control and Ca\textsuperscript{2+} paradox hearts with and without IP were determined by using the Western blot analysis and values are expressed as percentage of control (Fig. 3, B and C). The density of each band from the control heart was considered to be 100%, and the other groups CP, IP, and IP plus CP were compared with this control value. RyR, SERCA2a, and PLB protein levels did not change due to IP when compared with control value. On the other hand, RyR, SERCA2a, and PLB protein levels in the Ca\textsuperscript{2+} paradox hearts were decreased by 94.6%, 96.8%, and 44.7% of the control value, respectively. IP significantly prevented the depression in protein content of RyR and SERCA2a due to Ca\textsuperscript{2+} paradox. Although PLB content in the Ca\textsuperscript{2+} paradox hearts was 17.3% higher on treatment with IP, this change was not significant.

Modification of changes in cardiac function. The effects of IP on cardiac performance in Ca\textsuperscript{2+} paradox induced by 5-min Ca\textsuperscript{2+} depletion and 30-min Ca\textsuperscript{2+} repletion with or without some inhibitor treatments are shown in Table 1. Hearts subjected to Ca\textsuperscript{2+} paradox under control conditions showed significant depressions in LVDP, +dP/dt, and −dP/dt and a marked

![Fig. 2](image_url)

**Fig. 2.** Left ventricular (LV) function and sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-uptake activities in CP hearts with or without IP. Top: percentage recovery of LV developed pressure (LVDP). Values for LVDP are expressed as % of basal values (82.6 ± 3.42 mmHg). Middle: LV end-diastolic pressure (LVEDP). Bottom: SR Ca\textsuperscript{2+}-uptake activities. Hearts were examined before Ca\textsuperscript{2+} depletion, at 5 min after Ca\textsuperscript{2+} depletion, at 10 min after Ca\textsuperscript{2+} repletion after 5 min of Ca\textsuperscript{2+} depletion, and at 30 min after Ca\textsuperscript{2+} repletion after 5 min of Ca\textsuperscript{2+} depletion. Each value is a mean ± SE of 6 hearts in each group. *P < 0.05 vs. CP without IP.

![Fig. 3](image_url)

**Fig. 3.** Effect of ischemic preconditioning and CP on SR protein content in rat heart. Western blot autoradiograms and analysis of ryanodine receptor (A), sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA2a) (B), and phospholamban (PLB) (C) of SR protein content in control (C), IP, CP, and IP + CP. Each value is a mean ± SE of 4 separate experiments. *P < 0.05 vs. C and † vs. CP.
in the control and Ca\textsuperscript{2+} uptake activities were determined in SR preparations and Ca\textsuperscript{2+} uptake was measured at 30 min of Ca\textsuperscript{2+} repletion as described in Fig. 1. Each value is a mean ± SE of 5 different preparations in each group.

*P < 0.05 vs. control and †P < 0.05 vs. CP.

Fig. 4. SR Ca\textsuperscript{2+}-uptake activities in control and CP hearts with and without IP. SR Ca\textsuperscript{2+} uptake was measured at 30 min of Ca\textsuperscript{2+} repletion as described in Fig. 1. Each value is a mean ± SE of 5 different preparations in each group.

*P < 0.05 vs. control and †P < 0.05 vs. CP + IP.

Table 1. Left ventricular performance in the control and Ca\textsuperscript{2+} paradox hearts with or without ischemic preconditioning

<table>
<thead>
<tr>
<th>Group</th>
<th>Percent Recovery of Cardiac Function</th>
<th>LVDP, +dp/dt</th>
<th>LVDP, -dp/dt</th>
<th>LVEDP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>CP</td>
<td>0.7 ± 0.2*</td>
<td>0.9 ± 0.2*</td>
<td>1.3 ± 0.2*</td>
<td>76.5 ± 8.5*</td>
</tr>
<tr>
<td>8-SPT + CP</td>
<td>0.9 ± 0.1*</td>
<td>0.8 ± 0.1*</td>
<td>1.0 ± 0.2*</td>
<td>70.0 ± 7.0*</td>
</tr>
<tr>
<td>KN-93 + CP</td>
<td>0.7 ± 0.1*</td>
<td>0.9 ± 0.4*</td>
<td>1.1 ± 0.4*</td>
<td>67.8 ± 3.4*</td>
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<td>CHT + CP</td>
<td>0.8 ± 0.1*</td>
<td>0.7 ± 0.6*</td>
<td>0.9 ± 0.6*</td>
<td>65.9 ± 14.0*</td>
</tr>
<tr>
<td>IP + CP</td>
<td>27.4 ± 7.4†</td>
<td>38.3 ± 4.0†</td>
<td>52.1 ± 7.7†</td>
<td>41.9 ± 4.7†</td>
</tr>
<tr>
<td>8-SPT + IP + CP</td>
<td>0.6 ± 0.1*</td>
<td>0.8 ± 0.2*</td>
<td>1.2 ± 0.2*</td>
<td>65.6 ± 9.0*</td>
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<tr>
<td>KN-93 + IP + CP</td>
<td>0.7 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
<td>1.1 ± 0.1*</td>
<td>70.1 ± 6.4*</td>
</tr>
<tr>
<td>CHT + IP + CP</td>
<td>0.8 ± 0.2*</td>
<td>0.9 ± 0.7*</td>
<td>1.1 ± 0.1*</td>
<td>66.8 ± 12.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ca\textsuperscript{2+} paradox (CP) was induced by Ca\textsuperscript{2+}-free perfusion for 5 min and Ca\textsuperscript{2+} repletion for 3 min. Values for left ventricular developed pressure (LVDP), rate of pressure development (+dp/dt) and rate of pressure decay (-dp/dt) for control hearts were 82 ± 3.1 mmHg, 1,804 ± 76 mmHg/s, and 1,670 ± 81 mmHg/s, respectively; these values are expressed as % of control. IP, ischemic preconditioning; 8-SPT, adenosine antagonist 8-(p-sulfophenyl)-theophylline (10 µM); KN-93, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMK II) inhibitor (1 µM); CHT, PKC inhibitor chelerythrine (5 µM). *P < 0.05 vs. control and †P < 0.05 vs. CP.

Increase in LVEDP in comparison with the control values. 8-SPT, KN-93, or chelerythrine treatment did not affect the LV function of the Ca\textsuperscript{2+} paradox hearts significantly. On the other hand, IP improved the recovery of LVDP, +dp/dt, as well as -dp/dt significantly and markedly reduced the increase in LVEDP in the Ca\textsuperscript{2+} paradox hearts. Treatments with 8-SPT, KN-93, or chelerythrine abolished these beneficial effects of IP.

Modification of changes in SR Ca\textsuperscript{2+}-uptake and Ca\textsuperscript{2+} pump ATPase activities. Oxalate-supported SR Ca\textsuperscript{2+}-uptake activities were determined in SR preparations in the control and Ca\textsuperscript{2+} paradox hearts (5-min Ca\textsuperscript{2+} depletion and 30-min Ca\textsuperscript{2+} repletion) with or without some inhibitor treatments and the results are shown in Fig. 4. The Ca\textsuperscript{2+}-uptake activity was significantly reduced in Ca\textsuperscript{2+} paradox hearts in comparison with control hearts. 8-SPT prevented the decrease in SR Ca\textsuperscript{2+} uptake due to Ca\textsuperscript{2+} paradox significantly; this improvement was attenuated with 8-SPT, KN-93, or chelerythrine treatment. To show whether the observed changes in SR Ca\textsuperscript{2+} uptake were due to alterations in the SR Ca\textsuperscript{2+}-pump, Ca\textsuperscript{2+}-stimulated ATPase activities were determined. The results in Fig. 5 reveal that the SR Ca\textsuperscript{2+}-pump ATPase activity in the Ca\textsuperscript{2+} paradox hearts was significantly reduced in comparison to control hearts. IP improved Ca\textsuperscript{2+}-pump ATPase activity of the Ca\textsuperscript{2+} paradox hearts; this beneficial effect of IP was lost on treatment with 8-SPT, KN-93, or chelerythrine.

Modification of changes in SR Ca\textsuperscript{2+}-release activity. The Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+}-release activity was determined in SR preparations from the control and Ca\textsuperscript{2+} paradox hearts (5-min Ca\textsuperscript{2+} depletion and 30-min Ca\textsuperscript{2+} repletion) with or without some inhibitor treatments and results are shown in Fig. 6. Hearts subjected to Ca\textsuperscript{2+} paradox exhibited a significant depression of the SR Ca\textsuperscript{2+}-release activity in comparison with control; IP attenuated the reduction in SR Ca\textsuperscript{2+} release in Ca\textsuperscript{2+} paradox hearts. This improvement due to IP was lost when the hearts were treated with 8-SPT, KN-93, or chelerythrine.

Modification of changes due to mild Ca\textsuperscript{2+} paradox. Because perfusion for 5 min with Ca\textsuperscript{2+}-free medium and reperfusion for 30 min with Ca\textsuperscript{2+}-containing medium produced severe changes in the heart, some experiments were carried out to examine the effects of IP on changes in SR function due to a mild Ca\textsuperscript{2+} paradox induced by 3-min Ca\textsuperscript{2+} depletion and 30-min Ca\textsuperscript{2+} repletion (Table 2). The mild Ca\textsuperscript{2+} paradox was associated with a significant decrease in LVDP, a marked increase in LVEDP, as well as significant decreases in SR Ca\textsuperscript{2+}-uptake, Ca\textsuperscript{2+}-pump ATPase, and
Ca\textsuperscript{2+}-release activities in comparison to the control values. IP prevented the reduction in LVDP and the increase in LVEDP significantly in addition to preventing changes in SR Ca\textsuperscript{2+} uptake, Ca\textsuperscript{2+}-pump ATPase, and Ca\textsuperscript{2+}-release activities in the Ca\textsuperscript{2+} paradox hearts.

**DISCUSSION**

In this study we have provided evidence for a significant protection by IP against SR dysfunction due to Ca\textsuperscript{2+} paradox induced by perfusing the heart for 5 min with a Ca\textsuperscript{2+}-free medium followed by reperfusion with Ca\textsuperscript{2+}-containing medium for 30 min. The beneficial effects of IP were also evident under a mild Ca\textsuperscript{2+} paradox condition where the hearts were subjected to 3-min Ca\textsuperscript{2+}-free perfusion and 30-min reperfusion with a normal medium. Although several mechanisms have been put forward to explain the beneficial effects of IP against the ischemia-reperfusion-induced changes in the heart, attenuation of cytosolic Ca\textsuperscript{2+} overload appears to be a major factor (24). In fact, the intracellular Ca\textsuperscript{2+} overload is considered to be the hallmark of Ca\textsuperscript{2+} paradox injury (3) and is believed to be due to an alteration in the ability of the myocardium to regulate the level of intracellular Ca\textsuperscript{2+} (17). Such an alteration in the Ca\textsuperscript{2+} paradox heart has been shown to be in part due to a defect in the SR function (2), which is considered to be intimately involved in intracellular Ca\textsuperscript{2+} homeostasis and cardiac excitation-contraction coupling (8). Changes in the SR Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+}-release activities have also been shown to occur during other pathological conditions, resulting in the occurrence of intracellular Ca\textsuperscript{2+} overload and subsequent cardiac dysfunction (1, 10, 22). Thus the observed alterations in SR Ca\textsuperscript{2+}-pump and Ca\textsuperscript{2+}-release activi-
Table 2. Left ventricular performance and SR function in control and mild Ca2+ paradox with or without ischemic preconditioning

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>3-Min CP</th>
<th>IP + 3-Min CP</th>
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<tbody>
<tr>
<td>Recovery of LVDP, %</td>
<td>100</td>
<td>36.0 ± 8.0*</td>
<td>77.7 ± 3.1†</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5.3 ± 0.5</td>
<td>45.1 ± 2.4*</td>
<td>30.4 ± 4.4†</td>
</tr>
<tr>
<td>SR Ca2+ uptake, nM · mg−1 · min−1</td>
<td>49.4 ± 6.3</td>
<td>29.4 ± 3.9*</td>
<td>41.6 ± 2.0†</td>
</tr>
<tr>
<td>SR Ca2+-pump ATPase, nmol</td>
<td>131.6 ± 13.8</td>
<td>87.1 ± 5.0*</td>
<td>148.7 ± 13.0†</td>
</tr>
<tr>
<td>P1·mg−1·min−1</td>
<td>8.15 ± 0.95</td>
<td>1.15 ± 0.05*</td>
<td>4.78 ± 0.19*†</td>
</tr>
<tr>
<td>SR Ca2+ release, nM · mg−1 · 15 s−1</td>
<td>6.3 ± 2.4*†</td>
<td>3.9 ± 4.1*†</td>
<td>6.3 ± 2.4*†</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4–6 separate experiments in each group. Mild CP was induced by Ca2+-free perfusion for 3 min and Ca2+ repletion for 30 min. Values for LVDP are expressed as % of control. LVDP basal values for control hearts were 84 ± 3.7 mmHg. *P < 0.05 vs. control and †P < 0.05 vs. 3 min CP.

ties as well as SR Ca2+-pump and Ca2+-release channel proteins in hearts subjected to Ca2+ paradox are consistent with impaired cardiac function in this experimental condition. In addition, we have demonstrated that the recovery of cardiac functions in Ca2+ paradox hearts by IP was associated with the ability of IP to partially prevent changes in SR Ca2+-uptake, Ca2+-pump ATPase, and Ca2+-release activities as well as SR Ca2+-pump and Ca2+-release channel proteins. These observations allow us to speculate that IP rendered cardioprotection by reducing the intracellular Ca2+ overload via an improved Ca2+-handling ability of the SR in Ca2+ paradox hearts.

Since Murry et al. (19) observed the phenomenon of IP, many factors have been proposed to explain the beneficial effects of IP (29). In particular, the formation of adenosine and subsequent activation of adenosine receptors in the heart have been suggested to be an important mediator of IP (14). However, the role of adenosine in mediating IP in rat hearts has been questioned because pretreatment with an adenosine receptor blocker failed to prevent the ischemia-reperfusion damage (13). On the other hand, Ashraf et al. (4) reported that adenosine may be involved in Ca2+ preconditioning for conferring significant protection against the Ca2+ paradox injury in the rat heart (4). Moreover, adenosine acting via A1 receptors has also been shown to render significant cardioprotection against rat hearts subjected to Ca2+ paradox (25). The results in the present study have shown that the beneficial effects of IP on SR function were abolished by pretreatment of the heart with 8-SPT, an adenosine receptor antagonist. Thus it is likely that the formation of adenosine may be one of the mechanisms by which IP protects the heart against Ca2+ paradox injury. Although on the basis of the effects of 8-SPT, which is known to act on the adenosine A1 receptors (14, 25), it appears that A1 receptors may be involved in the IP-induced cardioprotection against Ca2+ paradox, the involvement of other types of adenosine receptors cannot be ruled out at this time.

In view of the fact that the activation of PKC plays a central role in IP (15, 29), Miyawaki et al. (16) demonstrated that a transient increase in intracellular Ca2+ concentration during IP represents the trigger for the activation of PKC and subsequent cardioprotection against the ischemic-reperfusion injury. Moreover, Movsesian et al. (18) reported that PKC may regulate Ca2+ uptake by cardiac SR via phosphorylation of PLB. In this study, we show that the treatment of hearts with a PKC inhibitor, chelerythrine, attenuated the beneficial effects of IP on SR function in Ca2+ paradox hearts. Our results regarding IP are consistent with a recent study demonstrating a role for PKC in the protection against Ca2+-paradox injury by Ca2+ preconditoning in the rat heart (28). Because Osada et al. (20) reported that IP prevented alterations in the SR CaMK II-mediated phosphorylation of SR Ca2+-cycling proteins as well as SR and cardiac functions in the ischemia-reperfusion injured hearts, the SR CaMK II can also be seen to represent an important mechanism underlying the beneficial effects of IP against the ischemic-reperfusion injury in the isolated rat heart. In the present study, we also show that the treatment of hearts with a CaMK II inhibitor, KN-93, attenuated the beneficial effects of IP on the SR function in the Ca2+ paradox hearts. Thus it appears that the activation of both PKC and CaMK II in addition to adenosine receptors may represent the mechanisms by which IP protects the hearts against the Ca2+ paradox injury. Extensive research is required to establish whether adenosine receptors, PKC, and CaMK II represent three parallel pathways or act sequentially leading to protection of the Ca2+ paradox heart by IP.

Although the observed reduction in SR Ca2+ uptake and Ca2+-release activities due to Ca2+ paradox and the protection of SR function due to IP can be explained on the basis of corresponding changes in SERCA2a and RyR protein contents of the SR membrane, the depressed levels of PLB protein in the Ca2+ paradox heart can be seen to relieve the inhibitory effect of PLB on the SR Ca2+ pump and thus increase the Ca2+-uptake activity. However, this effect due to the reduction of PLB content may not be sufficient to compensate for the depression in the SR Ca2+-uptake activity due to the loss of SERCA2a, because the reduction in the SERCA2a level was considerably greater than that in the PLB content in the Ca2+ paradox heart. The loss of SR proteins in hearts subjected to Ca2+ paradox may be due to the activation of proteases because intracellular Ca2+ overload under pathological conditions such as ischemia-reperfusion has been reported to result in SR protein degradation due to protease activation (30). It should be pointed out that the loss of proteins may not be a generalized phenomenon in the paradoxic hearts because the loss of PLB in the SR membrane due to Ca2+ paradox with or without IP was considerably smaller than that of RyR and SERCA2a proteins. Furthermore, other proteins such as inhibitory guanine binding proteins and β2-adrenergic receptor proteins were unaltered under similar conditions of Ca2+ paradox (unpublished observations). In view of the role of KATP channels in the protective effect of IP against the ischemia-reperfusion injury (23), it is possible that KATP
channels may also mediate the beneficial effects of IP against Ca\textsuperscript{2+} paradox with respect to changes in cardiac contractile activity. However, such a mechanism cannot be invoked to explain the beneficial effects of IP against the Ca\textsuperscript{2+} paradox-induced or ischemic-reperfusion-induced (20) changes in SR function because the relationship between K\textsubscript{ATP} channels and the SR Ca\textsuperscript{2+} transport system is not understood at the present time.

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