Status of Ca\(^{2+}\)/calmodulin protein kinase phosphorylation of cardiac SR proteins in ischemia-reperfusion

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Netticadan, Thomas, Rana Temsah, Mitsuru Osada, and Naranjan S. Dhalla. Status of Ca\(^{2+}\)/calmodulin protein kinase phosphorylation of cardiac SR proteins in ischemia-reperfusion. Am. J. Physiol. 277 (Cell Physiol. 46): C384–C391, 1999.—Although the sarcoplasmic reticulum (SR) is known to regulate the intracellular concentration of Ca\(^{2+}\) and the SR function has been shown to become abnormal during ischemia-reperfusion in the heart, the mechanisms for this defect are not fully understood. Because phosphorylation of SR proteins plays a crucial role in the regulation of SR function, we investigated the status of endogenous Ca\(^{2+}\)-calmodulin-dependent protein kinase (CaMK) and exogenous cAMP-dependent protein kinase (PKA) phosphorylation of the SR proteins in control, ischemic (I), and ischemia-reperfused (I/R) hearts treated or not treated with superoxide dismutase (SOD) plus catalase (CAT). SR and cytosolic fractions were isolated from control, I, and I/R hearts treated or not treated with SOD plus CAT, and the SR protein phosphorylation by CaMK and PKA, the CaMK- and PKA-stimulated Ca\(^{2+}\) uptake, and the CaMK, PKA, and phosphatase activities were studied. The SR CaMK and CaMK-stimulated Ca\(^{2+}\) uptake activities, as well as CaMK phosphorylation of Ca\(^{2+}\) pump ATPase (SERCA2a) and phospholamban (PLB), were significantly decreased in both I and I/R hearts. The PKA phosphorylation of PLB and PKA-stimulated Ca\(^{2+}\) uptake were reduced significantly in the I/R hearts only. Cytosolic CaMK and PKA activities were unaltered, whereas SR phosphatase activity in the I and I/R hearts was depressed. SOD plus CAT treatment prevented the observed alterations in SR CaMK and phosphatase activities, CaMK and PKA phosphorylations, and CaMK- and PKA-stimulated Ca\(^{2+}\) uptake. These results indicate that depressed CaMK phosphorylation and CaMK-stimulated Ca\(^{2+}\) uptake in I/R hearts may be due to a depression in the SR CaMK activity. Furthermore, prevention of the I/R-induced alterations in SR protein phosphorylation by SOD plus CAT treatment is consistent with the role of oxidative stress during ischemia-reperfusion injury in the heart.

calmodulin/calmodulin-dependent protein kinase; cAMP-dependent protein kinase; cardiac sarcoplasmic reticulum; oxidative stress

ALTHOUGH REPERFUSION IS essential for the survival of the ischemic myocardium, reperfusion of the ischemic heart, if not carried out within a certain period, has also been shown to induce further damage to the cardiac muscle (13). The deleterious effects of ischemia-reperfusion injury are evident under different clinical conditions including myocardial infarction and ischemic cardiomyopathy as well as during surgical interventions such as coronary angioplasty, aortocoronary bypass surgery, and cardiac transplantation (13). The occurrence of intracellular Ca\(^{2+}\) overload due to abnormal Ca\(^{2+}\) homeostasis in cardiomyocytes has been suggested as one of the mechanisms underlying ischemia-reperfusion injury (16). The generation of oxyradicals due to a sudden supply of oxygen to the ischemic myocardium has also been implicated as a mechanism responsible for ischemia-reperfusion injury (6). In fact, hearts reperfused after a prolonged period of global or regional ischemia, as well as those exposed directly to free radical-generating systems, have revealed similarities with respect to structural and functional alterations (5, 6, 9).

It is now well established that the sarcoplasmic reticulum (SR) plays a central role in the regulation of intracellular Ca\(^{2+}\) and cardiac contraction-relaxation processes. Although SR dysfunction has been shown to occur in ischemia-reperfused (I/R) hearts (7, 9, 23), the mechanisms underlying these alterations are not fully understood. In this regard, we have recently reported that ischemic preconditioning may render protection against the ischemia-reperfusion-induced damage to the myocardium by improving SR function (23). In view of the critical role of phosphorylations by endogenous Ca\(^{2+}\)-calmodulin-dependent protein kinase (CaMK) and exogenous cAMP-dependent protein kinase (PKA) in regulating SR function (3, 8, 12, 20), we have now examined changes in CaMK- and PKA-mediated phosphorylation as well as CaMK- and PKA-stimulated Ca\(^{2+}\) uptake activities in SR preparations from control, ischemic (I), and I/R hearts. Because treatment with superoxide dismutase (SOD) plus catalase (CAT) has been shown to significantly recover the depressed myocardial function in I/R hearts (24, 29), we also examined the effects of a SOD plus CAT treatment on the ischemia-reperfusion-induced changes in CaMK- and PKA-mediated phosphorylation as well as on CaMK- and PKA-stimulated SR Ca\(^{2+}\) uptake activities. Our results suggest that the regulation of the SR Ca\(^{2+}\) pump by protein phosphorylation is impaired in the I and I/R hearts. The observation that SOD plus CAT treatment prevented changes in SR protein phosphorylation in the I/R hearts indicates that oxyradicals may be involved in the ischemia-reperfusion-induced alterations in SR function.

METHODS

Perfusion of isolated heart. Male Sprague-Dawley rats weighing 320–350 g were used for experiments. The investi-
Measurement of phosphorylation by endogenous CaMK and exogenous PKA. For phosphorylation experiments, the SR was isolated in the presence of phosphatase inhibitors to prevent any dephosphorylation from occurring during the isolation procedure. The homogenization buffer contained 10 mM microcystin LR and 1 mM sodium pyrophosphate. SR protein phosphorylation by CaMK was determined according to the procedure described by Netticadan et al. (22). The assay medium (total volume 50 µl) for phosphorylation by endogenous CaMK contained (in mM) 50 HEPES (pH 7.4), 10 MgCl₂, 0.1 CaCl₂, 0.1 EGTA, 0.002 calmodulin, 0.8 γ-[³²P]ATP (sp act 200–300 cpm/pmol), and SR (30 µg protein). Phosphatase inhibitors microcystin LR (10 mM) and sodium pyrophosphate (1 mM) were also added to the reaction mixture to inhibit any endogenous phosphorylase activity. The initial concentration of free Ca²⁺ as determined by the computer program of Fabiato (10) was 3.7 µM. The Ca²⁺/calmodulin dependence of phosphorylation was monitored in parallel assays in which Ca²⁺ (1 mM EGTA present) and calmodulin were lacking in the assay medium. The assay medium (50 µl) for phosphorylation by PKA contained (in mM) 50 HEPES (pH 7.4), 10 MgCl₂, and 0.8 γ-[³²P]ATP (sp act 200–300 cpm/pmol), as well as SR (30 µg protein) and PKA (catalytic subunit from the bovine heart; 5.6 µg). The PKA dependence of phosphorylation was monitored in parallel assays without the PKA catalytic subunit. The phosphorylation reaction was initiated by the addition of γ-[³²P]ATP after preincubation of the assay medium for 3 min at 37°C. Reactions were terminated after 2 min by adding 15 µl of SDS sample buffer, and the samples were subjected to SDS-PAGE in a 4–18% gradient slab gels. The gels were stained with Coomassie brilliant blue, dried, and autoradiographed. The intensity of each phosphorylated band was scanned by an imaging densitometer with the aid of Molecular Analyst software, version 1.3 (Bio-Rad, Hercules, CA).

Measurement of CaMK and PKA activities. The CaMK and PKA activities of the cytosolic and SR preparations were measured by using Upstate Biotechnology (Lake Placid, NY) assay kits. The assay kit for CaMK activity is based on the phosphorylation of a specific substrate peptide (KKALR-QETVDAL) by the transface of the γ-phosphate of γ-[³²P]ATP by CaMK II. Because the exogenous substrate was also phosphorylated by the SR and cytosolic CaMK, the activity was calculated as the difference between the values obtained in the presence and absence of the exogenous substrate. The assay kit for PKA activity measurement is based on the phosphorylation of a specific substrate (kemptide) by using the transface of the γ-phosphate of γ-[³²P]ATP by PKA. The phosphorylated substrates in both assays were then separated from the residual γ-[³²P]ATP with P81 phosphocellulose paper and quantified by using a scintillation counter.

Measurement of Ca²⁺ uptake activity. The Ca²⁺ uptake activities of phosphorylated and unphosphorylated SR membranes were determined by the procedure of Hawkins et al. (12) with slight modifications. The standard reaction mixture
superoxide dismutase; CAT, catalase. *1,729 vs. I/R group.

LVDP, preischemic values. The control (C) preischemic values for LVDP, pressure decline (−dP/dt), and rate of ventricular pressure development (+dP/dt) are expressed as percentages of respective preischemic values. The control (C) preischemic values for LVDP, pressure decline (−dP/dt), and rate of ventricular pressure development (+dP/dt) were 83 ± 4.7 mmHg, 9.6 ± 0.5 mmHg/s, 1.729 ± 0.05 mmHg/s, and 1.569 ± 0.05 mmHg/s, respectively. SOD, superoxide dismutase; CAT, catalase. *P < 0.05 vs. control; †P < 0.05 vs. I/R group.

A typical SDS-PAGE protein profile of sarcoplasmic reticulum (SR) preparations in control (C), ischemic (I), ischemia-reperfused (I/R), and superoxide dismutase (SOD) + catalase (CAT) treatment groups. Ca2+/CM, Ca2+/calmodulin. B: endogenous Ca2+/CM-dependent protein kinase (CaMK)-mediated phosphorylation of ryano-dine receptor (RyR). Ca2+ pump ATPase (SERCA2a), high-molecular weight phospholamban (PLB(H)), and low-molecular weight PLB (PLB(L)) by endogenous CaMK. C: analysis of CaMK phosphorylation of RyR, SERCA2a, and PLB. PLB phosphorylation was sum of PLB(H) and PLB(L) phosphorylations. Cardiac SR was phosphorylated in presence and absence of Ca2+/CM and subjected to 4–18% SDS-PAGE as described in Methods. Results are means ± SE for 5 hearts in each group. *P < 0.05 vs. control; †P < 0.05 vs. I/R group.

Table 1. Cardiac performance of I and I/R groups with and without SOD plus CAT treatment

<table>
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<tr>
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<th>C</th>
<th>I</th>
<th>I/R</th>
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<tr>
<td>LVDP</td>
<td>100</td>
<td>2.08 ± 0.39*</td>
<td>27.4 ± 4.27*</td>
<td>89.4 ± 5.19†</td>
</tr>
<tr>
<td>LVEDP</td>
<td>100</td>
<td>411 ± 33.34*</td>
<td>1143 ± 7.07*</td>
<td>384 ± 41.72†</td>
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<tr>
<td>+dP/dt</td>
<td>100</td>
<td>4.18 ± 0.99*</td>
<td>15.9 ± 3.50*</td>
<td>86.3 ± 3.01†</td>
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<tr>
<td>−dP/dt</td>
<td>100</td>
<td>4.15 ± 0.19*</td>
<td>24.0 ± 4.52*</td>
<td>81.6 ± 5.38†</td>
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Results are means ± SE for 5 hearts in each group. Ischemia (I) was induced in isolated perfused rat hearts by occluding coronary flow for 30 min, whereas reperfusion of 30-min ischemic heart (I/R) was carried out for 60 min. Values for left ventricle developed pressure (LVDP), left ventricle end diastolic pressure (LVEDP), rate of ventricular pressure development (+dP/dt), and rate of ventricular pressure decline (−dP/dt) were statistically evaluated by ANOVA test with post hoc Scheffe test using Schaffe’s procedure. P < 0.05 was considered significant.
the threshold for statistical significance between the control and experimental groups.

RESULTS

Cardiac performance. Cardiac function in the isolated heart was monitored by measuring LVDP, LVEDP, +dP/dt, and −dP/dt. Hearts subjected to global ischemia for 30 min failed to generate LVDP, +dP/dt, and −dP/dt but showed a marked increase in LVEDP (Table 1). Reperfusion of the I hearts for 60 min resulted in the recovery of the contractile function (LVDP, +dP/dt, and −dP/dt) to 16–25% of the respective preischemic values; however, LVEDP was further increased markedly (Table 1). The recovery of contractile activity in I/R hearts was markedly improved by SOD plus CAT treatment; this was reflected by the 80–85% recovery of LVDP, +dP/dt, and −dP/dt compared with the control heart preparations, as well as a marked reduction of LVEDP compared with the I/R hearts (Table 1). It was interesting to observe that nearly complete restoration of changes in LVDP, +dP/dt, and −dP/dt due to I/R occurred after treatment with SOD plus CAT, whereas the reduction in LVEDP was not as pronounced; in fact, LVEDP in these treated hearts was not appreciably different from that observed in the I hearts (Table 1). Although LVEDP is considered to be a major determinant of LVDP, the observations in this study suggest differences in the underlying mechanisms for these changes in contractile parameters due to ischemia-reperfusion injury and SOD plus CAT protection.

Phosphorylation by CaMK. Endogenous CaMK phosphorylation of SR proteins in control, I, and I/R hearts, in addition to that in I/R hearts treated with SOD plus CAT, was studied. Figure 1A shows the typical SDS-PAGE protein profile of SR preparations in control, I, I/R, and SOD plus CAT treatment groups. From Fig. 1A it can be seen that a 97-kDa protein band, unlike most of the other protein bands, was markedly diminished in I and I/R hearts. Although the identity of this band remains to be established, the reduction in its intensity was prevented by treatment of the I/R hearts with SOD plus CAT. Figure 1B is an autoradiogram depicting SR protein phosphorylation; the analysis of the phosphorylation of the ryanodine receptor (RyR), the Ca2+ pump ATPase (SERCA2a), and phospholamban (PLB) in the different groups is given in Fig. 1C. Identification of RyR, SERCA2a, and PLB as the phosphorylated substrates was carried out as described previously (12, 22, 23). CaMK phosphorylation of all three SR proteins was significantly decreased in the I and I/R hearts in comparison to controls. Treatment of the I/R hearts with SOD plus CAT significantly recovered the phosphorylation of all three SR proteins when compared with the phosphorylation of I/R heart proteins. Because CaMK phosphorylation has been shown to regulate the activity of cardiac SR Ca2+ uptake (12), we examined the effect of CaMK phosphorylation on Ca2+ uptake. For the purpose of comparison, Ca2+ uptake in the unphosphorylated SR preparations in the presence of 10 µM W-7 and 1 mM EGTA to inhibit endogenous calmodulin and chelate endogenous Ca2+, respectively, was also studied. Figure 2A shows Ca2+ uptake activities of SR membranes phosphorylated in the presence and absence of CaMK activators Ca2+ and calmodulin. CaMK-stimulated Ca2+ uptake was depressed significantly in the I and I/R hearts compared with that in controls. SOD plus CAT treatment of the I/R hearts significantly improved the CaMK-stimulated Ca2+ uptake compared with that for I/R hearts; the levels of stimulation in control, I, I/R, and SOD plus CAT treatment hearts were 303, 142, 212, and 338%, respectively. The low Ca2+ uptake values for the unphosphorylated (without Ca2+ or calmodulin) SR membranes from control, I, I/R, and SOD plus CAT treatment hearts indicate that the calmodulin present in all groups was fully inhibited by the presence of W-7 and that endogenous Ca2+ was chelated by the presence of EGTA in the assay medium.

In view of the observed decrease in both endogenous CaMK phosphorylation and CaMK-stimulated Ca2+ uptake in the I and I/R hearts, the endogenous SR CaMK activity was measured. The cytosolic CaMK
activity was estimated to account for any changes in the endogenous CaMK activity. Figure 2B shows the SR and cytosolic CaMK activities for the different groups. Although the endogenous CaMK activity in the SR was significantly depressed in the I and I/R groups compared with controls, the cytosolic CaMK activity was unaffected. Treatment of I/R hearts with SOD plus CAT significantly prevented changes in endogenous SR CaMK activity compared with results for the I/R group.

Phosphorylation by PKA. To test if the observed changes in SR protein phosphorylation were limited to the CaMK system, the phosphorylation of SR proteins by PKA in control, I, I/R, and SOD plus CAT treatment I/R hearts was also studied. Because appreciable PKA activity was not detectable in the SR, exogenous PKA was used to examine the PKA phosphorylation of SR proteins. Figure 3A shows the typical SDS-PAGE protein profile of SR preparations in control, I, I/R, and SOD plus CAT treatment groups. Figure 3B is an autoradiogram depicting SR protein phosphorylation, and Fig. 3C shows the analysis of the phosphorylation of PLB for the different groups. The identification of PLB as the phosphorylated substrate was established as previously shown (23). PKA phosphorylation of PLB was significantly decreased only in the I/R hearts compared with controls. Treatment of the I/R hearts with SOD plus CAT significantly prevented changes in the phosphorylation of PLB when compared with results for the I/R group.

DISCUSSION

Our results show a depression of cardiac function and decreased SR Ca^{2+} uptake in hearts subjected to ischemia as well as ischemia-reperfusion. Impaired cardiac performance and depressed SR Ca^{2+} uptake have been reported to occur in I/R hearts (9, 23). In this study we have shown that the CaMK-mediated phosphorylation of SR proteins is depressed in both the I and I/R groups of rat hearts, whereas the PKA-mediated phosphoryla-

![Image](https://example.com/image.png)

**Fig. 3.** A: SDS-PAGE protein profile of SR preparations in C, I, I/R, and SOD + CAT treatment groups. B: exogenous cAMP-dependent protein kinase (PKA)-mediated phosphorylation of PLB(H) and PLB(L). C: analysis of PKA phosphorylation of PLB. PLB phosphorylation was sum of PLB(H) and PLB(L) phosphorylations. Cardiac SR was phosphorylated in presence and absence of exogenous PKA and subjected to 4-18% SDS-PAGE as described in METHODS. Results are means ± SE for 5 hearts in each group. *P < 0.05 vs. control; #P < 0.05 vs. I/R group.
It is pointed out that CaMK is endogenous to the SR membrane and has been identified as the δ-isoform (4). The decrease in CaMK-mediated phosphorylation of SERCA2a and PLB during ischemia for a period of 30 min and during reperfusion for 1 h is in agreement with our previous report (23). Such a depression may explain the observed decrease in the CaMK-stimulated Ca^{2+} uptake in the SR vesicles in the I and I/R hearts. In fact, the decrease in CaMK activity in the SR membrane due to ischemia may account for the reduced phosphorylation of the SERCA2a and PLB. Because cytosolic CaMK activity was not altered during ischemia, our results indicate a specific impairment of the SR CaMK only. Because phosphorylation and dephosphorylation are complementary regulatory mechanisms, the decreased SR phosphorylation activity observed in the I and I/R hearts may be due to an increase in the endogenous phosphatase activity. Contrary to our expectations, the endogenous phosphatase activity in the SR membrane was depressed in the I and I/R hearts. It has been shown that the endogenous phosphatase is a type 1 phosphatase (28), which dephosphorylates PLB at both the PKA and the CaMK sites, resulting in decreased SR Ca^{2+} uptake (19). Our results indicate that ischemia for 30 min did not affect the phosphorylation of PLB by exogenous PKA or the PKA-stimulated Ca^{2+} uptake activity. This observation is in agreement with another report (27) showing that there was no change in the PKA phosphorylation of PLB in the pig heart when the ischemic period was of 30-min duration; however, longer periods of ischemia depressed the SR PLB phosphorylation. In our study PKA phosphorylation of PLB and PKA-stimulated Ca^{2+} uptake activities were found to be depressed in the I/R hearts. The reduced phosphorylation of PLB by PKA observed in the I/R heart is also consistent with another report indicating a similar decrease after 30 min of myocardial ischemia followed by 2 h of reperfusion (31). Although PKA phosphorylation of PLB was reduced in the I/R heart, the cytosolic PKA activity was unaltered because of ischemia-
The depressed phosphorylation by CaMK and PKA in the I/R hearts may be due to a decrease in the PLB protein content of the SR membrane (data not shown). Because the endogenous CaMK and endogenous phosphatase activities were depressed in both the I and I/R hearts, it may be suggested that the balance of the phosphorylation-dephosphorylation cycle in the I and I/R hearts was not altered. To the best of our knowledge, this is the first report demonstrating simultaneous impairment of the SR CaMK and CaMK-stimulated Ca\textsuperscript{2+} uptake as well as the SR phosphatase in the I and I/R hearts.

It is now well accepted that SR Ca\textsuperscript{2+} cycling in the cardiac muscle is dependent on the rate of Ca\textsuperscript{2+} uptake and release. Although defects in SR Ca\textsuperscript{2+} release in the I/R hearts have been reported (7, 9), the mechanisms underlying these alterations are not yet clear. The RyR has been shown to undergo phosphorylation by several protein kinases, and maximal incorporation of 32P\textsuperscript{2+} has been achieved with CaMK phosphorylation (15). Besides, a unique phosphorylation site for CaMK (serine 2809 residue) on the cardiac RyR has been identified (33). Although CaMK- and PKA-mediated phosphorylation has been shown to occur under physiological conditions (32), PKA phosphorylation of the SR membranes under the experimental conditions used in this study resulted in weak phosphorylation of the RyR. In the present study we observed that the phosphorylation of RyR by endogenous CaMK was depressed in both the I and I/R hearts. An increase in the Ca\textsuperscript{2+} release channel activity in intact cardiac myocytes during excitation-contraction coupling resulting from SR CaMK-mediated phosphorylation of RyR has been recently reported (21). In view of the role of endogenous CaMK phosphorylation in Ca\textsuperscript{2+} release, it may be suggested that depressed RyR phosphorylation in both the I and I/R hearts may account for the impaired Ca\textsuperscript{2+} release from the SR vesicles.

Although the beneficial effects of antioxidative treatment for the I/R hearts have been documented (29), there is some controversy regarding the effectiveness of SOD plus CAT in preventing contractile dysfunction due to ischemia-reperfusion. There are reports showing beneficial effects in the I/R hearts (11) and in the oxyradical-perfused hearts (14), but no protection for the I/R hearts has also been reported (25). In the latter study, 15 min of ischemia depressed SR Ca\textsuperscript{2+} uptake, which was normalized completely by reperfusion with or without SOD plus CAT treatment; however, the contractile dysfunction due to ischemia-reperfusion was not prevented. The difference in the results obtained in this and our study with respect to contractile dysfunction could be due to 1) the ischemic models used, i.e., global ischemia vs. regional ischemia, 2) the animals used (rats vs. dogs), 3) the durations of ischemia and reperfusion (30 min of ischemia and 1 h of reperfusion vs. 15 min of ischemia and 15 min of reperfusion, and 4) the duration of SOD plus CAT treatment, 80 vs. 16½ min. In our study, not only did SOD plus CAT treatment improve the cardiac performance but also the reductions in endogenous CaMK activity, CaMK phosphorylation, and CaMK-stimulated Ca\textsuperscript{2+} uptake, as well as exogenous PKA phosphorylation and PKA-stimulated Ca\textsuperscript{2+} uptake in the I/R hearts were attenuated. In addition to preventing changes in the endogenous CaMK activity, SOD plus CAT also attenuated alterations in the endogenous phosphatase activity; these effects would render protection to the SR phosphorylation-dephosphorylation cycle in the I/R hearts. Furthermore, SOD plus CAT treatment was effective in recovering the endogenous CaMK phosphorylation of RyR in the I/R hearts, suggesting an improved SR Ca\textsuperscript{2+} release. Our results indicating beneficial effects of SOD plus CAT treatment are further supported by a recent report showing the protective effects of membrane phosphorylation on the cardiac SR SERCA2a against chlorinated oxidants in vitro (2). Nonetheless, in view of the well-accepted role of SOD plus CAT in scavenging oxyradicals and active species of oxygen (11, 14, 29), it is likely that oxidative stress may be playing a crucial role in inducing SR dysfunction in the I/R hearts. This is a new observation because there is no report showing the beneficial effects of SOD plus CAT in preventing alterations in SR phosphorylation and dephosphorylation in the I/R hearts.

In conclusion, the SR CaMK, unlike the cytosolic PKA, is affected by ischemia and ischemia-reperfusion. Moreover, alterations in SR protein phosphorylation may result in altered SR Ca\textsuperscript{2+} uptake and release. Treatment with SOD plus CAT may improve SR Ca\textsuperscript{2+} uptake and release in the I/R hearts by protecting changes in the SR protein phosphorylation. Because SOD plus CAT treatment is known to scavenge oxyradicals and other species of active oxygen metabolites (6, 24, 29), it is reasonable to presume that the observed beneficial effects of this intervention are due to attenuation of oxidative stress in the I/R hearts. However, it should be noted that the depressions of CaMK- and PKA-mediated phosphorylations, SR CaMK activity, SR Ca\textsuperscript{2+} uptake activities in the absence or presence of PKA, and SR phosphatase activity in the I/R hearts were not greater than those observed in the I hearts. In view of the fact that the degree of oxidative stress in the I/R hearts is greater than that in the I hearts (6, 13, 24), it can be argued that the changes in the above-mentioned parameters in both I and I/R hearts may not be entirely due to the function of oxyradicals and oxidants. Thus the involvement of other ischemic effects such as a decrease in the intracellular pH, depletion of high-energy phosphate stores, the occurrence of intracellular Ca\textsuperscript{2+} overload, and proteolysis (9, 13, 14, 16, 29) cannot be ruled out when indicating the role of oxidative stress in cardiac depression and SR dysfunction.

This work was supported by a grant from the Medical Research Council of Canada (MRC Group in Experimental Cardiology). R. Temsah was a postdoctoral fellow of the Heart & Stroke Foundation of Canada and N. S. Dhalla held the MRC/PMAC Chair in Cardiovascular Research supported by Merck Frosst Canada.

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