Depressed Levels of Ca\textsuperscript{2+}-Cycling Proteins May Underlie Sarcoplasmic Reticulum Dysfunction in the Diabetic Heart

Thomas Netticadan, Rana M. Temsah, Ardeep Kent, Vijayan Elimban, and Naranjan S. Dhalla

In view of the depressed sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-pump and Ca\textsuperscript{2+}-release activities in the diabetic heart and the critical role of phosphorylation in regulating the SR function, we examined the status of Ca\textsuperscript{2+}-calmodulin–dependent protein kinase (CaMK) and cAMP-dependent protein kinase (PKA)-mediated phosphorylations in the diabetic heart. Diabetes was induced in male Sprague-Dawley rats by an injection of streptozotocin (65 mg/kg i.v.), and the animals were killed 6 weeks later for assessment of the ventricular SR function. Depressed cardiac performance and SR Ca\textsuperscript{2+}-uptake and -release activities in diabetic animals were accompanied by a significant decrease in the level of SR Ca\textsuperscript{2+}-cycling proteins, such as ryanodine receptor, Ca\textsuperscript{2+}-pump ATPase, and phospholamban. On the other hand, the CaMK- and PKA-mediated phosphorylations of these Ca\textsuperscript{2+}-cycling proteins, the endogenous SR CaMK and PKA activities, and the endogenous SR and cytosolic phosphatase activities were increased in the diabetic heart. Treatment of 3-week diabetic animals with insulin partially or fully prevented the diabetes-induced changes in cardiac performance, SR Ca\textsuperscript{2+}-uptake and -release activities, and SR protein content, whereas the diabetes-induced changes in SR CaMK- and PKA-mediated phosphorylations and activities, as well as phosphatase activities, were not significantly affected. These results suggest that the reduced content of the Ca\textsuperscript{2+}-cycling proteins, unlike alterations in PKA and phosphatase activities, appear to be the major defect underlying SR dysfunction in the diabetic heart. Diabetes 50:2133–2138, 2001

Diabetic cardiomyopathy has now been well documented in both clinical and experimental settings (1,2), and cardiac dysfunction in chronic diabetes has been linked to defective Ca\textsuperscript{2+}- handling by cardiomyocytes (3,4). Although the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+}-pump and -release activities, which play a central role in regulating the intracellular level of Ca\textsuperscript{2+}, are decreased in the diabetic heart (5,6), the mechanisms underlying these alterations are not fully understood. In this regard, it is noted that the SR Ca\textsuperscript{2+}-pump and -release activities are determined by the status of Ca\textsuperscript{2+}-pump ATPase (SR Ca\textsuperscript{2+}-ATPase [SERCA2a]) and Ca\textsuperscript{2+}-release channels (ryanodine receptor [RyR]), respectively, whereas phospholamban (PLB) is known to affect the SERCA2a and Ca\textsuperscript{2+}-uptake function of the SR membrane. Thus, the depression in SR Ca\textsuperscript{2+}-uptake and -release activities in the diabetic heart can be attributed to changes in RyR, SERCA2a, and/or PLB levels. Therefore, the present investigation examined the content of Ca\textsuperscript{2+}-cycling proteins in the cardiac SR membrane by using a rat model of streptozotocin (STZ)-induced diabetes. Because of earlier observations (2,5,6) that changes in the SR function in the diabetic animal are reversible on treatment with insulin, the effects of insulin on cardiac SR proteins in the diabetic animal were also studied.

Because protein phosphorylation/dephosphorylation is known to regulate various cellular processes (7,8), two major signaling pathways, namely Ca\textsuperscript{2+}-calmodulin–dependent protein kinase (CaMK) and cAMP-dependent protein kinase (PKA) (9), have been implicated in the control of SR function. However, very little information regarding the status of these mechanisms in the diabetic heart is available. We have recently reported that defects in SR protein phosphorylation may be partly responsible for SR dysfunction in the ischemic-reperfused heart (10–12), as well as for heart failure caused by myocardial infarction (13). Accordingly, it is hypothesized that decreased phosphorylation of the SR Ca\textsuperscript{2+}-cycling proteins by CaMK and PKA may be a contributing factor for the depressed SR function in the diabetic heart. In view of these considerations, we investigated the status of CaMK- and PKA-mediated phosphorylations of RyR, SERCA2a, and PLB proteins in the control and diabetic SR membrane. In this study, we provide evidence for decreased SR Ca\textsuperscript{2+}-cycling protein contents and increased SR CaMK- and PKA-mediated phosphorylations in the diabetic heart. Furthermore, the activities of SR and cytosolic phosphatases, which are involved in the dephosphorylation process, are increased in the diabetic heart.

RESEARCH DESIGN AND METHODS

The experimental protocol in this investigation was approved by the Animal Care Committee of the University of Manitoba and conforms with the guidelines of the Canadian Council on Animal Care Concerning the Use of Experimental Laboratory Animals.
TABLE 1
General characteristics, hemodynamic parameters, and SR Ca\(^{2+}\)-uptake and -release activities of control, diabetic, and diabetic plus insulin-treated hearts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic plus insulin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>515 ± 10.5</td>
<td>348 ± 11.3*</td>
<td>425 ± 15†</td>
</tr>
<tr>
<td>Heart weight (g)</td>
<td>1.10 ± 0.05</td>
<td>1.02 ± 0.03</td>
<td>1.08 ± 0.09</td>
</tr>
<tr>
<td>Heart/body weight (mg/g)</td>
<td>2.1 ± 0.05</td>
<td>2.9 ± 0.03*</td>
<td>2.5 ± 0.06†</td>
</tr>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>151 ± 13</td>
<td>322 ± 24*</td>
<td>200 ± 15†</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td>118.5 ± 10.7</td>
<td>27.3 ± 2.2*</td>
<td>75 ± 2.6†</td>
</tr>
<tr>
<td>+ dP/dt (nmHg/s)</td>
<td>5615 ± 223</td>
<td>3408 ± 198*</td>
<td>4871 ± 240†</td>
</tr>
<tr>
<td>− dP/dt (nmHg/s)</td>
<td>5345 ± 143</td>
<td>3188 ± 231*</td>
<td>4636 ± 151†</td>
</tr>
<tr>
<td>Ca(^{2+}) uptake (nmol Ca(^{2+})·mg(^{-1})·min(^{-1}))</td>
<td>58.2 ± 6.3</td>
<td>30.6 ± 3.2*</td>
<td>45.9 ± 5.1†</td>
</tr>
<tr>
<td>Ca(^{2+}) release (nmol Ca(^{2+})·mg(^{-1})·15 s(^{-1}))</td>
<td>8.1 ± 0.7</td>
<td>2.8 ± 0.2*</td>
<td>6.1 ± 0.6†</td>
</tr>
</tbody>
</table>

Data are means ± SE of six independent experiments for each group. Diabetic animals were killed 6 weeks after the STZ injection. Heart weight represents weight of both ventricles; + dP/dt, rate of pressure development; − dP/dt, rate of pressure decay. *P < 0.05 vs. control; †P < 0.05 vs. diabetic.

RESULTS
Cardiac performance, SR Ca\(^{2+}\)-transport activities, and SR Ca\(^{2+}\)-cycling proteins. STZ-injected diabetic animals showed a significant decrease in body weight and an increase in heart/body weight ratio compared with controls (Table 1). The development of diabetes was confirmed by a marked elevation in the plasma glucose level and a decrease in the plasma insulin level in STZ-injected animals. The results in Table 1 also show that the rate of pressure development and the rate of pressure fall were significantly decreased in the 6-week diabetic animals. Furthermore, the SR Ca\(^{2+}\)-uptake and -release activities were significantly decreased in the hearts of diabetic animals compared with those of controls. To test whether the depressed SR activities in the diabetic hearts were caused by a decrease in the content of SR Ca\(^{2+}\)-cycling proteins, the levels of RyR, SERCA2a, and PLB were estimated in the control and diabetic hearts. Figure 1 shows that the contents of all of these proteins were decreased (P < 0.05) in the diabetic hearts. Treatment of 3-week diabetic animals with insulin was found to significantly reverse the diabetes-induced changes in the general characteristics, cardiac performance, SR function, and levels of SR Ca\(^{2+}\)-cycling proteins (Table 1 and Fig. 1).

SR protein phosphorylation by CaMK and PKA. Because SR Ca\(^{2+}\)-uptake and -release activities are regulated by CaMK-mediated phosphorylation of the Ca\(^{2+}\)-cycling proteins (11), the endogenous SR CaMK phosphorylation of SR proteins was determined. Figure 2 shows the SR protein profile (panel A), the corresponding autoradiogram depicting SR protein phosphorylation (panel B), and the analysis of the RyR, SERCA2a, and PLB phosphorylations in the control, diabetic, and insulin-treated diabetic hearts, respectively (panel C). CaMK phosphorylation of all three proteins was significantly increased in the hearts of diabetic animals compared with those of controls, and this increase was not attenuated on insulin treatment; the
identity of the phosphorylated proteins has been previously established (13,14). There was no clear decrease in the protein content of the bands representing RyR and SERCA2a in the diabetic group compared with the control group (Fig. 2A). This is in contrast to the significant decrease detected by Western blot analysis (Fig. 1). This discrepancy may be a result of the fact that the bands observed in Fig. 2A may contain other proteins comigrating with the RyR and SERCA2a proteins. Moreover, Western blot analysis uses specific monoclonal antibodies recognizing RyR and SERCA2a and thus is an accurate method for determining the content of proteins. To understand the reason for the increase in the endogenous CaMK phosphorylation in the diabetic hearts, the SR and cytosolic CaMK activities were assessed. Table 2 shows that the SR CaMK activity was significantly increased in the hearts of diabetic animals, whereas the cytosolic CaMK activity was unaltered when compared with the hearts of controls. Insulin treatment did not reverse the diabetes-induced increase in SR CaMK activity.

Because a defect in PKA-mediated phosphorylation may also contribute to depressed SR function (10,13), PKA phosphorylation of SR proteins was examined in the control, diabetic, and insulin-treated diabetic hearts. Figure 3 shows the SR protein profile (panel A), the corresponding autoradiogram depicting SR protein phosphorylation (panel B), and the analysis of RyR and PLB phosphorylation by PKA in the control, diabetic, and insulin-treated diabetic groups (panel C). PKA-mediated phosphorylation of RyR and PLB was significantly increased in the hearts of diabetic animals compared with those of controls, and this increase was not attenuated on insulin treatment; the identity of these phosphorylated proteins has been previously established (11,13). To determine the mechanisms for the increase in PKA-mediated phosphorylation in the diabetic hearts, the SR and cytosolic PKA activities were assessed. Table 2 shows that the SR PKA activity was significantly increased in the hearts of diabetic animals compared with those of controls, whereas the cytosolic PKA activity was unaltered. Insulin treatment did not reverse the diabetes-induced increase in SR PKA activity.

**DISCUSSION**

Our results show impaired cardiac function and decreased SR Ca$^{2+}$-uptake and -release activities in the diabetic heart. Although a reduction of SR Ca$^{2+}$-uptake, Ca$^{2+}$-release, and ryanodine binding has been previously demonstrated in the diabetic heart (5,6), the mechanisms underlying alterations in SR function were not fully established. In this regard, the decrease in protein contents of RyR, SERCA2a, and PLB observed in the diabetic heart and other studies (15,16) could explain the depression in SR Ca$^{2+}$-uptake and -release activities. However, it should be pointed out that the depression of the Ca$^{2+}$-cycling protein levels may represent one of several factors, such as increased phospholipid and fatty acid content in...
the SR vesicles (5,17), which have been proposed to explain SR dysfunction in the diabetic heart. Nonetheless, in view of the role of SR in cardiac contraction and relaxation processes, the observed reduction in SR function may explain the depressed cardiac performance in diabetic animals. Such a view does not exclude other mechanisms, such as depressed myofibrillar ATPase (18), sarcolemmal Na\(^+\)-K\(^+\) ATPase (19), and Ca\(^{2+}\)-pump and Na\(^+\)-Ca\(^{2+}\) exchange activities (20), which have been shown to contribute to cardiac dysfunction in the diabetic heart.

Current evidence favors enhanced SR Ca\(^{2+}\)-uptake under physiological conditions as a consequence of phosphorylation of the SERCA2a by CaMK (14) and phosphorylation of PLB by both CaMK and PKA (21–23). Phosphorylation of PLB by both CaMK and PKA has been shown to relieve the inhibitory action of PLB on SERCA2a, resulting in an increased affinity of SERCA2a for Ca\(^{2+}\) (21–23). CaMK phosphorylation of SERCA2a has been reported to increase \(V_{\text{max}}\) of SR Ca\(^{2+}\) transport (14). Although Odermatt et al. (24) failed to demonstrate the CaMK-mediated stimulation of \(V_{\text{max}}\) of Ca\(^{2+}\) transport, the data from our previous study (10) supported the observation of other investigators (14). SR Ca\(^{2+}\)-release was also shown to be regulated by RyR phosphorylation directly because both CaMK and PKA were reported to promote SR calcium release (25–27). Thus, the impairment in the SR Ca\(^{2+}\)-uptake and -release can be seen as a result of a decrease in SR protein phosphorylations. However, we observed an increase in the in vitro endogenous CaMK-mediated phosphorylation of RyR, SERCA2a, and PLB, as well as the PKA-mediated phosphorylation of RyR and PLB in the diabetic heart. A recent study (28) has linked RyR hyper-phosphorylation by PKA to defective Ca\(^{2+}\)-release channel function in the failing heart. Because CaMK and PKA have been observed to be endogenous to SR (29,30), the observed increase in substrate phosphorylation may be explained on the basis of an increase in endogenous CaMK and PKA activities. Moreover, these increases in SR protein kinase activities were specific because the cytosolic CaMK and PKA activities were unaltered in the diabetic heart. Although other investigators (31) did not observe any change in the levels of CaMK and PKA activities in the hearts of diabetic rats, it is noted that whole-heart homogenate was used in their study (31), whereas SR vesicles were used in the present investigation.

In view of the role of endogenous phosphatases in dephosphorylation of membrane proteins, the increased endogenous CaMK- and PKA-mediated phosphorylations in the diabetic heart may be attributed to depressed endogenous phosphatase activities. On the contrary, we observed an increase in the endogenous and the cytosolic phosphatase activities in the diabetic heart. Such an increase in the total phosphatase activity may be caused by increases in protein phosphatase-1 and -2 activity in the diabetic heart (32). It is likely that the enhanced endogenous phosphatase activities may compensate for the marked elevation in the endogenous CaMK and PKA activities in such a way that the SR phosphorylation/depshorylation cycle is unaffected in the diabetic heart. This view is consistent with an earlier report (33) suggesting that the SR regulatory mechanisms may not play any role in the cardiac SR dysfunction observed in the diabetic heart. It is possible that the diabetic heart may respond to the depressed SR function by enhancing the activities of the endogenous kinases; however, these compensatory mechanisms may be inadequate to maintain the SR function because of increased dephosphorylation.

The results in this study indicate that a depression in the

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic plus insulin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR CaMK activity (pmol Pi/µg·min(^{-1}))</td>
<td>51.0 ± 4.0</td>
<td>105.3 ± 6.2*</td>
<td>109.6 ± 3.6*</td>
</tr>
<tr>
<td>Cytosolic CaMK activity (pmol Pi/µg·min(^{-1}))</td>
<td>191.1 ± 11.0</td>
<td>190.7 ± 7.6</td>
<td>ND</td>
</tr>
<tr>
<td>SR PKA activity (pmol Pi/µg·min(^{-1}))</td>
<td>53.4 ± 2.8</td>
<td>103.9 ± 7.7*</td>
<td>105.3 ± 4.7*</td>
</tr>
<tr>
<td>Cytosolic PKA activity (pmol Pi/µg·min(^{-1}))</td>
<td>3,364.2 ± 153.7</td>
<td>3,811.8 ± 233.8</td>
<td>ND</td>
</tr>
<tr>
<td>SR phosphatase activity (pmol Pi/µg·min(^{-1}))</td>
<td>372.1 ± 50.5</td>
<td>536.3 ± 35.3*</td>
<td>522.9 ± 18.2*</td>
</tr>
<tr>
<td>Cytosolic phosphorylase activity (pmol Pi/µg·min(^{-1}))</td>
<td>5,052.3 ± 35.8</td>
<td>5,904.3 ± 136.1*</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are means ± SE of four independent experiments for each group. *P < 0.05 vs. controls. ND, not determined.

![FIG. 3. Exogenous PKA phosphorylation of RyR and PLB in the control (C), diabetic (D), and insulin-treated diabetic (D + I) hearts.](Image)
content of SR Ca\(^{2+}\)-cycling proteins may contribute to SR dysfunction in the diabetic heart. Because the treatment of STZ-induced diabetic rats with insulin has been reported to prevent diabetes-induced changes in the heart (3–6,17–20), it is likely that insulin deficiency may play a role in regulating cardiac protein content. Increased proteolysis shown to occur in the diabetic heart (34) is reduced by insulin (35). Our results showing partial or full recovery of the SR Ca\(^{2+}\)-cycling proteins contents in the diabetic heart on insulin treatment support the ideas mentioned above. However, it must be mentioned that insulin treatment did not completely normalize diabetes-induced alterations in general characteristics, hemodynamic parameters, and SR Ca\(^{2+}\)-uptake and -release activities, and SERCA2a content. Earlier studies (5,36,37) have also reported an incomplete recovery of some of the above parameters in the insulin-treated diabetic animals. Although the insulin dose (3 units per day) used in this study was selected based on our previous experience (5,32,36), it is possible that a higher dose of insulin might recover all of the above parameters to control values. Furthermore, the 3-week duration of treatment with insulin in this study may not be sufficient for full recovery of all subcellular changes in the diabetic heart. These views are consistent with partial recovery of plasma insulin and glucose levels in diabetic animals treated with insulin. Nonetheless, it should be noted that the diabetes-induced changes in SR, RyR, and PLB contents were reversed completely by treatment of diabetic animals with 3 units of insulin per day for 3 weeks. On the other hand, insulin treatment did not affect the diabetes-induced increase in CaMK and PKA activities or phosphorylation of their substrates. A recent study has also reported the ineffectiveness of insulin treatment in reversing the diabetes-induced increase in CaMK and PKA activities in liver tissue homogenates (31). Thus, recovery of SR function as a result of insulin treatment may be mediated via protection of the SR Ca\(^{2+}\)-cycling proteins rather than through regulatory mechanisms, and, therefore, SR proteins could be potential targets for the treatment of diabetes.

By using the hearts of diabetic rats, our study is the first to demonstrate enhanced in vitro CaMK and PKA phosphorylation of SR Ca\(^{2+}\)-cycling proteins, specific increases in the endogenous CaMK and PKA activities, and increased SR and cytosolic phosphatase activities. Furthermore, we have shown partial or full recovery of SR Ca\(^{2+}\)-cycling proteins, unlike CaMK- and PKA-mediated SR phosphorylations and their activities in the diabetic heart, upon treatment with insulin. Thus, these results provide new information regarding the mechanisms underlying cardiac dysfunction in chronic diabetes.

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