REGULATION OF SARCOPLASMIC RETICULUM IN DIABETIC CARDIOMYOPATHY

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For the Degree of Master of Science

by

Ardeep Kent

Institute of Cardiovascular Sciences

And Department of Physiology, Faculty of Medicine

University of Manitoba

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Regulation of Sarcoplasmic Reticulum in Diabetic Cardiomyopathy

BY

Ardeep Kent

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

Master of Science

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ABSTRACT

Although Ca^{2+}-transport activities in the cardiac sarcoplasmic reticulum (SR) have been shown to be depressed in chronic diabetes, the status of its regulatory mechanisms is not fully understood. Since Ca^{2+}-calmodulin and cAMP-dependent protein kinases (CAMK and PKA) are known to stimulate SR function, it is possible that these enzymes may be altered in the diabetic heart. For this purpose, rats were made diabetic by an injection with streptozotocin; vehicle injected animals served as control. Some of the 4 week diabetic animals were treated with insulin (3 U/day) for 2 weeks. Hearts were removed at 6 weeks after the induction of diabetes and the ventricular tissue was used for either SR preparation or other biochemical determination. The decreased level of glucose, increased level of insulin and depressed ventricular function in diabetic animals were prevented by insulin treatment. Both Ca^{2+}-uptake and Ca^{2+}-release activities in SR preparations from diabetic hearts were decreased. The SR protein content as estimated by Western blot analysis for Ca^{2+}-pump ATPase, Ca^{2+}-release channels and phospholamban proteins were also decreased in the diabetic hearts. Both CAMK- and PKA-mediated protein phosphorylations were increased in the diabetic SR. These changes in the diabetic heart were associated with increased SR CAMK, PKA and phosphatase activities. Although insulin treatment of diabetic animals provided partial recovery of SR function, it had no effect on changes in CAMK and PKA activities. These results suggest marked changes in the regulatory mechanisms for SR function in the diabetic hearts.

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I. INTRODUCTION

Diabetes is a disease which is associated with high levels of blood glucose. Diabetes mellitus is of two types: insulin-dependent diabetes mellitus (IDDM) and noninsulin-dependent diabetes mellitus (NIDDM). IDDM, or Type I, results from lack of insulin due to the inability of pancreatic β-cells to produce sufficient insulin; the elevated glucose levels are usually maintained by the administration of exogenous insulin. Although the use of insulin by diabetics has minimized the risk of death from ketotic coma, cardiovascular dysfunction still remains a major cause of death in patients with diabetes. As force generation by the heart is a cellular event, it is believed that subcellular defects are involved in the pathogenesis of heart dysfunction. Studies carried out in the past 25 years have focused on the role of Ca^{2+} in the contraction-relaxation coupling process for muscle contraction. By virtue of its ability to release and accumulate Ca^{2+}, the sarcoplasmic reticulum (SR) is considered to intimately participate in the process of cardiac contraction and relaxation. Furthermore, the SR function is regulated by protein phosphorylation. In view of the central role of SR in cardiac contractile function, alterations in cardiac function in chronic diabetes may be due to SR dysfunction. Although SR dysfunction in diabetes has been documented, the mechanisms underlying these alterations are not completely understood. In view of the critical role of phosphorylation in regulating the SR function, we have investigated the role of CAMK and PKA-mediated phosphorylation in control and diabetic rats.

II. REVIEW OF LITERATURE

1. Clinical classification of diabetes mellitus

Diabetes mellitus (DM) has been known for many centuries. In fact, the ancient people from India were first to characterize the urine of diabetic patients to be "honeyed" in approximately 400 B.C.[1] It is a serious disease that seems to make afflicted individuals more susceptible to heart dysfunction, independent of atherosclerosis, and hypertension. Diabetes mellitus involves defects in protein, carbohydrate and fat metabolism; these abnormalities are associated with the deficiency in systemic insulin or inability of insulin to act on the cell and thus creating a hyperglycemic status in the body. According to the National Diabetes Data Group[2] and the World Health Organization,[3] diabetes mellitus can be divided in two groups, namely the insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). The IDDM (Type I diabetes) or Juvenile-onset diabetes involves the destruction of the β-cells of the pancreas and their inability to produce insulin. The IDDM patients are required to take exogenous insulin to control their blood glucose levels and prevent ketoacidosis. Ketoacidosis occurs when the ketone levels in the body are high enough that it leads to metabolic acidosis, diabetic coma and sometimes death. Symptoms that accompany IDDM are excessive thirst, unexplained weight loss, polyuria, and ketosis. The NIDDM (Type II diabetes) or Adult-onset diabetes involves the body's inability to utilize insulin due to receptor defects or genetic predisposition, and therefore individuals are usually hyperinsulinemic. The NIDDM patients are not required to take exogenous insulin, and their risk of ketosis is rare in comparison to IDDM. It is estimated

that approximately 80-85% of all diabetics are NIDDM;[4] the majority of these patients are also obese.

2. Diabetes mellitus and its complications

Even with the discovery of insulin by Banting and Best for the treatment of diabetes, the diabetic patients still suffer a higher mortality than the general population. Insulin has increased the life expectancy of diabetic patients, but the question remains why about 80% of deaths in diabetic patients are related to cardiovascular disease.[5,6] Even in the absence of hypertension, atherosclerosis and vascular complications, diabetics still suffer more cardiovascular dysfunction. The following are some of the cardiovascular complications which are commonly associated with diabetes:

a. Hypertension

Elevated blood pressure seems to be quite common in diabetes and in fact, hypertension precedes the microvascular and macrovascular changes seen in diabetics and is considered to exacerbate the increased mortality. Hypertension in diabetics makes these individuals more susceptible to the occurrence of renal, stroke, coronary artery disease, retinal and cardiovascular dysfunction. Studies conducted in the past have estimated that diabetics with hypertension are twice that of non-diabetics.[7] Factor et al[8] have confirmed that hypertensive-diabetics suffer increased myocellular damage which may account for their higher mortality than non-diabetics. Hyperinsulinemia may provide an answer for the increased sodium retention seen in diabetics since increased endogenous insulin was shown

to increase sodium reabsorption.[9] An increase in intracellular sodium poses a threat, because it can lead to increased vasoconstriction. The use of anti-hypertensive therapy such as α- and β-adrenoceptor blockers, angiotensin converting enzyme (ACE) inhibitors, calcium channel blockers, vasodilators and diuretics may provide some benefit in reducing blood pressure and coronary artery disease. However, these agents are also known to cause some adverse effect on glucose and lipid metabolism and may in some accelerate vascular disease.[10-12] Nonetheless, hypertension is of great concern to health practitioners searching for the reason why diabetics show increased mortality and cardiovascular disease than non-diabetics.

b. Myocardial infarction

Diabetic patients have been shown to suffer from more frequent (2.5 - 5 times) and severe myocardial infarction (MI) versus non-diabetics.[13-15] Studies conducted in the past demonstrated that male diabetics increase the likelihood of cardiovascular problems by 2 times, whereas female diabetics increase the chance of cardiovascular problems by 3 - 5 times the normal risk.[16] Dhalla et al[16] have indicated three risk factors that may account for the increased incidence of cardiovascular dysfunction in diabetics: atherosclerosis, microvascular alterations and primary myopathic disorder in cardiac muscle. Studies in the past have demonstrated that two months after an MI the mortality in diabetic patients was approximately 41%, in comparison to 15% in non-diabetics.[17] Even more alarming is the evidence that regardless of infarct size, diabetics still suffer a higher mortality than non-diabetics.[18] The increased incidence of MI in diabetics has been linked to glycemic

status. Studies have shown that when the hyperglycemic state of diabetics was stringently controlled the incidence of MI fell significantly.[19] Hyperglycemia has also been linked to endothelial dysfunction and hypertension. Another danger that increases the mortality in diabetics is the occurrence of a silent MI which has been suggested to be due to the damage of cardiac nerves and the inability of afferent nerves to transmit information as a result of visceral neuropathy.[20] Silent MI was shown to be more common in the diabetic population[21,22] and is of great concern because the patients are unaware that they have suffered an MI and thus may not summon the proper medical attention.[23] The survival of diabetic patients with MI after 1, 2 and 5 years is 82%, 78% and 58% whereas that for non-diabetic patients with MI is 94%, 92% and 82% respectively. Thrombolytic therapy with aspirin and/or heparin seems to be the standard treatment for diabetic patients with MI.[24] The poor outcome in diabetics is the result of advanced coronary artery disease present in the diabetic population; however, β-adrenoceptor blockers have proven to provide long-term benefit to diabetics with MI.[24] Nonetheless, it has been shown that diabetes mellitus increases the prevalence of coronary artery disease.[25] It has been suggested that factors such as age, cholesterol and hypertension amplify the affect of diabetes on the prevalence of coronary artery disease. It has also been established that diabetics suffer from 4 - 5 times more incidences of congestive heart failure following MI independent of age, weight, cholesterol level, blood pressure and coronary artery disease.[14,26-29]

c. Coronary thrombosis and stroke

Atherosclerosis involves dysfunction of cerebral arteries and peripheral vasculature.

As a result of poor lipoprotein metabolism and hypercholesterolemia, plaque formation or thrombosis has been shown to occur. It has been recognized that high level of LDL (low-density lipoprotein) and low level of HDL (high-density lipoprotein) lead to the initiation and progression of arterial lesions. It has been suggested that the atherosclerotic process is similar in the general population; however, in diabetic patient it proceeds at a faster pace.[30] Thrombosis seems to be initiated by injury to the arterial wall; this will result in alterations in platelet coagulation and fibrin activity in the diabetic patient. It is now believed that thrombosis may occur prior to vascular injury and endothelial cell dysfunction in the diabetic patient. One possible explanation for the increased prevalence of thrombosis in diabetics could be due to the increased platelet hyperaggregability seen in patients with IDDM and NIDDM.[31] Some studies have confirmed that if patients do not control their diabetes, their platelets release more vascular growth factors and this results in increased progression of lesion via smooth muscle cell proliferation.[32] It has been suggested that the production of thromboxane (a potent vasoconstrictor) is increased in the insulin deficient diabetic and this may hasten the thrombolytic process.[33] Treatment of diabetic patients with coronary thrombosis as well as stroke may involve proper glycemic status via insulin regulation, diet and weight reduction, exercise, use of antiaggregant agents such as aspirin, and also use of anticoagulants. Postmortem studies have shown stroke to be a major cause of death in diabetic patient.[34-36] It has been estimated that 7% of deaths in diabetics are related to stroke, and 25% to cerebrovascular disease.[37] Women diabetics have also shown to be at more risk than their female counterparts.[38]

3. Cardiac dysfunction during diabetes mellitus

Studies conducted around the world have concluded that the heart is indeed compromised in diabetic patients. The diabetic heart has been shown to have lower ejection fractions and stroke volume.[38-40] It has also been suggested that the compromised heart is due to decreased compliance of the left ventricle[38] and a slower relaxation process.[41] It has also been suggested that a lack of systemic insulin may result in loss of membrane integrity. This may alter membrane permeability, allowing for increased entry of cations such as calcium and resulting in dysfunction of contractile units; this eventually leads to arrythmias and heart failure. Factors such as isovolumic contraction or relaxation time and left ventricular ejection time have been shown to increase in diabetics, a clear indication of cardiac dysfunction.[42,43] A larger ejection fraction than normal indicates contractile dysfunction whereas a lower ejection fraction indicates the presence of heart disease.[44] Experimental data has pinpointed a direct linear relationship between serum glucose levels and ejection fraction levels.[45] Since diabetes mellitus has been linked to other vascular complications such as hypertension and atherosclerosis, these factors may attenuate the afterload on the heart resulting in higher filling pressures.[38] It has been indicated that if the hyperglycemic state of these patients was regulated with insulin to approximately control levels, their cardiac performance also improved to control levels.[46] When myocardial shortening was examined it was also found to be subnormal in diabetics.[47,48] Systolic and diastolic dysfunction have been discussed in the past in correlation with diabetes. Studies have also shown that in most cases diastolic dysfunction or impaired isovolumic relaxation, preceded

systolic dysfunction or impaired isovolumic contraction;[48-50] this is important clinically, because it allows for the early detection of cardiac disease in diabetic patients. Studies on isolated diabetic ventricular tissue have shown that diabetes mellitus itself results in compromised cardiac function.[26] Experiments on animal models of diabetes mellitus have shown decreased force generation, as well as decrease in cardiac output and other hemodynamic factors.[50-61] The duration of diabetes may also have a bearing on the recovery of contractile function once insulin is administered. It has been indicated that in the chronically diabetic rat, insulin treatment only led to the partial recovery of function to normal levels but contractile dysfunction was still observed.[52,62,63]

Treatment of diabetics in general has been mostly centered around correcting the hyperglycemic state of diabetics with insulin. It has been shown that if insulin treatment is given immediately after induction of diabetes, insulin will allow recovery of cardiac function to normal levels, as well as the restoration of physical attributes such as heart and body weight.[61,64] While assessing the effectiveness of insulin treatment, it has been concluded that factors such as dosage of insulin, severity and duration of diabetes should be taken into account since insulin did not reverse dysfunction in trials conducted over longer periods of time.[65] Other studies have suggested that insulin itself and not the systemic glucose level may play a role in recovery of contractile dysfunction.[66] Various investigators have also examined the possible role of depressed thyroid hormone levels in diabetics.[51,66-68] Restoration of thyroid hormone levels did not result in correction of contractile dysfunction to control values but in fact cardiac dysfunction was still evident.[51,53,66,67,69] Therefore, the

possible role of hypothyroidism in generation of cardiac dysfunction in diabetics has not been validated.

4. Subcellular defects in diabetes mellitus

It has been noted that the heart function in the diabetic population is compromised but the question still remains on exactly what causes this dysfunction. Some invesetigators have concentrated on the excitation-contraction coupling mechanism because the force generation by the heart is a cellular event. Other researchers have focused solely on the importance of Ca^{2+} in the contraction process. Intracellular Ca^{2+} homeostasis is crucial to the viability of the heart as a pump, as concentrations of Ca^{2+} are seen to fluctuate from 10^{-5} M in contraction to 10^{-7} M in relaxation. Investigators have also focused their attention on the subcellular organelles such as the sarcoplasmic reticulum (SR), sarcolemmal membrane (SL) and the mitochondria (Mt), since these organelles regulate cations such as Ca^{2+} and play a vital role in the process of excitation-contraction coupling. The process of excitation-contraction coupling involves the binding of Ca^{2+} to troponin C, allowing the release of tropomyosin and the crossbridge cycling of myosin with actin. The myofibrillar Ca^{2+}-stimulated ATPase mediates the crossbridge cycling[70,71] which was found to be decreased in the diabetic myocardium in comparison to control values.[72-75] The activities of actomyosin and myosin were also depressed in the diabetic heart;[76,77] this defect was corrected upon receiving insulin treatment.[73] It has been suggested that the most likely explanation for a decrease in myofibrillar ATPase activity may be the conformational modification at or in the vicinity of

the enzymatic activity.[72,73] The deficiency in circulating thyroid hormones was also examined but ruled out as a primary cause for the decrease in ATPase activity.[76,78] This defect was considered to be of importance clinically because ATPase activity is associated with force generation in the heart, and a decrease in its activity is indicative of heart dysfunction in diabetes.[70,79]

a. Changes in the sarcoplasmic reticulum in diabetes

The sarcoplasmic reticulum (SR) is the major source of Ca^{2+} storage in the heart, and is primarily responsible for the release and subsequent uptake of Ca^{2+} for the cardiac contraction and relaxation phases. Ca^{2+}-pump ATPase protein (SERCA2a) accounts for approximately 50-90% of the SR proteins[80] and Ca^{2+}-uptake is an energy dependent process. It has been postulated that a slower relaxation time in the diabetic heart may be due to the slower removal of cytosolic Ca^{2+}. Since, the SR is the major storage site of Ca^{2+} in the myocardium, it was speculated that the depressed Ca^{2+}-uptake may be due to an abnormality in the SR in the diabetic condition. Penpargkul et al[81] were the first to confirm that indeed the ability of SR to accumulate Ca^{2+} was decreased in the diabetic heart. Other investigators over the years have also confirmed these findings and also have found that SR Ca^{2+} binding was also depressed in the diabetic heart.[51,82,83] It was found that the Mg^{2+}-dependent ATPase activity was unaffected;[51] treatment of diabetic animals with insulin corrected the depression in SR Ca^{2+}-pump ATPase and Ca^{2+}-uptake activities.[51,83] In searching for an answer for the depressed function of the SR in diabetes, it was suggested that hypothyroidism may cause defect in cardiac SR Ca^{2+}-transport,[84] however, treatment of diabetic animals with thyroxine

did not alter the depressed SR Ca^{2+}-transport.[51] It has also been suggested that the defect in SR Ca^{2+}-transport could be due to a change in lipid accumulation, especially long-chain acyl-carnitines observed in the diabetic hearts.[85] Chronic treatment with carnitine prevented the accumulation of long-chain acyl-carnitines and allowed for recovery of SR function but not cardiac function in diabetics.[83]

The contractile state of the myocardium is regulated via protein phosphorylation and dephosphorylation.[86,87] This process includes phosphorylation and dephosphorylation of various intracellular proteins and it is when these regulatory mechanisms fail cardiac dysfunction occurs. Initially researchers only focused on the cAMP-dependent processes mediated by the cAMP-dependent protein kinase (PKA),[88-90] but now they have expanded to include Ca^{2+}-calmodulin dependent protein kinase (CAMK),[91,92] protein kinase C (PKC),[93,94] cGMP-dependent protein kinase (PKG),[95] tryosine protein kinases,[96] extracellularly signal-regulated kinases,[97] mitogen-activated protein kinases[96,98] and c-jun N-terminal protein kinases.[99] It should be pointed out that the cardiac SR is involved in the regulation of intracellular Ca^{2+} and therefore it affects contraction and relaxation through the phosphorylation of various regulatory proteins such as SERCA2a, Ca^{2+}-release channel or ryanodine receptor (RyR) and phospholamban (PLB).

It has been recently shown that direct phosphorylation of SERCA2a by CAMK results in an increased rate of Ca^{2+}-uptake by the SR.[100] It is believed that in the unphosphorylated state PLB is bound to SERCA2a in an inhibitory complex, preventing Ca^{2+}-uptake from occurring.[101] This view is substantiated in PLB deficient SR hearts which show increased

Ca^{2+}-uptake activity.[102] It has been suggested that phosphorylation is a key factor in the mechanism of Ca^{2+}-uptake. It has been proposed that upon phosphorylation PLB undergoes a conformational change allowing for the activation of SERCA2a pump.[103,104] Kirchberger et al[105] were the first to show that PKA-dependent phosphorylation of cardiac SR resulted in an increase in Ca^{2+}-transport activity. It has been suggested that PKA may accomplish this by increasing the affinity of the SERCA2a pump for Ca^{2+}.[106,107] The increase in SERCA2a pump activity by PKA-dependent phosphorylation of PLB could also be due to the increased coupling ratio of SERCA2a for Ca^{2+} or an increased turnover of SERCA2a.[108] Numerous studies have also confirmed the role of phospholamban as a regulator of cardiac relaxation in response to catecholamine or sympathetic stimulation. For example, β-adrenergic stimulation increased PLB phosphorylation and SR Ca^{2+}-transport in addition to shortening the myocardial relaxation.[88,89,109-111] In order to confirm the effects of β-adrenergic stimulation, some researchers inhibited this stimulation via muscarinic and cholinergic mediated processes and found that the parasympathetic stimulation can reverse the effects of β-adrenergic stimulation on PLB phosphorylation.[111-115]

CAMK has also been shown to phosphorylate PLB, and increase Ca^{2+}-uptake.[106,116,117] The increase in SR Ca^{2+}-uptake activity seems to be due to the increase in affinity of SERCA2a pump for Ca^{2+}.[116,117] It is speculated that both PKA and CAMK are involved in the phosphorylation of PLB and seem to act independently of each other, but when these regulatory mechanisms operate together their effect is additive.[106,116,118] It should also be noted that it is estimated that approximately 50% of PLB phosphorylation is accounted for

by CAMK.[119] It has also been observed that PKG can also increase the phosphorylation of PLB but the significance of this mechanism is not clearly understood.[120] PKC has also been observed to increase PLB phosphorylation, and therefore Ca^{2+}-uptake activity.[121,122] Protein phosphatases dephosphorylate proteins and regulate a variety of signal transduction pathways.[123] Some researchers have suggested the presence of a 'PLB-specific' phosphatase that can dephosphorylate PKA and CAMK phosphorylation sites thus resulting in a decrease in Ca^{2+}-uptake activity.[124-126]

SR Ca^{2+}-release occurs through the RyRs. The influx of Ca^{2+} from voltage-gated sarcolemmal Ca^{2+} channels in the sarcolemmal membrane leads to further release of Ca^{2+} from the SR (via RyR) by a process called calcium-induced-calcium-release.[127] RyR has been suggested to be regulated by PKA, CAMK and Ca^{2+};[128-131] PKA and CAMK phosphorylation of RyR promote SR Ca^{2+}-release. Calsequestrin is a Ca^{2+}-binding protein involved in the binding of large amount of Ca^{2+} that is sequestered by the SERCA2a pump. It is believed that this phosphoprotein is phosphorylated by casein kinase II, but its effect on calsequestrin still has to be identified.[132,133] PKA and CAMK have also been implicated in the phosphorylation of phospholipids present in cardiac muscle.[134]

b. Changes in the sarcolemmal membrane in diabetes

The sarcolemmal membrane plays a crucial role in the regulation of membrane potential and thus excitation-contraction coupling. Ca^{2+} enters the cytoplasm through the voltage-gated Ca^{2+} channels in the SL membrane. Upon entry, a small amount of Ca^{2+} can act as trigger Ca^{2+} and allow the release of more Ca^{2+} from the SR through calcium-induced

calcium release.[135,136] Any alterations of the SL may therefore result in cardiac dysfunction in diabetic animals. A decrease in β-adrenergic receptor number rather than changes in the receptor affinity has been reported in the diabetic hearts.[137] The result would be a decrease in adrenergic stimulation of cardiac function in the diabetic heart. If the density of β-adrenergic receptors on the SL membrane is decreased in the diabetic animal, then it must follow that the ability of catecholamines to bind these receptors and initiate adenylate cyclase to produce cyclic AMP (cAMP) to further phosphorylate other protein kinases to release more Ca^{2+} for force generation will also be compromised. It is believed that diabetes itself is responsible for the depression in adrenergic receptor density; the severity of depression is dependent upon the duration of diabetes in animal.[138] Also in support of this hypothesis is the fact that insulin treatment was able to rectify this defect in the diabetic animal.[139] And lastly, the weight loss experienced by these animals was not a factor, because food restrictions had no bearing on the receptor density.[140] It has been suggested that the hypothyroid state of the diabetic may be a causal factor in the occurrence of adrenergic receptor depression.[139]

The Na^+-K^+ pump allows for the exchange of 3 Na^+ for 2 K^+ against their concentration gradients. The Na^+-K^+ pump of the cardiac sarcolemmal membrane plays a critical role in the regulation of membrane depolarization and repolarization. Ion homeostasis is essential for the maintenance of proper cardiac function. Schwartz et al[141] have indicated that if the Na^+-K^+ pump was inhibited the intracellular concentration of Na^+ will rise. This would activate the Na^+-Ca^{2+} exchanger, increase the intracellular Ca^{2+} and

thus result in the contractile dysfunction observed in diabetes. Experiments conducted on diabetic dog hearts by Onji and Liu,[142] provided the first data that the Na^+-K^+ ATPase enzyme system was significantly decreased in comparison to control values. Other studies have also confirmed this depression in Na^+-K^+ ATPase in the diabetic animals.[143-145] By using the purified cardiac SL from diabetic animals, Pierce and Dhalla[146] showed that the Na^+-K^+ ATPase activity and K^+-pNPPase activity, or the dephosphorylation of Na^+-K^+ ATPase were significantly decreased. The decrease in enzyme activity and subsequent Na^+-pump activity was reversed by the administration of insulin to diabetic animals.[147] Some investigators focused on the Na^+-Ca^{2+} exchanger as a possible mechanism to explain the contractile dysfunction observed in diabetics. This exchanger is of importance, because Ca^{2+} is directly involved in the contraction and relaxation of cardiac function and the Na^+-Ca^{2+} exchanger is involved in the influx and efflux of Ca^{2+}.[148,149] It was demonstrated that the Na^+-Ca^{2+} exchanger and Ca^{2+}-pump activities are decreased in the diabetic heart SL but were normalized with insulin administration.[142-145,150] The role of the Na^+-H^+ exchanger in the cardiac SL has also been examined as the mechanism for influx of Na^+ and efflux of H^+. If there is a depression in Na^+-H^+ exchange, as confirmed by Pierce et al,[145] then the diabetic heart will show a marked depression in recovery due to acidosis; the combined effect of sodium concentration and intracellular pH will clearly result in an altered myocardium.

c. Changes in the mitochondria in diabetes

The mitochondria is an important storage site for calcium. Although the SR is considered the primary source of calcium, the mitochondria is a secondary source of

Ca^{2+}-uptake when a surplus of Ca^{2+} is present in the cytoplasm under pathological conditions.[151] This defense mechanism of the mitochondria will act to prevent any cardiac contractile dysfunction that may occur.[151] It was found that the Ca^{2+}-uptake activity in the mitochondria of diabetic hearts was decreased in comparison to control animals, and administration of insulin reversed the decrease in activity observed in diabetics.[152,153] This may be indicative of cardiac dysfunction present in diabetes, especially since insulin treatment corrected the defect. Mitochondrial oxidative metabolism has been found to be depressed in the hearts of diabetic animals; this change was corrected with insulin treatment.[154,155] It should be noted that these studies were conducted on acute diabetic animals, and thus their relevance to the chronic diabetic animal is questionable. Studies on strepzotocin (STZ)-induced diabetic rats by Pierce and Dhalla[152] demonstrated decreased oxidative phosphorylation, Mg^{2+}-dependent ATPase, and respiratory control index activities. Insulin treatment reversed the decrease in activities observed in diabetic animals. Examination of other variables to find the solution to the decreased activity of the mitochondria in diabetes, uncovered the decrease in ATP content and ATP synthesis observed in diabetic animals.[152] ATP and phosphocreatine play a vital role in the energy production and utilization by cardiac muscle; a defect in ATP synthesis could manifest itself as a dysfunction in contractility and thus leading to the occurrence of heart dysfunction.[159] If insulin treatment was administered, then these alterations were reversible.

III. METHODS

1. Streptozotocin-diabetic rats

Male Sprague-Dawley rats weighing approximately 175-210 g were made diabetic with a single injection of streptozotocin (65 mg/kg) in citrate buffer (pH 4.5) into the tail vein.[51,146] After 12-24 hr a dose of 50% dextrose-saline solution was administered subcutaneously to reduce mortality. Three days after the streptozotocin injection, urine glucose levels were assessed using Keto-sticks (Bayer) to confirm the high levels of urine glucose in experimental animals. The control animals were injected with citrate buffer.

After 6 weeks both control and diabetic animals were sacrificed and the ventricular tissue and plasma were collected for further experiments. Serum glucose levels were assessed using instructions from Sigma kit. One group of 4 week diabetic animals were treated with insulin (3 U/day) for a period of 2 weeks to examine if the diabetes-induced changes were reversible with insulin treatment.

2. Hemodynamic studies

Using a procedure as described elsewhere,[156] experimental animals were anesthetized using sodium pentobarbital (50 mg/kg) administered i.p. Hemodynamic assessment was conducted using a microtip pressure transducer (model SPR-249, Miller Instruments, Houston, TX). Readings from a dynograph recorder (model R5HA, Beckman; Fullerton, CA) were taken for determining the rate of pressure development (+dP/dt) and rate of pressure decay (-dP/dt) in the left ventricle.

3. Isolation of SR vesicles

The SR vesicles were prepared as described by Osada et al[157] with slight modifications. Left ventricular tissue was minced and homogenized twice at 20 sec intervals using half-maximal setting on a Polytron homogenizer (Brinkman, Westbury, NY). The homogenization buffer contained (in mmol/L): 15 Tris-HCl, pH 6.8, 10 $NaHCO_3$, 5 NaN_3 and protease inhibitors (in μmol/L: 1 pepstatin, 100 phenylmethylsulfonylfluoride and 1 leupeptin). The homogenate was centrifuged for 20 min at 9,500 rpm and the supernatant thus obtained was centrifuged at 19,000 rpm for 45 min to obtain the pellet. A buffer containing 20 mmol/L Tris-HCl, pH 6.8, and 0.6 mol/L KCl was used to suspend the pellet, which was then centrifuged at 19,000 rpm for 45 min. A mixture of 10 mmol/L histidine, pH 7.0 and 0.25 mol/L sucrose was used to suspend the resultant pellet and this was the SR fraction. The entire procedure was performed at 0-4°C in the cold room. Various assays were conducted using the SR suspension. The protein content and the membrane purity were assessed by the measurement of the activity of marker enzymes such as cytochrome c oxidase, rotenone-insensitive NADPH cytochrome c reductase, glucose-6-phosphatase, and ouabain-sensitive Na^+-K^+ ATPase were determined by the methods used previously.[157]

4. Measurement of Ca^{2+}-uptake activity

The Ca^{2+}-uptake activity was determined by the method described by Hawkins et al.[158] 250 μL of standard reaction mixture was used, containing in mmol/L: 0.1 $^{45}CaCl_2$ (12,000 cpm/nmol), 5 NaN_3, 5 ATP, 5 K-oxalate, 5 $MgCl_2$, 120 KCl, 50 Tris-Maleate (pH 6.8) and 25 μmol/L ruthenium red. The initial Ca^{2+} measured by using Fabiato's[127] program was 8.2

μmol/L, Ca^{2+}-release was inhibited by the use of ruthenium red. SR membranes (6 μg) were added to the reaction mixture, the reaction was terminated after 1 min by filtering a 200 μL aliquot of the reaction mixture through 0.45 μm HAWP filters (Millipore, Mississauga, Canada). The filters were washed, dried at 60°C for 1 hr and the radioactivity was determined in the beta liquid scintillation counter.

5. Measurement of ryanodine sensitive Ca^{2+}-release

Ca^{2+}-release activity was measured as described by Temsah et al.[159] 625 μL of loading buffer was used to suspend the SR fraction (62 μg protein). The loading buffer consisted of (in mmol/L): 20 Tris-HCl (pH 6.8), 5 K-oxalate, 100 KCl, 5 $MgCl_2$ and 5 NaN_3. Incubation was conducted at room temperature for 45 min, with 5 mmol/L ATP and 10 μmol/L $^{45}CaCl_2$ (20 mCi/L). Ca^{2+}-induced Ca^{2+}-release was initiated by the addition of 1 mM $CaCl_2$ plus 1 mM EGTA to the reaction mixture. After 15 sec the reaction was terminated by filtering through 0.45 μm HAWP filters (Millipore, Mississauga, Canada). The filters were washed, dried and then the radioactivity was determined in a beta scintillation counter. Approximately 95 to 98% of Ca^{2+}-induced Ca^{2+}-release was prevented via 20 μM ryanodine treatment of SR preparations.

6. Western blot analysis

Using the method described by Osada et al,[157] the protein content of RyR, SERCA2a and PLB in SR preparations were examined in control, diabetic and insulin-treated diabetic rats. Immunoassay was conducted by subjecting SR (20 μg protein/lane) to sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 5, 10 and 15% gels for detecting RyR, SERCA2a and PLB, respectively. For RyR, proteins from the gels were transferred electrophoretically to nitrocellulose membrane, and for SERCA2a or PLB to a polyvinylidene difluoride membrane. These membranes were probed with anti-ryanodine receptor (1:1,400), anti-SERCA2a (1:1,400) and anti-phospholamban (1:2,000) antibodies. Peroxidase-linked anti-mouse IgG was used as a secondary antibody for SERCA2a and PLB (1:5,000). Anti-RyR and anti-PLB antibodies were purchased from Upstate Biotechnology (Lake Placid, NY) while anti-SERCA2a antibody was purchased from Affinity Bioreagents (Golden, CA). For RyR, biotinylated anti-mouse IgG (1:2,500) was used as the secondary antibody and then incubated with streptavidin-conjugated horseradish solution (1:5,000). Visualization of protein bands with antibodies was conducted using enhanced chemiluminescence detection system from Amersham (Amersham, UK). Intensity of each band was assessed using Imaging Densitometer with the aid of Molecular Analyst Software version 1.3 (Bio-Rad, Hercules, CA).

7. Measurement of phosphatase activity

The phosphatase activity of SR was measured as described by Upstate Biotechnology Ser/Thr phosphatase assay kit. The assay was based on the dephosphorylation of synthetic phosphopeptide (KRpTIRR). The resultant release of inorganic phosphate was measured at a wavelength of 660 nm. Assays were carried out in the presence and absence of exogenous substrate. Phosphatase activity was calculated by subtracting the value in the absence of exogenous substrate from that in the presence of exogenous substrate.

8. Measurement of endogenous CAMK activity

The CAMK activity of the SR was determined using Upstate Biotechnology (Lake Placid, NY) assay kits. This assay is based on the phosphorylation of specific substrate peptide (KKALRRQETVDAL) by transfer of γ-phosphate of adenosine-5'-[^{32}P] triphosphate [γ-^{32}P] ATP) by CAMK II. The phosphorylated substrate was separated from the residual [γ-^{32}P]ATP with P81 phosphocellulose paper and quantified using a scintillation counter. Assays were performed in the presence and absence of the substrate peptide. CAMK activity was determined by subtracting the value in the absence of exogenous substrate from that in the presence of exogenous substrate.

9. Measurement of SR PKA activity

The PKA activity in the SR fraction was determined using Upstate Biotechnology (Lake Placid, NY) assay kits. This assay is based on the phosphorylation of specific substrate (kemptide) using the transfer of the γ-phosphate of [γ-^{32}P] ATP) by PKA. The phosphorylated substrate was separated from the residual [γ-^{32}P] ATP with P81 phosphocellulose paper and quantified using a scintillation counter. Assays were performed in the presence and absence of kemptide. PKA activity was determined by subtracting the value in the absence of exogenous substrate from that in the presence of exogenous substrate.

10. Measurement of phosphorylation by endogenous CAMK and exogenous PKA

In order to prevent any dephosphorylation during isolation of the SR, phosphatase

inhibitors were incorporated. The homogenization buffer contained 1 mmol/L sodium pyrophosphate and 10 nmol/L microcystin-LR. SR protein phosphorylation by CaMK was determined using procedures as described by Netticadan et al.[160] Phosphorylation by endogenous CaMK was conducted using a total volume of 50 μL assay medium containing: 100 μmol/L EGTA, 0.8 mmol/L [γ-^{32}P] ATP (specific activity 200-300 cpm/pmol), SR (30 μg protein), 2 μmol/L calmodulin, 100 μmol/L $CaCl_2$, 50 mmol/L HEPES (pH 7.4) and 10 mmol/L $MgCl_2$. Initial free Ca^{2+} was 3.7 μmol/L, as determined by the program of Fabiato.[127] In order to monitor Ca^{2+}/calmodulin dependent phosphorylation, parallel assays were conducted in the absence of calmodulin and Ca^{2+} (1 mmol/L EGTA present). Phosphorylation by PKA was conducted in a 50 μL assay medium containing in mmol/L: 10 $MgCl_2$, 0.8 [γ-^{32}P] ATP (specific activity 200-300 cpm/pmol), 50 HEPES (pH 7.4), SR (30 μg protein) and PKA (catalytic subunit from bovine heart; 5.6 μg). Assays lacking PKA catalytic subunit were carried out, in order to monitor PKA-dependent phosphorylation. The assay medium was preincubated for 3 min at 37°C, then [γ-^{32}P] ATP was added to start phosphorylation reaction. After 2 min the reaction was terminated by the addition of 15 μL of SDS-sample buffer. SDS-PAGE was then performed using 4-18% gradient slab gels. The gels were stained with Coomassie Brilliant Blue, dried and autoradiographed. Using an Imaging Densitometer and Molecular Analyst software version 1.3 (Bio-Rad, Hercules, CA), the intensity of each phosphorylated band was analyzed.

11. Statistical analysis

Data was evaluated according to the ANOVA test followed by Students' t-test, and expressed as mean ± standard error. Statistical significance was assessed at $P < 0.05$ between control, diabetic and insulin treated groups.

IV. RESULTS

1. General characteristics of control and experimental animals

As shown in Table 1, the body weight and heart weight of the diabetic rats were significantly depressed in comparison to control values whereas the heart weight/body weight ratio was significantly increased in comparison to control values. Insulin treatment of diabetic animals resulted in partial recovery of these parameters towards the control values. Assessment of plasma glucose and insulin revealed a significant increase in glucose and a significant decrease in insulin levels in the diabetic animals; these changes were reversed by insulin treatment. Hemodynamic assessment of the diabetic animals revealed depressions in the left ventricular developed pressure (LVDP), rate of pressure development (+dP/dt) and rate of pressure decay (-dP/dt) in diabetic animals in comparison to control values. When diabetic animals were given insulin, cardiac function recovered towards the control values.

2. SR Ca^{2+}-uptake and -release activities

The oxalate-supported SR Ca^{2+}-uptake in the diabetic heart was significantly depressed in comparison to control values (Fig. 1A). An approximate 42% reduction in Ca^{2+}-uptake was observed in the diabetic animals in comparison to control. Insulin treatment (3 U/day) of the diabetic animals for two weeks produced a partial recovery of SR function in Ca^{2+}-uptake. Measurement of ryanodine sensitive Ca^{2+}-release (Figure 1B) in diabetic animals also showed a marked depression in the activity in comparison to control animals.

TABLE 1: General Characteristics and Ventricular Function of Control and Experimental Animals

	Control	Diabetic	Diabetic + Insulin
Body weight (BW; g)	515 ± 10.5	348 ± 11.3*	425 ± 15#
Heart weight (HW; g)	1.20 ± 0.05	1.02 ± 0.03*	1.08 ± 0.09#
HW/BW ratio (mg/g)	2.2 ± 0.05	2.9 ± 0.03*	2.5 ± 0.06#
Plasma glucose (g/dL)	1.5 ± 0.13	3.2 ± 0.24*	2.0 ± 0.12#
Plasma insulin (mU/dL)	3.0 ± 0.28	0.8 ± 0.09*	3.4 ± 0.15#
LVDP (mm Hg)	82 ± 3.4	64 ± 2.9*	75 ± 2.6#
+dP/dt (mm Hg/sec)	5,515 ± 223	3,498 ± 198*	4,871 ± 240#
-dP/dt (mm Hg/sec)	5,348 ± 143	3,188 ± 231*	4,636 ± 151#

*$P < 0.05$ in comparison to control values. Each value is the mean ± SE of 6 animals in each group. #$P < 0.05$ in comparison to diabetic values.

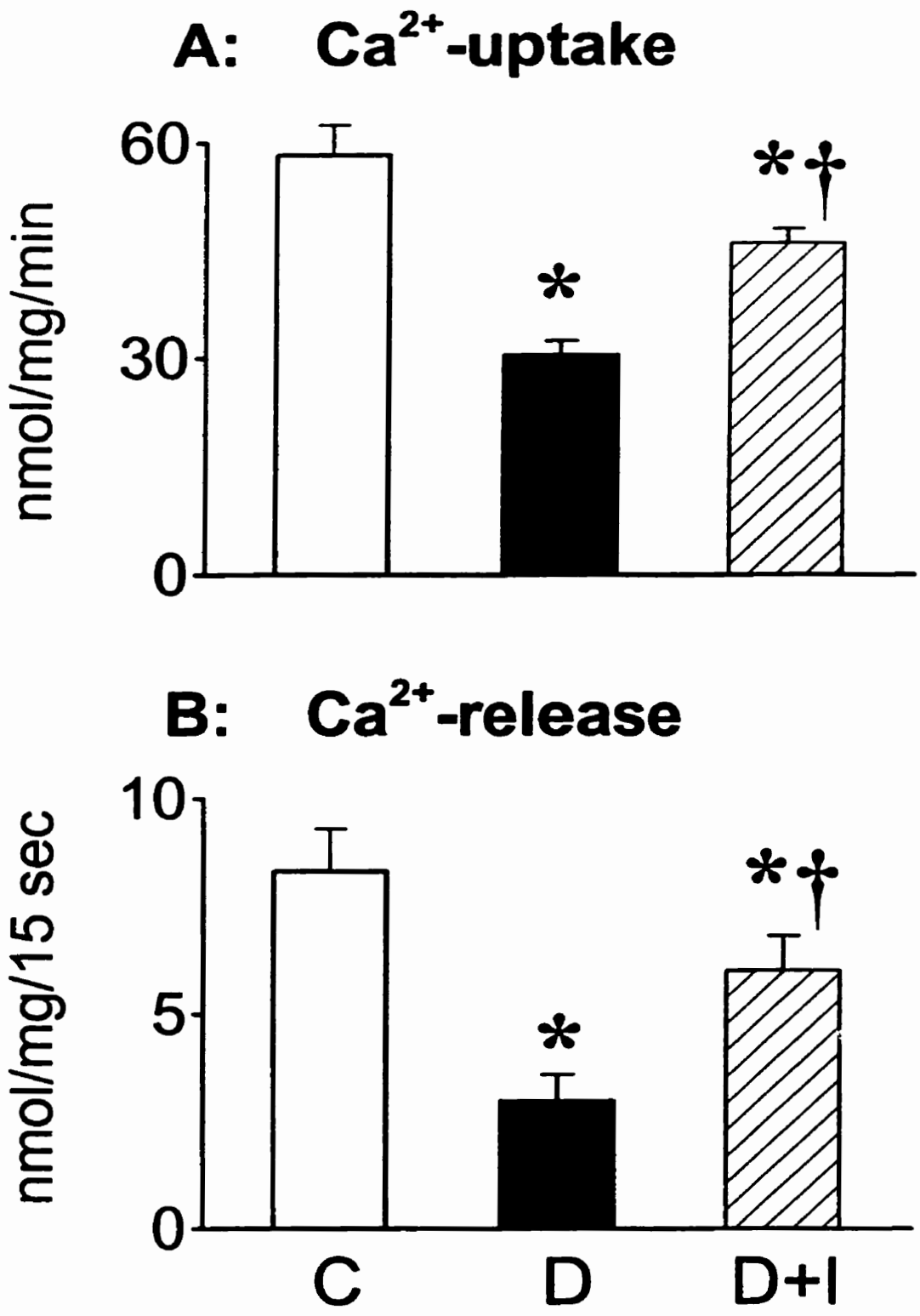

Figure 1: **Oxalate supported Ca^{2+}-uptake of the SR vesicles (A) and ryanodine-sensitive Ca^{2+}-release by the SR vesicles (B), in control (C), diabetic (D) and insulin treated diabetic (D + I) groups. Each value is a mean ± SE of 6 preparations in each group. *P < 0.05 in comparison with control; †P < 0.05 in comparison to diabetic group.**

Administration of insulin to diabetic animals for two weeks reversed this defect in SR function partially.

3. Analysis of SR protein content

In order to assess the mechanism of the depression in Ca^{2+}-transport by the SR in the diabetic heart, protein content of SERCA2a, RyR and PLB were examined by the Western blot analysis. The data (Figure 2) indicate that for SERCA2a there was a significant depression in SR content of this protein in diabetic animals. Upon administration of insulin (3 U/day) for two weeks to the diabetic animals, the protein content of SERCA2a showed a significant improvement in comparison to diabetics but was still significantly lower than control value. The data for the protein content of RyR in Figure 2 indicate a significant depression in comparison to control animals; insulin treatment of diabetic animals significantly improved the RyR protein content. Finally, the analysis of PLB revealed that there is a significant reduction in the protein content in diabetic animals in comparison to control animals. Insulin treatment for 2 weeks in diabetic animals significantly reversed this depression in PLB protein content.

4. Ca^{2+}-calmodulin dependent protein kinase phosphorylation of SR proteins

Examination of Ca^{2+}-calmodulin dependent protein kinase phosphorylation of RyR, SERCA2a, and PLB was conducted to see if any alterations in protein phosphorylation in the diabetic heart would account for the depressed uptake and release of Ca^{2+} from the cardiac SR. Analysis of the data (Figure 3) demonstrate that the phosphorylation of RyR by CAMK

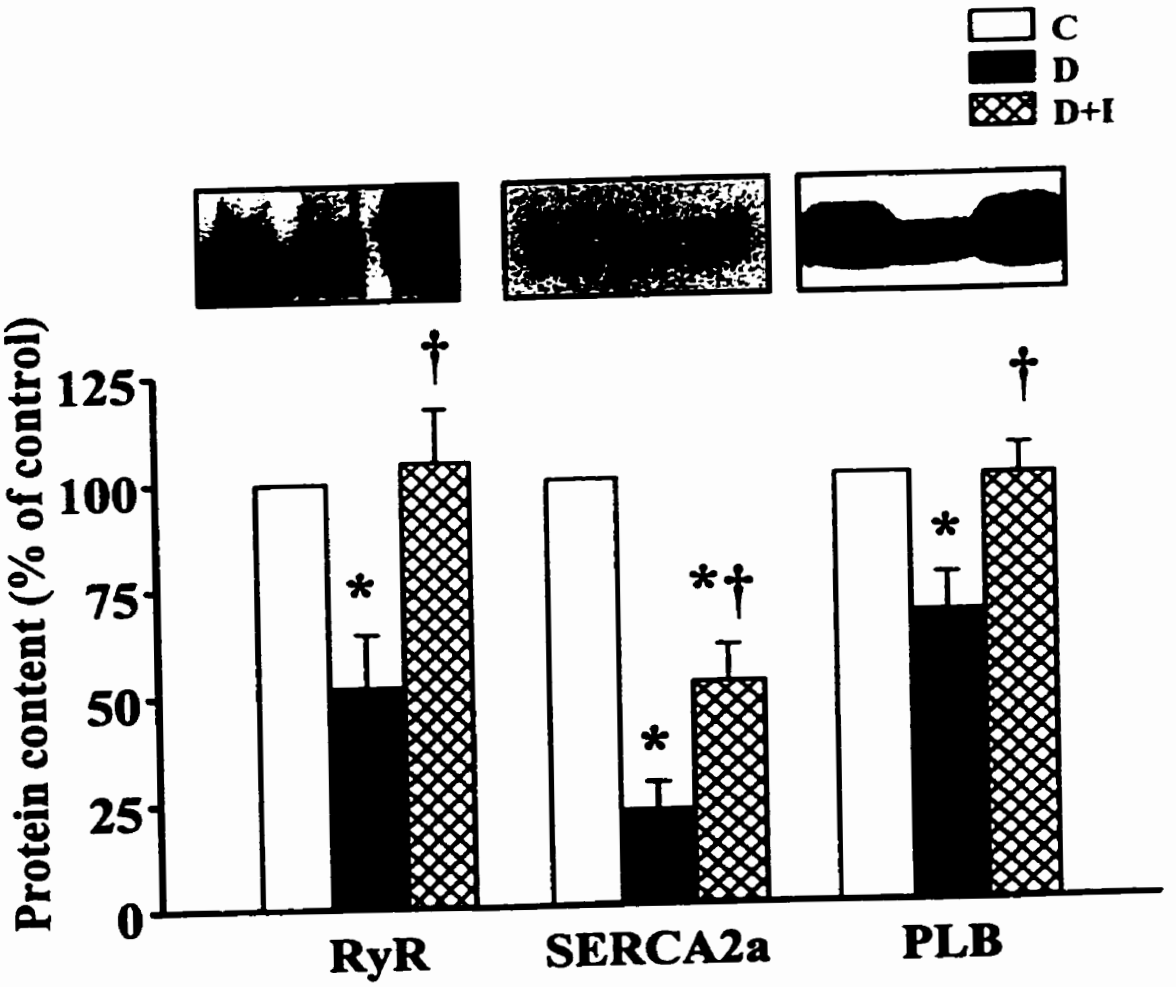

Figure 2: **Western blot analysis of cardiac SR proteins RyR, SERCA2a and PLB in control (C), diabetic (D) and insulin treated diabetic (D + I) groups. Each value is a mean ± SE of 6 experiments in each group. *P < 0.05 in comparison to control; †P < 0.05 in comparison to diabetic group.**

was significantly increased in diabetic animals in comparison to control. Furthermore, upon treatment of diabetic animals with insulin, a further increase in phosphorylation of RyR by CaMK was observed. The quantification of bands in the autoradiogram (Figure 3) also show an increased phosphorylation of the CAMK phosphorylation of SERCA2a and PLB in diabetic animals and an even further increase in diabetic-treated groups in comparison to control values was apparent. The identity of the phosphoproteins have been established earlier by immunoblotting as well as immunoprecipitation techniques.[100,157-160]

5. CAMK activity

The data for endogenous CAMK activity in Figure 4 show that the CAMK activity in cardiac SR was significantly increased in comparison to control animals. This increase in the SR CaMK activity in the diabetic heart was not affected upon treatment with insulin.

6. Phosphorylation of SR protein PLB

The data in Figure 5 indicate that PKA dependent phosphorylation of PLB in cardiac SR was significantly increased in diabetic animals in comparison to control. Moreover, in the insulin-treated diabetic group there was a further increase in PKA-dependent phosphorylation of PLB in comparison to the diabetic and control groups.

7. PKA activity

The examination of PKA activity in the SR fraction (Figure 6) indicates the presence of increased PKA activity in diabetic and insulin-treated diabetic animals in comparison to control animals.

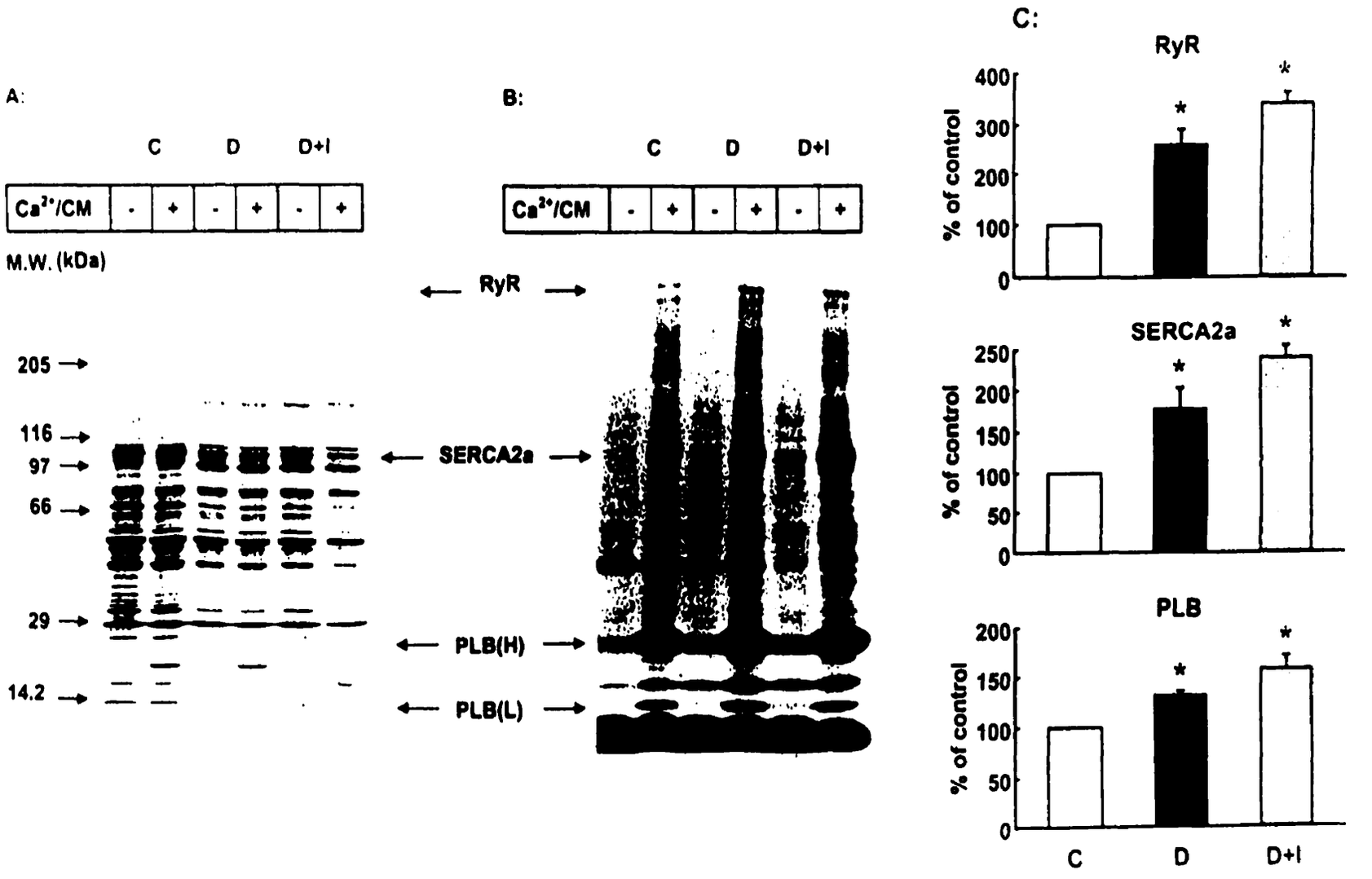

Figure 3: SR CAMK phosphorylation of SR proteins in control (C), diabetic (D) and insulin treated diabetics (D+I) groups. Panel A depicts SR protein bands resolved by SDS-PAGE. Panel B is the corresponding autoradiogram of phosphorylated SR proteins. Panel C is the analysis of RyR, SERCA2a and PLB. PLB phosphorylation is the sum of the high molecular weight PLB (H) plus the low molecular weight PLB (L). Phosphorylation data are mean ± SE from 4 preparations in each group. *P < 0.05 in comparison to control group.

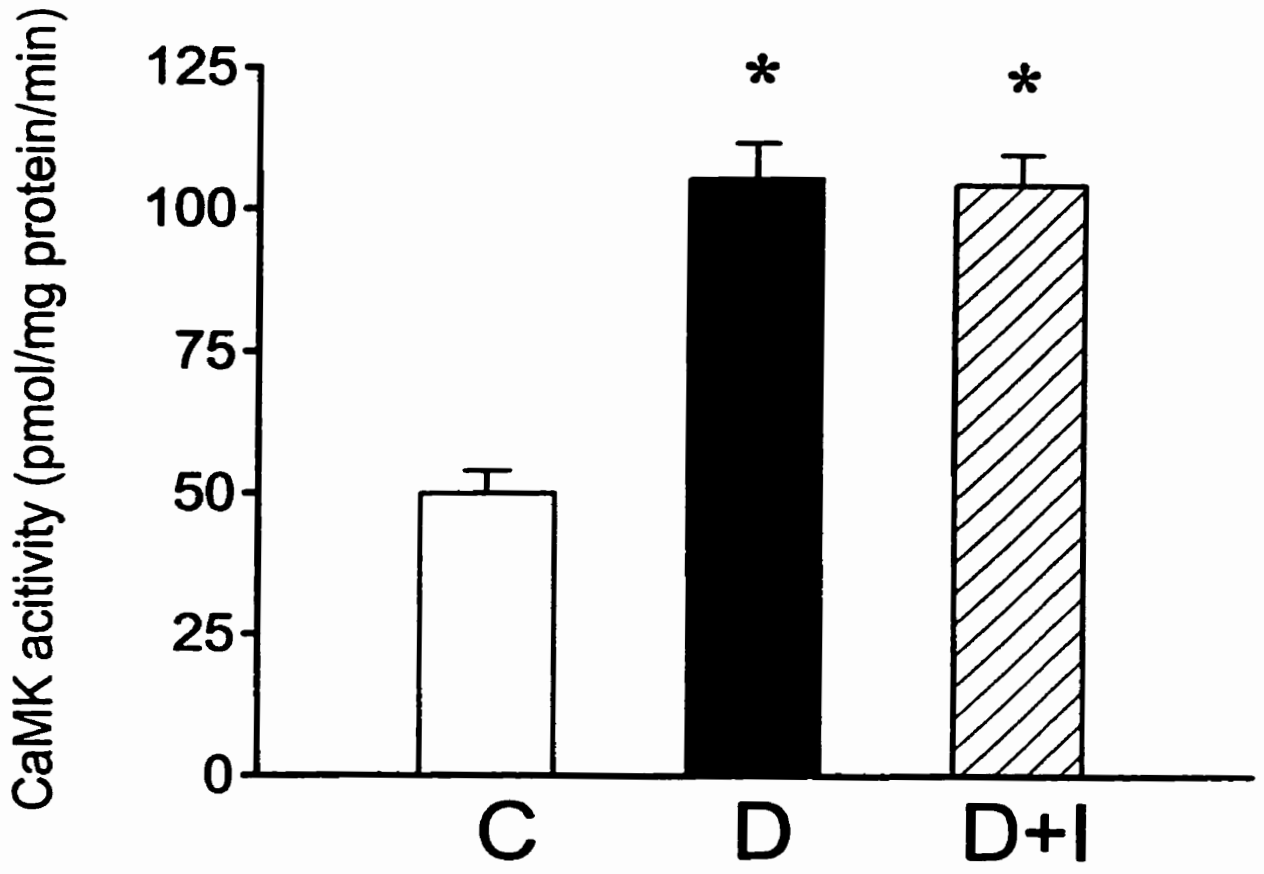

Figure 4: **SR CAMK activity in control (C), diabetic (D) and insulin treated diabetic (D+I) groups. Activities were assayed using Upstate Biotechnology Kits. Each value is a mean ± SE of 4 preparations in each group. *P < 0.05 in comparison to control group.**

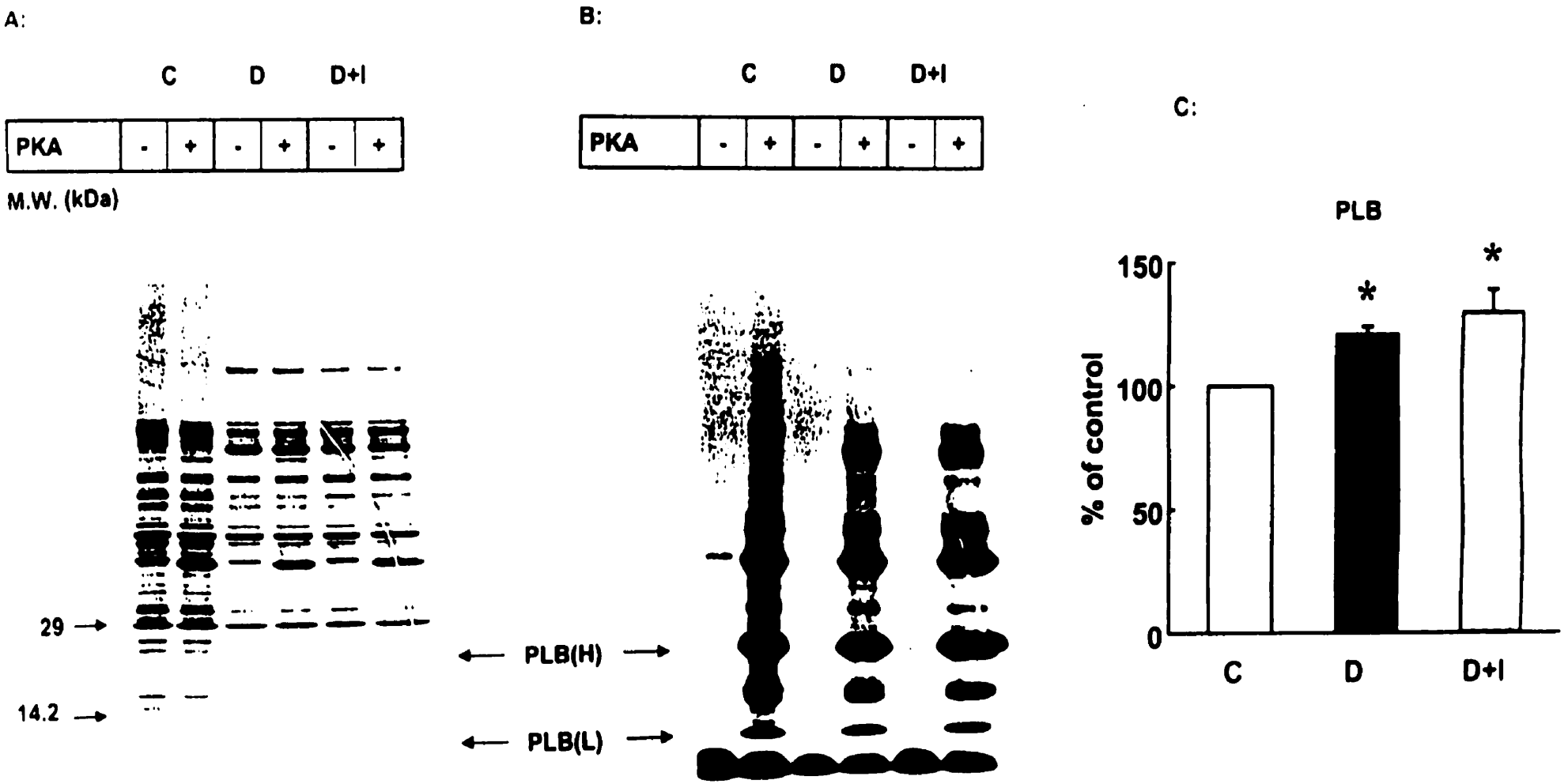

Figure 5: PKA phosphorylation of SR proteins in control (C), diabetic (D) and insulin treated diabetic (D+I) groups. Panel A depicts SR proteins resolved by SDS-PAGE. Panel B is the corresponding autoradiogram of phosphorylated SR proteins. Panel C is the analysis of PLB. PLB phosphorylation is the sum of high molecular weight PLB (H) plus the low molecular weight PLB (L). The phosphorylation data are mean ± SE from 4 preparations in each group. *$P < 0.05$ in comparison to control group.

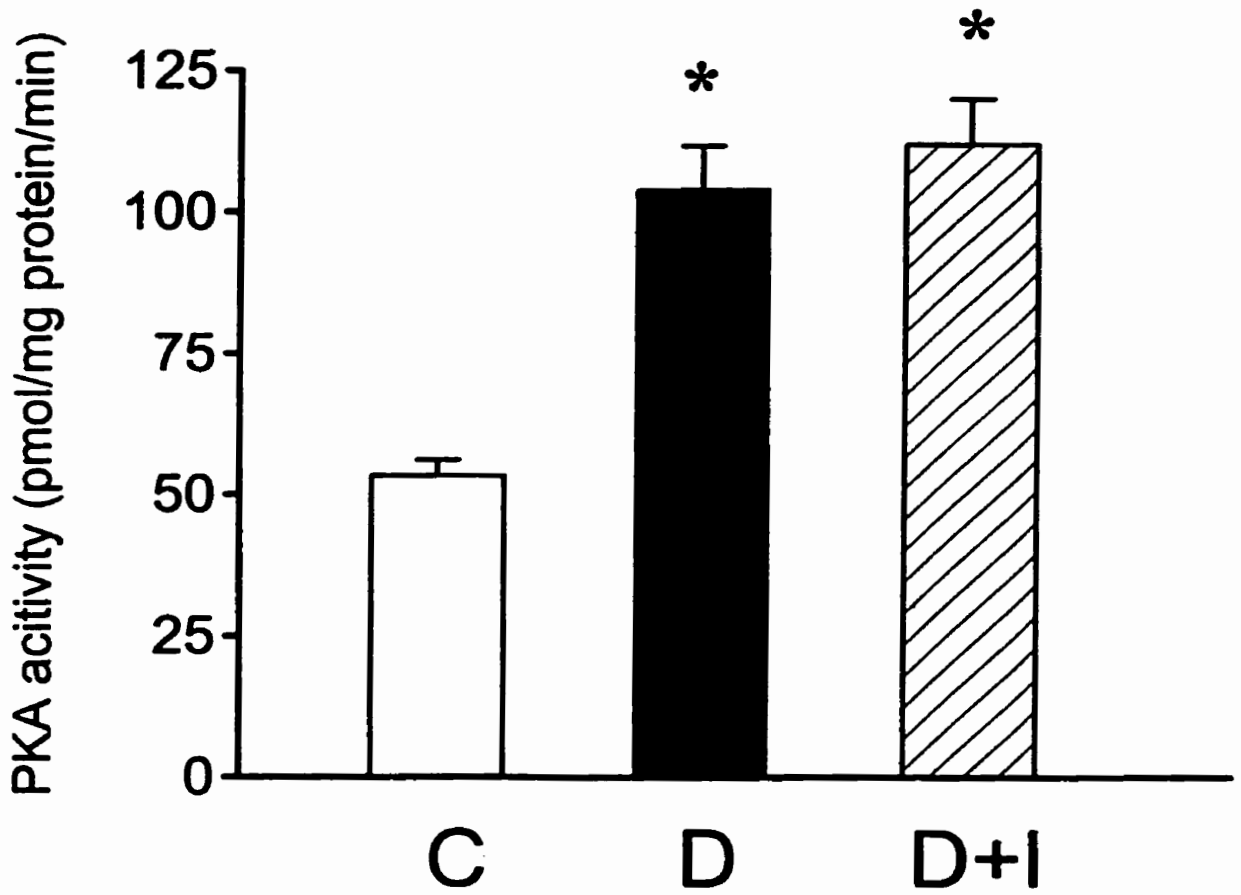

Figure 6: **SR PKA activity in control (C), diabetic (D) and insulin treated diabetic (D+I) hearts. Activities were assayed using Upstate Biotechnology Kits. Each value is a mean ± SE of 4 experiments in each group. *P < 0.05 in comparison to control.**

8. SR phosphatase activity

In order to ensure that the observed increase in PKA and CaMK phosphorylation activities were not due to any changes in the SR phosphatase activity we also examined this parameter. However, as depicted in Figure 7, the phosphatase activity in diabetic and insulin-treated animals was significantly increased in comparison to control animals.

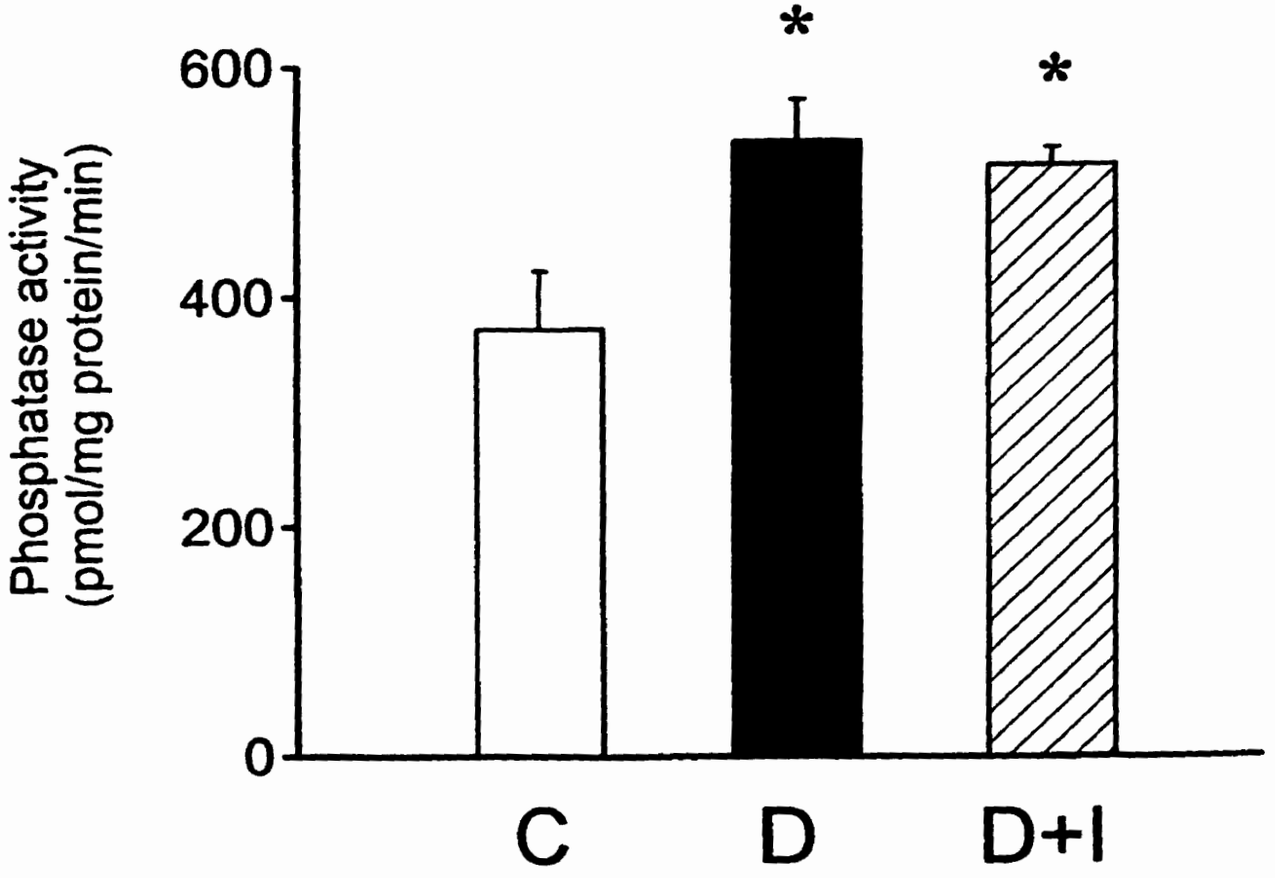

Figure 7: **SR phosphatase activity in control (C), diabetic (D) and insulin treated diabetic (D+I) hearts. Phosphatase activities were assayed using Upstate Biotechnology Kits. Each value is a mean ± SE of 4 preparations in each group. *P < 0.05 in comparison to control.**

V. DISCUSSION

The data obtained for the general characteristics of control and experimental animals are consistent with the results of other investigators.[151,161-164] The decrease in body weight observed in the diabetic rat may indicate an alteration in metabolism due to the diabetic state. It has been suggested that normal carbohydrate, lipid and protein metabolism are disrupted during diabetes mellitus; this metabolic dysfunction could be correlated to the decrease in energy production and thereby explain the depressed cardiac contractile performance.[165-172] We have shown that in the diabetic heart the rate of contraction and relaxation were significantly depressed in comparison to control values. This is in agreement with observations in other studies.[161-164] The reduction in body weight in diabetic animals may be due to a disruption in protein metabolism. It has been demonstrated that diabetic animals exhibit an increased rate of protein excretion.[169] Other investigators have shown an approximate 31-44% reduction in protein synthesis in cardiac muscle and a dramatic 70% reduction from skeletal muscle of diabetic animals.[170,171] Some investigators have focused on the role of insulin in stimulating protein synthesis as a possible mechanism for the observed defect, since diabetic animals were hypoinsulinemic.[172] Since insulin treatment of diabetic animals resulted in partial recovery of protein synthesis, other mechanisms may play a role in protein synthesis dysfunction.[170] It should also be noted that the increased content of circulating lipids in diabetic animals have a detrimental effect on carbohydrate metabolism in the heart, and also alter the synthesis of high energy phosphate compounds responsible for contractile force generation.[167,168] Thus, the increased lipid concentration may also account

for the depressed body weight and contractile function in diabetic animals.

1. SR Ca^{2+}-uptake and -release activities

We have observed a marked reduction in SR Ca^{2+}-uptake and -release activities in diabetic heart in comparison to control animals. It has been suggested that defects in Ca^{2+}-uptake are the cause of dysfunction in cardiac relaxation;[51,83,173] this correlates with the depressed relaxation rate (-dP/dt) in diabetic animals. Upon insulin administration to diabetic animals, a recovery of cardiac function and SR Ca^{2+}-uptake function was observed similar to other investigators.[84] SR Ca^{2+}-release was also found to be depressed in diabetic animals. Since SR Ca^{2+}-release is related to the rate of cardiac contraction (+dP/dt), our data correlates well with the depression in cardiac contraction observed in diabetic animals. Insulin treatment of diabetic animals was also shown to reverse the depression in SR Ca^{2+}-release to approximately normal SR function. Our results have shown a decrease in SERCA2a, RyR and PLB protein content in diabetic animals. This is consistent with the observed depression in SR Ca^{2+}-uptake and release activities in diabetic animals and suggests that these reductions may critically impair the Ca^{2+}-uptake and -release mechanisms in the diabetic heart. Since insulin treatment of diabetic animals demonstrated a recovery of SR protein content of SERCA2a, RyR and PLB, it is possible that insulin may render its cardioprotective effect by improving the SR function in the diabetic heart.

2. Phosphorylation of SR proteins

We examined the CAMK phosphorylation of RyR, SERCA2a and PLB to investigate the possibility that a defect in the phosphorylation of these regulatory proteins by CAMK in the diabetic animal may be the mechanism for cardiac dysfunction. Analysis of the data revealed that in diabetic animals phosphorylation of RyR, SERCA2a and PLB by Ca^{2+}-dependent CAMK phosphorylation was increased in comparison to control animals. What surprised us was the fact that insulin treatment of diabetic animals did not reverse this trend to control values, but demonstrated a further increase in CAMK phosphorylation in comparison to diabetic animals. Examination of CAMK activity also showed the parallel increase in activity in diabetic animals and in insulin-treated diabetic animals. The increase in CAMK phosphorylation was consistent with enhanced SR CAMK activities in the diabetic and insulin treated hearts. These results suggest that the upregulation of phosphorylation in the diabetic heart may be in response to decreased SR function. This upregulation may however not be effective in correcting the SR function because of the depression of the RyR, SERCA2a and PLB protein content. Insulin treatment of diabetic animals resulted in a further increase in CAMK phosphorylation in comparison to diabetic animals suggesting a compensatory role for insulin by upregulating phosphorylation in the diabetic heart.

In conducting further experiments to examine the effect of PKA dependent phosphorylation on PLB, we found that PKA phosphorylation of PLB was increased in the diabetic animal in comparison to control. This increase related well with enhanced SR PKA activity in the diabetic heart. Furthermore, we found that there was a further increase in PKA-dependent phosphorylation of PLB and PKA activity in insulin-treated diabetic animals

in comparison to diabetic animals. It thus appears that the increased PKA phosphorylation of PLB may also be a compensatory mechanism for the loss in the Ca^{2+}-uptake function and insulin may enhance this compensatory mechanism in the diabetic heart. Elevated phosphorylation of SR proteins by CAMK and PKA suggested a depression in phosphatase activity. In order to confirm this possible defect, we examined the activity of phosphatase and found to our surprise that in diabetic and insulin-treated animals there was a parallel increase in phosphatase activity in comparison to the control. It thus appears that increased phosphorylation is not a result of decreased dephosphorylation and the delicate balance of SR phosphorylation and dephosphorylation is unaffected in the diabetic heart.

VI. CONCLUSIONS

From the data that we have collected in this study, the findings are summarized in the following statements:

- The SR function with respect to Ca^{2+}-uptake and -release activities was depressed in the diabetic heart; insulin treatment partially reversed these defects.

- The SR protein contents of RyR, SERCA2a and PLB were depressed in the diabetic hearts and showed recovery upon insulin treatment towards the control values.

- CAMK and PKA phosphorylation of SR proteins were enhanced in the diabetic heart and these increases were not significantly affected by insulin treatment.

- The abnormalities in SR function in the diabetic heart may in part be responsible for alterations in cardiac function in the diabetic animals whereas increased phosphorylation of SR proteins may be a compensatory mechanism.

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