Calcium-Handling Proteins in Diabetic Cardiomyopathy

Vijayan Elimban, Adriana Frota P. Pinto, and Naranjan S. Dhalla

Abstract Diabetes mellitus increases the risk of cardiomyopathy independently of underlying comorbidities, and heart failure is a major cause of death in diabetic patients. The development of this distinct cardiomyopathy in both type 1 and type 2 diabetes is associated with complex and multifactorial cellular and molecular perturbations. It is widely recognized that cardiac dysfunction in chronic diabetes involves hormonal imbalance, oxidative stress, proteases activation, defects in Ca^{2+} cycling, and varying degrees of subcellular remodeling of organelles.

Ca^{2+} -handling abnormalities in diabetic cardiomyocytes have primarily been attributed to changes in the sarcolemmal Na^{+}–Ca^{2+} exchanger, L-type Ca^{2+} channel, Na^{+}–K^{+} ATPase, and Na^{+}–H^{+} exchanger proteins as well as Ca^{2+}-release channels and Ca^{2+}-pump proteins embedded in the sarcoplasmic reticulum. Intracellular Ca^{2+} overload has been implicated in the impairment of excitation–contraction coupling as a result of alterations in Ca^{2+}-entry, Ca^{2+}-removal, Ca^{2+}-uptake, and Ca^{2+}-release processes in the diabetic heart. These observations are consistent with the view that defects in Ca^{2+}-handling proteins play a critical role in the pathogenesis of cardiac dysfunction during the development of diabetic cardiomyopathy.

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1 Introduction

Cardiovascular disease is the leading cause of death in the diabetic population. Although diabetic cardiomyopathy is associated with several comorbidities including atherosclerosis, hypertension, coronary artery disease, and valvular malfunction, it has been demonstrated that chronic diabetes impairs ventricular function independently of other risk factors [1, 2]. This distinct diabetic cardiomyopathy is characterized by reduced diastolic compliance and rate of myocardial relaxation as well as a decrease in absolute force development [3, 4]. The exact underlying pathological mechanisms are not clear; however, several studies have suggested that cardiac dysfunction in chronic diabetes is intimately associated with varying degrees of defects in subcellular organelles such as sarcolemma (SL), sarcoplasmic reticulum (SR), mitochondria (MT), myofibrils (MF), and extracellular matrix (ECM) [3, 5, 6]. Remodeling of these components in the diabetic heart primarily occurs in response to hormonal imbalance, oxidative stress, activation of different proteases, changes in gene expression, and metabolic shift caused by increased levels of cholesterol and fatty acids. It is worthwhile to note that remodeling of SL and SR along with altered calcium metabolism has been shown to be an early sign in the process for the development of diabetic cardiomyopathy [7–9].

It is well known that intracellular Ca^{2+} is a major regulator of excitation–contraction coupling, and multiple aspects of calcium handling are considered to underlie the subcellular mechanisms responsible for the impaired cardiac contraction and relaxation in diabetic cardiomyopathy [6]. Indeed, several studies have reported the occurrence of intracellular Ca^{2+} overload in diabetic cardiomyocytes [3, 7, 10]. This alteration have been mostly attributed to the SL and SR remodeling, leading to depressed SL Na^+–Ca^{2+} exchanger activity, decreased SR Ca^{2+}-pump ATPase (SERCA2a) activity, reduced SR Ca^{2+} load, and Ca^{2+}-release channel (ryanodine receptor) dysfunction [11–13]. It is pointed out that the inward Ca^{2+} current is the critical initiator of the contractile and relaxation cycle in the heart. Cardiac depolarization opens L-type Ca^{2+} channels in the SL membrane and allows the entry of Ca^{2+} into cardiomyocytes. This transient increase in cytoplasmic Ca^{2+} concentration triggers Ca^{2+} release from SR, mainly through the Ca^{2+}-release channel or ryanodine receptor2 (RyR2) and by inositol triphosphate receptors (InsP3R) to a lesser extent. This event, described as calcium-induced calcium release (CICR), is crucial for excitation–contraction coupling in cardiac muscle [14, 15]. Following the opening of a RyR2 cluster on the SR, Ca^{2+} sparks are generated; this local, rapid, and brief elevation in $[Ca^{2+}]_i$ elevates cytosolic-free Ca^{2+} by tenfold or more and initiates contraction. The relaxation of cardiac muscle occurs upon lowering the concentration

of free Ca^{2+} by intracellular SR uptake via SERCA2a as well as SL efflux via the Na^{+}–Ca^{2+} exchanger and the SL Ca^{2+} pump in the SL membrane [6, 14]. Although the MT and nucleus are also known to accumulate a significant amount of Ca^{2+} in cardiomyocytes, their role in the regulation of cytoplasmic concentration of free Ca^{2+} during the contraction and relaxation processes is not well established [6, 14]. This chapter is therefore focused on discussion regarding the status Ca^{2+}-handling proteins in SL and SR during the development of diabetic cardiomyopathy.

2 SL Defects in Diabetic Heart

Alterations in SL L-type Ca^{2+} channels, Na^{+}–Ca^{2+} exchanger, Na^{+}–K^{+} ATPase, and Na^{+}–H^{+} exchanger proteins, which are involved in Ca^{2+} handling directly or indirectly, have been shown to occur in diabetic cardiomyopathy [2, 6]. L-type Ca^{2+} channels are voltage-gated channels mostly located in the transverse tubules in proximity with RyR in SR, thereby suggesting the existence of a physical coupling between both Ca^{2+}-entry and Ca^{2+}-release channels [16]. SL Ca^{2+} channels in cardiomyocytes are modulated by several pathways including calmodulin (CaM), β-adrenergic receptors, phosphatidylinositol-3-kinase (PI3K), protein kinase A (PKA), and protein kinase C (PKC) [17, 18]. Although most of the calcium for cardiac contraction is provided by the SR, the activity of L-type Ca^{2+} channels is of critical importance for heart function. For instance, genetic mutation of SL Ca^{2+} channels leading to their impaired function has been linked with short QT syndrome, arrhythmia, and sudden death [19]. One of the early alterations detected in diabetic hearts was the prolongation of the ventricular action potential, which was attributed mainly to depressed transient outward K^{+} current and to L-type Ca^{2+} current [3, 20]. Experimental investigations in diabetic animals have revealed an unaltered [21, 22] or decreased [23–26] SL Ca^{2+}-channel density. These disparities in results seems to reflect differences in models used, especially regarding the progression of disease, because alteration of the Ca^{2+} current has been shown to occur only in later stages of diabetes. The reduced Ca^{2+}-channel density has been attributed to decreased levels of protein content [23, 25], depressed cell-surface expression [24–26], and changes in the phosphorylation status [23]. Lu et al. [24], after a series of investigations using type 1 (*Ins2*^{Akita} rats) and type 2 (*db/db* rats) [23] diabetes models, have reported decreased Ca^{2+}-current density in both groups of diabetic animals, although the reduction was more intense in *db/db* than in *Ins2*^{Akita} myocytes as compared to nondiabetic cells. Because reduced phosphorylation status of the L-type Ca^{2+} channel was observed, it was hypothesized that Ca^{2+}-current alteration could be related to a lack of insulin in type 1 diabetes and downregulation of the Akt pathway.

Following intracellular infusion of phosphatidylinositol-3,4,5-trisphosphate (PIP3), a second messenger produced by PI3K, and consequently because of stimulation of the Akt pathway, depression in Ca^{2+}-current density was fully restored in *Ins2*^{Akita} myocytes in contrast with the partial restoration seen in *db/db* myocytes. The reduced levels of SL Ca^{2+}-channel protein in the *db/db* cardiomyocytes were not seen

in *Ins2*^{Akita} cardiomyocytes, thereby leading to the hypothesis that hyperglycemia in combination with obesity and insulin resistance in type 2 diabetes could cause more damage to SL Ca^{2+}-channel function than hyperglycemia and lack of insulin in type 1 diabetes [23, 24]. It is important to highlight that another study has revealed that the activation of the PI3K-dependent Akt signaling pathway by insulin-like growth factor 1 restored L-type Ca^{2+} channels function in type 1 diabetic animals [27]. Taken together, these data suggest that insulin may have a positive inotropic effect and could explain how insulin resistance can affect heart function in several pathological states [3]. Despite the fact that most of the investigations support the idea that L-type Ca^{2+}-channel activity is not impaired in cardiac hypertrophy, Ca^{2+} transients trigged by Ca^{2+}-channel current have shown to be desynchronized, presenting a decreased amplitude and slow kinetics. These findings support the view that the intermolecular failure state would also apply to SL Ca^{2+} channels, the SR Ca^{2+}-release channels, considering that the Ca^{2+} current becomes less effective in triggering SR Ca^{2+} release in the diabetic heart [16, 28–30].

It has become clear that Na^+–Ca^{2+} exchanger 1 (NCX1) is the major SL protein for extruding Ca^{2+} that enters the cardiac cell via SL Ca^{2+} channels [31, 32]. This exchanger promotes the influx of 3 Na^+ for the extrusion of each Ca^{2+}, and its activity is controlled by both internal and external Na^+ and Ca^{2+} levels as well as by the membrane potential. Under certain pathological conditions, NCX1 also works in the reverse mode, contributing to the development of intracellular Ca^{2+} overload in cardiomyocytes. It is noteworthy that the direction and amplitude of NCX1 current relies on the activity of SL Na^+–K^+-ATPase, which is responsible for maintaining the intracellular Na^+ concentration at a low level [31, 32]. In type 1 diabetes, both depressed NCX activity [10, 33] and expression [10, 34, 35] were observed in the heart. It has been suggested that NCX1 dysfunction is related to alterations in the phospholipid composition of SL and reduced stimulation of the transporter by protein kinase C [36]. Furthermore, marked depression in SL Na^+–K^+ ATPase activity in insulin-dependent diabetes animals is considered to stimulate the NCX activity in a reverse mode to normalize the cytosolic Na^+ concentration [37–39]. Depressed SL activity of Ca^{2+}-pump ATPase was also reported in the diabetic heart [40, 41]. Consequently, a net gain of Ca^{2+} would occur as a result of the impaired efflux and increased Ca^{2+} entry, leading to intracellular Ca^{2+} overload as well as mechanical and electrical dysfunction in diabetic cardiomyocytes. On the other hand, in some studies involving type 2 diabetes, the NCX1 activity was either increased [25] or unchanged [42, 43], and no difference in mRNA level or protein content was detected [36, 42, 43]. Thus, the role of NCX in the etiology of cardiomyocyte dysfunction is complex, and changes in its expression or activity are viewed as compensatory or causal, depending upon the stage and severity of diabetes.

SL Na^+–K^+ ATPase plays a key role in maintenance of the resting membrane potential in cardiac cells by removing intracellular Na^+ in exchange for extracellular K^+. It has been demonstrated that Na^+–K^+ ATPase dysfunction in diabetic cardiomyopathy is related to downregulation of its subunit expression as well as alteration in the enzyme kinetics [37, 39]. The activity of this enzyme may also be influenced by alterations in composition of SL membrane observed in diabetes [44]. The abnormality in Na^+–K^+

ATPase activity in the diabetic heart results in cytosolic Ca^{2+} overload involving the NCX exchanger. It is important to emphasize that treatment of diabetic animals with insulin upregulates the expression of Na^+–K^+ ATPase and improves cardiac function [45]. Moreover, antioxidant agents, including vitamin E [46] and fish oil containing n-3 fatty acids [47], were able to attenuate and even prevent the diabetic-induced changes in SL Na^+–K^+ ATPase and cardiac dysfunction. These observations suggest the role of the observed depression in Na^+–K^+ in Ca^{2+}-handling abnormalities in cardiomyocytes during the development of diabetic cardiomyopathy.

Another integral SL protein, Na^+–H^+ exchanger (NHE), is involved in intracellular Ca^{2+} modulation. NHE-1, which isoform is mostly expressed in cardiac cells, regulates intracellular pH by exchanging one intracellular H^+ ion for an extracellular Na^+ ion. In addition, NHE-1 participates in the regulation of Na^+ fluxes and cell volume. Although emerging evidence supports NHE-1 involvement in diabetic cardiomyopathy, the results are controversial, and its potential role has not been established [48]. The NHE-1 activity has been shown to be decreased in isolated cardiomyocytes as well as the SL membranes of the diabetic heart [49, 50]. In another study, the reduced activity of NHE-1 in diabetes has been considered responsible for resistance of diabetic hearts to ischemia–reperfusion injury [51]. An increase in NHE-1 activity in cardiomyocytes of the Goto-Kakizaki rat model of type 2 diabetes has also been detected [52]. It has been suggested that intracellular acidification in cardiac cells stimulates the Akt signaling pathway, which could represent a likely mechanism that mediates the myocardial hypertrophy observed in the diabetic animals. In addition, chronic treatment with cariporide, a NHE-1-selective inhibitor, has been shown to prevent the phenotype of hypertrophy [52]. It is worth noting that some studies have also indicated that chronic administration of NHE-1-selective inhibitors may prevent vascular hypertrophy in diabetic rats [53] and also attenuate or even reverse the development of cardiac hypertrophy and its progression to heart failure in different animal models [54–56]. Thus, the observed alterations in SL Na^+–H^+ exchanger in diabetes can be seen to indirectly affect the Ca^{2+} handling by cardiomyocytes and participate in the development of diabetic cardiomyopathy.

3 SR Changes in Diabetic Heart

Several studies have revealed that different Ca^{2+}-handling proteins embedded in the SR membrane become abnormal during the development of diabetic cardiomyopathy [2, 6, 46]. SR channel or RyR is a key component in Ca^{2+} handling and excitation–contraction coupling in the heart. Cardiac cells express mostly the RyR2 isoform, which is regulated by proteins such as calmodulin (CaM), Ca^{2+}-CaM-dependent kinase (CaMKII), and PKA [57]. Following the opening of a RyR2 cluster on the SR, Ca^{2+} sparks are generated and result in local, rapid, and brief elevation in cytosolic-free Ca^{2+} by tenfold or more and trigger cardiac contraction. It has been demonstrated that RyR2 function in diabetic cardiomyocytes is compromised, becoming leaky to Ca^{2+} during diastole and accounting for a reduced SR Ca^{2+} load.

In addition, a leaky RyR would promote Ca^{2+} accumulation in the cytosol, resulting in increased SL NCX activity to remove the intracellular excess Ca^{2+} in exchange for Na^{+}. Consequently, the increased Na^{+} influx would induce cell membrane depolarization, thereby leading to extrasystolic depolarizations and development of premature beats [58–60]. It has been suggested that these abnormalities may be linked to reduced levels of FKBP12.6 and increased activity of PKA [25, 61]. It should be mentioned that FKBP 12.6 is an accessory protein that plays a role in coordinating the opening and closing of individual RyRs in an array. The hyperphosphorylation of RyR2 by PKA leads to the dissociation of FKBP 12.6 and increasing the open probability of the RyR2 receptor [43]. This increased phosphorylation at Ser2809 and Ser2814 of RyR2 is also observed in stress/exercise-induced cardiac arrhythmias, sudden death, and catecholaminergic ventricular tachycardia [62, 63].

In a model of diabetic cardiomyopathy, Bidasee et al. [58] have reported that RyR2 proteins of 6-week streptozotocin (STZ)-induced diabetes rats bound less [^{3}H]ryanodine in comparison to control, although the affinity of this specific ligand and protein expression of the receptor remained unchanged in comparison to control. In a later study using 6- and 8-week STZ-induced diabetes rats [64], they also observed impaired binding ability of RyR2 to [^{3}H]ryanodine, which was even more pronounced in 8-week STZ-induced diabetes cardiomyocytes. In addition, 8-week STZ-induced diabetes rats showed a decrease in RyR2 expression (mRNA and protein). In both studies [58, 64], 2 weeks of insulin treatment initiated after 4 and 6 weeks of untreated diabetes was able to minimize the loss in function and expression of RyR2. Taken together, the findings indicate that the loss of functional integrity of the receptor precedes reduction in its expression and that the severity depends on the duration of untreated disease. The underlying mechanisms for RyR2 dysfunction remain unclear, but it has been shown that it could be caused by oxidative stress, nonenzymatic glycation reactions, and increased formation of disulfide bonds between adjacent sulfhydryl groups of the receptor [65–67].

The InsP3R plays a minor role in excitation–contraction coupling compared to the RyR in ventricular cardiomyocytes, but in atrial myocytes InsP3Rs are much more numerous and coexist with RyR on the SR, suggesting a prominent role in atrial contraction [68]. Several studies have shown that the InsP3R pathway is involved in progression of heart failure and delayed after depolarizations arrhythmias [69, 70]. In an experiment involving animals with obesity and type 2 diabetes, InsP3R expression was unaltered in ventricles from *ob/ob* mice [71], but in other diabetes studies it was shown to be decreased in diabetic rats [72] and in the atrium from diabetic patients [73]. The existing data indicate that altered InsP3R signaling may account for impaired Ca^{2+} handling and arrhythmogenesis in diabetic cardiomyopathy. However, the precise role of InsP3R in such pathological conditions requires further study.

Most of the intracellular Ca^{2+} is stored in SR via SERCA, which transfers two Ca^{2+} ions from the cytosol to the lumen at the expense of the hydrolysis of one ATP molecule. SERCA2a, the isoform predominately expressed by cardiomyocytes, is regulated by phosphorylation of a SR protein, phospholamban (PLB) [32, 74]. In its dephosphorylated form, PLB interacts with the pump, reducing its affinity for Ca^{2+}. However, when phosphorylated by PKC or CAMK, PLB is not able to inhibit

SERCA2a activity [75, 76]. SR function in diabetic cardiomyocytes has been shown to be compromised, presenting a reduced Ca^{2+} uptake that could explain the prolonged cardiac relaxation observed. As a consequence, SR calcium storage declines, resulting in reduced systolic calcium release and therefore a weaker cardiac contraction [74]. In this regard, some investigations with STZ-induced type 1 diabetes rats have reported decreased protein content and SERCA2a pumping dysfunction, which might be partly associated with an upregulation of activity and inhibitory PLB expression [35, 77]. In addition, it was proposed that products from advanced glycosylation reactions would form irreversible crosslinks within many proteins, leading to impairment of SERCA2a activity in diabetes [78, 79]. Thus far, it has been difficult to establish a general conclusion regarding myocardial SERCA2a and PLB changes in type 2 diabetes, most likely because of data limitation and ambiguity, especially taking in consideration the differences in animals models used.

Several studies using different type 2 diabetes animal models observed compromised SERCA2a function [25, 42, 43, 80]. SERCA2a expression was shown to be downregulated in Otsuka Long-Evans Tokushima fatty rats [80] and *db*/*db* mice [43] but unaltered in sucrose (SU)-fed rats [42]. Increased protein level of inhibitory PLB was only detected by one study [43]. Furthermore, Fredersdorf et al. [81] evaluated cardiac function and protein expression of Zucker diabetic fatty (ZDF) rats in the early stages of type 2 diabetes. They were able to demonstrate that animals in transition from insulin resistance to type 2 diabetes developed significant myocardial hypertrophy initially characterized by an increased systolic function and an intense SR Ca^{2+} uptake. In addition, myocardial expression of SERCA2a was markedly elevated and PLB expression was depressed. These changes were attributed to Akt signaling pathway activation induced by high levels of insulin, thereby supporting the view that upregulation of myocardial SERCA2a expression may be seen as a feedback mechanism in handling volume overload in the early phase of diabetes type 2. Taken together, the conflicting results regarding gene and protein expressions for SERCA2a and PLB can be explained by differences in the duration and severity of diabetes in various studies. Nonetheless, these observations are consistent with the view that alterations in SR function and SR remodeling occur in the diabetic heart [74]. Moreover, the critical role of SERCA2a in excitation–relaxation coupling is reinforced with the evidence that upregulation of its expression is able to reverse contractile dysfunction and abnormal calcium flux in established diabetic cardiomyopathy [82–84].

4 Mechanisms of SL and SR Alterations in the Diabetic Heart

It has been suggested that hyperglycemia along with metabolic shift, as a result of the hormonal imbalance caused by elevated plasma levels of catecholamines and angiotensin II, leads to oxidative stress and contributes to diabetic injury to multiple organs, especially the cardiac muscle [2, 4, 46]. The shift in myocardial metabolism, marked by decreased use of glucose and excessive utilization of long-chain fatty acids as an energy substrate, intensify the production of reactive oxygen

species (ROS) that damage the respiratory and oxidative phosphorylation activities of mitochondria, contributing to decreased myocardial efficiency [14, 85]. In addition, there is experimental evidence to suggest that mitochondria under several pathological conditions can act as a Ca^{2+} sink [86, 87]. Although this mechanism initially seems to play an important compensatory role in Ca^{2+} regulation by preventing or delaying intracellular Ca^{2+} overload in cardiomyocytes, it also accounts for the development of oxidative stress at late stages of diabetes. The generation of ROS can lead to leakage of toxic proteins through opening of mitochondrial pores and further damage of cardiomyocytes [65, 88]. Another mechanism of oxidative stress is mediated by advanced glycation end products (AGE), which are able to activate signaling pathways that induce ROS production, and its accumulation is related to structural and functional alterations of proteins in chronic diabetic tissues. It is worthwhile to note that hyperglycemia can impair and decrease the antioxidant system capacity in the heart and other organs in diabetes [85, 89, 90]. Thus, both the intense generation of ROS and reduced antioxidant capacity contribute significantly to oxidative stress and therefore myocardial damage in chronic diabetes.

It is now well established that genomic alterations lead to myocardial dysfunction in diabetic cardiomyopathy. Numerous studies have also been relating the diabetic state with activation of proteases and changes in signal transduction pathways, including PKC, PKA, CaM kinase, and mitogen-activated protein kinase, contributing to subcellular remodeling [91]. With respect to Ca^{2+} cycling, downregulation of SERCA2a expression, as well as its promoter activity were reported. Some investigations also detected reduced protein levels of SL Ca^{2+} channels, NCX1, Na^{+}–K^{+} ATPase, and RyR2 [74, 92]. These alterations have been attributed to an increased nuclear O-GlcN acylation, as a result of oxidative stress induced by hyperglycemia and enhanced activity of the PKC signaling pathway [93]. Moreover, genomic alterations also seem to underlie myosin dysfunction [94, 95]. In models of diabetic cardiomyopathy, abnormal myosin isozyme distribution, shift in myosin content from V1 to V3, and increased troponin I phosphorylation via the PKC pathway have been detected. Taken together, this could contribute to the decrease in Ca^{2+} sensitivity of myofilaments [96–101].

5 Conclusions

From the foregoing discussion it can be appreciated that diabetes is a complex pathology and that a wide variety of mechanisms contributes to cardiac dysfunction. The hormonal imbalance along with metabolic shift enhances oxidative stress, which leads to several abnormalities including activation of proteases, increased intracellular concentration of free Ca^{2+}, and alterations in cardiac gene expression (Fig. 1). Intracellular Ca^{2+} overload has been implicated not only in the process of excitation–contraction impairment but also in subcellular remodeling of organelles in cardiac cells. This event has been attributed to decreased SR Ca^{2+} load, depressed SERCA2a activity, and RyR2 dysfunction as well as changes in L-type Ca^{2+} channels. Abnormalities of SL proteins such as SL NCX, Na^{+}–K^{+} ATPase, NHE-1, and

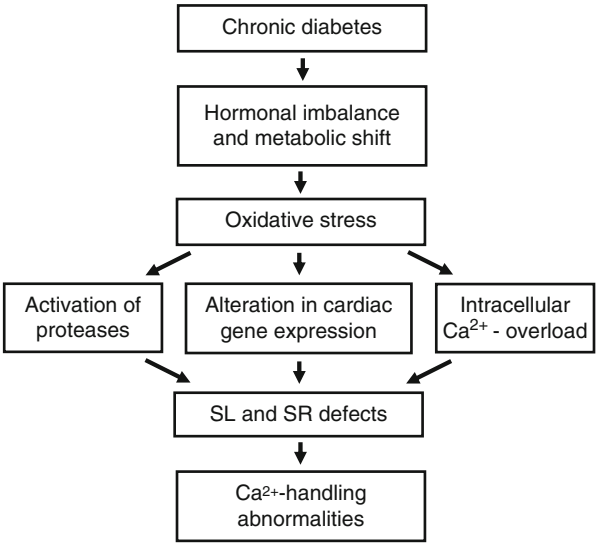

Fig. 1 Role of hormonal imbalance in sarcolemma (*SL*) and sarcoplasmic reticulum (*SR*) defects and subsequent Ca^{2+}-handling abnormalities in chronic diabetes

Ca^{2+}-pump ATPase have also been shown to be involved in diabetic cardiomyopathy. Molecular targeting approaches to revert or even attenuate alterations in proteins associated with Ca^{2+} handling hold promise as a new therapeutic modality. In addition, recent data have suggested that the insulin signaling pathway and Ca^{2+} regulatory processes are clearly interrelated, although many of these relationships are yet to be defined. Thus, further in-depth studies regarding the interactions between these pathways should lay the foundations for the design of new therapeutic approaches for diabetic heart disease.

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