MECHANISMS OF DEFECTS IN THE SARCOPLASMIC RETICULUM AND CARDIAC FUNCTION IN CARDIOMYOPATHIC HAMSTERS

A thesis presented to the

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In partial fulfilment of the requirements

For the degree of

MASTER OF SCIENCE

By

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FACULTY OF GRADUATE STUDIES

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ABSTRACT

Cardiomyopathy is defined as a disease of the heart muscle. Dilated cardiomyopathy (DCM) is a form of cardiomyopathy that is characterized by chamber dilation and impaired cardiac pump function. Although DCM is known to result in cardiac contractile dysfunction, the underlying mechanisms are unclear. As the sarcoplasmic reticulum (SR) is the main regulator of intracellular Ca^{2+} concentration in cardiac contraction and relaxation, we hypothesize that abnormalities in both the SR function and its regulation contribute to cardiac contractile dysfunction in cardiomyopathy. Accordingly, we examined cardiac performance, SR function and its regulation in the J2N-k cardiomyopathic hamster, an appropriate model of DCM. Echocardiographic assessment of the cardiomyopathic hamsters indicated contractile dysfunction, as the ejection fraction (EF), fractional shortening (FS%), cardiac output (CO) and heart rate (HR) were all significantly reduced in comparison to controls. Depressed cardiac function was associated with a decrease in cardiac SR Ca^{2+}-uptake in cardiomyopathic hamsters. In addition, a decrease in SR Ca^{2+}-release was observed in the cardiomyopathic hamster, further confirming that the disruption in Ca^{2+} homeostasis in the cardiac muscle. Reduced SR Ca^{2+}-uptake was associated with a decrease in the expression of SR ATPase (SERCA2a), the major Ca^{2+} handling protein in the cardiomyopathic heart. This directly corresponded to a decrease in the PLB/SERCA2a ratio of the SR. However, reduced SR Ca^{+}-uptake was also associated with a specific reduction in the regulation of SR function by cAMP-dependent protein kinase (PKA) mediated phospholamban (PLB) phosphorylation at ser-16, as Ca^{2+}/calmodulin-dependent protein kinase (CaMK) phosphorylation at thr-17 was unchanged in cardiomyopathic hearts. Depressed PLB

phosphorylation could perhaps be a result of the reduction in the SR associated PKA activity in cardiomyopathic hearts. Elevated protein phosphatase activity also contributed to reduced PLB phosphorylation in the cardiomyopathic hamsters. To complement the protein content studies, mRNA studies evidently revealed a decrease in the gene expression of PLB, SERCA, and CQS. The results of this study suggest that the impairment of SERCA2a may lead to altered SR function and subsequent cardiac dysfunction in the J2N-k cardiomyopathic hamsters.

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LIST OF ABBREVIATIONS

ANOVA.....analysis of variance

ATP.....adenosine triphosphate

BSA.....bovine serum albumin

$[Ca^{2+}]_I$.....concentration of intracellular calcium

CaMK.....calcium/calmodulin-dependent protein kinase

CK.....creatine kinase

CM.....cardiomyopathy

CO.....cardiac output

CQS.....calsequestrin

EF.....ejection fraction

FS .....percent fractional shortening

IVSd.....interventricular septum (diastole)

IVSs.....interventricular septum (systole)

LVIDd.....left ventricular internal dimension (diastole)

LVIDs.....left ventricular internal dimension (systole)

LVPWd.....left ventricular posterior wall (diastole)

LVPWs.....left ventricular posterior wall (systole)

PLB.....phospholamban

PKA.....cAMP-dependent protein kinase

RyR.....ryanodine receptor

SERCA2a.....sarcoendoplasmic reticulum Ca^{2+}ATPase

SR.....sarcoplasmic reticulum

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LITERATURE REVIEW

1. Introduction

Cardiomyopathy is classified as one of the most critical health disorders that cause disability and death among children and adults (1,2). It is now known that cardiomyopathy leads to heart failure, where over four and a half million individuals are currently affected and 400,000 more are targeted annually (1-3). Heart failure, which is a complex pathophysiological condition, can cause a range of symptoms from debilitating symptoms to ultimate death (4). In the gravest situations, the number of patients that succumb to heart failure greatly surpasses that of many known cancers (5). The transition of cardiomyopathy to heart failure is not well understood, and the definite boundaries of the two states have not yet been fully elucidated (6). However, years of vigorous research have offered insight into the possibility that the process of excitation-contraction coupling in the heart is a strong physiological candidate for potential therapeutic treatment (7). Although it has been said that heart failure has mysteriously entered this world, the biomedical community is constantly upgrading their knowledge and technology to alleviate the greatest health care challenge of all time (8).

2. Cardiomyopathy

a. Definition and Types of Cardiomyopathy

Cardiomyopathy is characterized as a disease of the heart muscle that causes cardiac contractile dysfunction with symptoms ranging from minor inconveniences to major health issues (9). Cardiomyopathy impairs efficient heart relaxation and contraction, thereby causing a discrepancy between organ blood supply and organ blood demand (10,11). It is a common chronic condition, in which the diseased heart diminishes one's ability to perform everyday

physical tasks (12). Cardiomyopathy differs from many other contractile dysfunctions in that, this disease can, and often does occur in the young (1). Cardiomyopathy can be classified as primary, where the cause of the disease is unknown, also known as idiopathic; or secondary, which is due to a known and definable cause (13). Through careful examination of patterns of symptoms, several types of cardiomyopathic disorders have been diagnosed thus far (9). They include hypertrophic cardiomyopathy, dilated cardiomyopathy, restrictive cardiomyopathy, ischemic cardiomyopathy, idiopathic cardiomyopathy, diabetic cardiomyopathy, alcoholic cardiomyopathy, and peripartum cardiomyopathy (1,9). Each category of cardiomyopathy displays unique features and can be identified by various haemodynamical and morphological characteristics (9,14). Essentially, cardiomyopathies affect several intricate systemic communications, thereby creating massive internal disorganization of homeostatic circuits.

The most common type of cardiomyopathy is dilated cardiomyopathy (1). Approximately 30-40% of all individuals with dilated cardiomyopathy have inherited the familial form that is primarily transmitted through autosomal dominant inheritance, but can also be inherited through X-linked, autosomal recessive and mitochondrial inheritance (15). In this state, the disease-affected muscle fibers result in an augmentation of one or more heart chambers, thereby weakening the heart's ability to pump due to a reduced contractile force of each chamber (9). Dilated cardiomyopathy is a syndrome of insufficient systolic function as a result of ventricular dilatation and moderate ventricular hypertrophy, followed by gradual congestive heart failure (16). It exhibits deficient contractility through reduced ejection fraction, reduced cardiac output and increased systemic vascular resistance (17). The course of dilated cardiomyopathy is progressive and has a poor prognosis (18). Dilated cardiomyopathy is one of the most prominent causes of congestive heart failure, and most often is the principal cause of hospitalization in the elderly (19). It has also been estimated that about half of the patients that suffer from dilated

cardiomyopathy, die within two years of diagnosis (16). Though the primary cause of dilated cardiomyopathy remains unclear, clinical evidence suggests that gene mutations or viral infections are involved in the preliminary stages of this disease (16). Other factors include inflammation, vascular diseases, alcohol induction, and abuse of drugs such as anthracyclines (20). Due to the plethora of etiopathogenetic hypotheses, it has been proposed that dilated cardiomyopathy may possibly represent the final stage of various myocardial diseases (21).

Specifically referring to genetic mutations, several studies have shown that a defect in the dystrophin gene in patients who suffer from Duchenne and Becker muscular dystrophy, results in a high occurance of dilated cardiomyopathy (22). Dystrophin is best described as a large cytoskeletal protein that offers structural integrity to the cell by creating a physical network linking the sarcomeric contractile unit to the extracellular matrix through the sarcolemma (23) (24-26). In particular, dystrophin binds to the actin protein of the sarcomere intracellularly, continues its path to the sarcolemma by binding to a member of the dystroglycan family, and then connects this complex to the laminin protein, located outside the cell in the extracellular matrix (1). Mutations in dystrophin would therefore disrupt the cytoskeleton, creating an environment for the cell that is not optimal for sustaining heavy mechanical stress from the continuous contraction and relaxation of the cardiac cycle (20).

The second most common type of cardiomyopathy is hypertrophic cardiomyopathy. It has been observed that ~1 in 500 live births are associated with this disease, making hypertrophic cardiomyopathy as prevalent as cystic fibrosis (27,28). A congenitally hypertrophic heart has an abnormal growth rate and an asymmetrical arrangement of muscle fibers, which ultimately leads to thickening of the ventricular septum and possible left ventricular hypertrophy(29-31). This thickening not only reduces the size of the ventricular cavity, but also blocks blood flow and

prevents proper filling during diastole (1,32). Characteristically, patients that suffer from hypertrophic cardiomyopathy experience reduced chamber compliance, delayed relaxation and extremely high levels of resting systolic function (33). Moreover, it has been discovered that hypertrophic cardiomyopathy shows genetic inheritance, and is called the disease of the sarcomere (16,28). This strong familial influence to develop hypertrophic cardiomyopathy suggests a certain genetic character that has been recently confirmed as a Mendelian autosomal dominant inheritance (9,16,30,34). Mutations in the sarcomeric genes include: cardiac troponin T, β-myosin heavy chain, α-tropomyosin, actin, titin and myosin binding protein (11,28,30,32). Due to this genetic inheritance, hypertrophic cardiomyopathy often unexpectedly and tragically causes unforeseen death amongst the young (35).

The third major form of cardiomyopathy is known as restrictive cardiomyopathy. It is the rarest case of all the cardiac disorders, as it offers the worst survival options due to its association with its poor response to therapeutic treatments (1). As the name suggests, restrictive cardiomyopathy impairs left ventricular filling by creating restrictive forces in the myocardium (16). The ventricular walls become extremely rigid and hinder proper filing, resulting in diastolic dysfunction along with a decrease in the heart's ability to pump (18). Diastolic dysfunction can be evident with chronic general symptoms or it may be present with altered hemodynamical characteristics (36).

b. Clinical Symptoms and Statistics

Various studies have shown that the most prevalent causes of heart dysfunction include coronary heart disease, myocardial infarcts, long-term hypertension, alcohol and drug abuse, congenital defects, toxic agents, metabolic disorders, mutations of sarcomeric or cytoskeletal genes, viral infections, valvular dysfunction, and other sources that are currently unknown

(9,12,35). The symptoms resulting from these conditions can be categorized into clinical, epidemiological, pathophysiological and exercise-related groups (37). In particular, the pathophysiological aspect of cardiomyopathy consists of altered function at the level of the myocardium (30), chamber remodelling (38), neurohormonal activation (39,40) as well as altered loading condititions (38). As heart failure develops from to the progression of cardiomyopathy, the following symptoms manifest: shortness of breath, fatigue, dizziness, swelling of the legs, decreasd urine output, decreased alertness, irregular heart beat, high blood pressure, chest pain, and abdominal swelling (9,32,41). Major health problems that have been documented include thromboembolism, arrhythmia and sudden cardiac death (9).

c. Different Animal Models of Dilated Cardiomyopathy

The advancement in the understanding of the various pathophysiologic and therapeutic methods of heart failure have been made a reality through the use of various animal models exhibiting heart disease (38). By using strains of cardiomyopathic hamsters and mice that were genetically programmed to develop dilated cardiomyopathy, one can study the cellular abnormalities that are proven to be analogous to this disease of the human heart (19,42). A vast array of cardiomyopathic hamster models that exist include: UM-X7.1 (43,44), BIO 14.6 (45-47), BIO 53.58 (47), Bio TO-2 (48), BIO 82.62 (49), Golden Syrian Hamsters (CHF 146) (50), and the J2N-k hamster (51). The following models offer some insight into the various areas of dilated cardiomyopathy that is being studied by researchers everywhere.

A very interesting mouse model of dilated cardiomyopathy focuses on mutations associated with genes in the intrasarcomeric cytoskeleton involved in force generation. Gene deletions of the α-cardiac actin gene, specifically in the immobilized portion of the filament that has been created in the α-cardiac actin knockout mouse show correlations with human hereditary

dilated cardiomyopathy (52). Other mouse knockout models that involve intrasarcomeric cytoskeletal proteins such as the myosin binding protein-C (53), or mice that overexpress tropomodulin (54), also display characteristic features of dilated cardiomyopathy that are observed both with and without a hypertrophic response. In contrast to the intrasarcomeric cytoskeleton, investigations of the extrasarcomeric cytoskeleton are also of equal importance. One of the earliest engineered models involving the extrasarcomeric cytoskeleton is the muscle LIM protein knockout mouse that displays characteristic phenotypic features of dilated cardiomyopathy (55). The muscle LIM protein is primarily involved in the cytoskeleton region that is known to co-localize with vinculin at the z-lines of the sarcomere (19). Though this model shows promise, the mechanisms by which this disease advances still remain unclear. Likewise, mice that suffer from the deletion of both dystrophin and MyoD (a transcription factor of skeletal muscle), as well as mice that are deficient in γ-sarcoglycan (56) or δ-sarcoglycan (57), but not α-sarcoglycan, also exhibit severe dilated cardiomyopathy followed by heart failure.

Another aspect of animal models manipulated to simulate dilated cardiomyopathy involves the regulation of intracellular calcium ions. Though mutations of the intracellular Ca^{2+} regulatory proteins have not yet been characterized in the human dilated cardiomyopathy (19), it has been found that mice models that do not contain FK Binding Protein (FKBP12.6) in association with the ryanodine receptor develop ventricular septal defects (58) and fatal dilated cardiomyopathy. In conjunction with the proteins of Ca^{2+} release, studies have also revealed that the high affinity Ca^{2+} binding protein calsequestrin when overexpressed in mice results in the development of cardiac hypertrophy followed by the advancement of dilated cardiomyopathy due to the defects in the Ca^{2+}-induced-Ca^{2+} release cycle (59). Other pathways that can be affected to initiate dilated cardiomyopathy include the manipulation of the genes involved in the G-protein coupled receptor pathways. A key member of the G-protein family that is of particular

interest is Gsα. When overexpressed in transgenic mice, Gsα is believed to be responsible for cardiac hyperfunction, gradual development of dilated cardiomyopathy and its corresponding association with myocardial fibrosis (60). Due to the fact that the β-adrenergic system utilizes the Gsα component in its pathway, this model can essentially provide compelling evidence that supports the fact that dilated cardiomyopathy is partially related to the prolonged stimulation of the β_1-adrenergic receptors in the overexpression of Gsα, which can ultimately be a contributing factor in the onset of dilated cardiomyopathy (61).

Transcription factors can also play an important role in cardiomyopathy. Recently, studies have shown that mice expressing the dominant-negative form of $CREB_{A133}$, exhibit symptoms related to the onset of dilated cardiomyopathy, without having inially gone through the hypertrophic phase (62). These transgenic mice display a high level of myocyte dropout a (low levels of myocytes due to myocardial cell death and hypertrophy), followed by consequent fibrosis and eventual death at an early age of 5-6 months. Alterations of several factors outside the nucleus, such as mutations in the mitochondria, are also associated with and may contribute to dilated cardiomyopathy. Due to the physiological importance of normal cardiac respiratory oxidative chain function, changes in the mitochondrial transcription factors can result in fatal consequences. Transgenic mice created with a deficiency in *Tfam* (the cardiac and skeletal muscle mitochondrial transcription factor) display a decline in the respiratory chain specific to the heart, in addition to the onset of dilated cardiomyopathy in association with atrioventricular block (63).

Recent breakthrough studies have investigated what happens to the survival of the cell when biochemical stress is placed upon a metabolic pathway. Knockout mice of GP130, a signal transducing receptor used to activate the Ras/MAPK and JAK-STAT pathways, showed

increased mortality due to defects of the skeletal, neural and metabolic systems (64). Further studies of this knockout model proved that these mice displayed normal cardiac function, but upon the introduction of stress via transverse aortic constriction, these mice rapidly transitioned into the dilated cardiomyopathic state and then further continued into heart failure and mortality, as a result of myocardial apoptosis (65).

Investigations involving cardiomyopathic hamster models have revealed intracellular changes of the cardiomyocyte. Cardiomyopathic hamster myocytes are considerably longer and wider than normal cells, possibly as a consequence of the increase in T-tubule and sarcoplasmic systems in the cell (45). This increase in membrane systems resulted in myocytes that were more sensitive to Ca^{2+}, as irregular spontaneous beating was reported, and was then followed by hypercontractility, which resulted in myocytes that became larger in width than in length (45). These effects therefore significantly decreased the contractile ability of the cardiomyopathic hearts as compared to control hearts (45,46), thereby showing cardiac systolic and diastolic dysfunctions (66). These diseased myocytes are not only structurally different, but also display differences in external regulation. Giudice *et al.* (67) have stated that an imbalance in the autonomic nervous system of the cardiomyopathic hamster exists, as elevated sympathetic and decreased parasympathetic tones have been observed. As a result of the fluctuating autonomic regulation, the following can also also be observed: insufficient yield of cyclic adenosine monophosphate (67), a decline in the Na^+/K^+ ATPase pump (44,67), insufficient uptake (47) and release of calcium (67,68), and an inefficient Na^+/Ca^{2+} exchange (67,69). Alteration of the activity of these proteins has not only disrupted the integrity of the cell, but also has caused an imbalance in the regulation of the ions involved in the chemical communication between contractile myocytes.

It has been shown that most of the early work of dilated cardiomyopathy has been researched in mice. However, a new era of study focuses on dilated cardiomyopathy in Syrian cardiomyopathic hamsters. Though both the hamster and mouse models are commonly used, in that they both are easily breeded with a reasonable survival rate, both can be maintained efficiently and both give a sufficient amount of offspring, the hamster model does prove to be more advantageous for this study. In measuring the affects of DCM (an autosomal recessive cardiomyopathy), the penetrance of cardiomyopathy in the hamster offspring is 100% (248). Recent studies have established δ-sarcoglycan mice, yet these animals developed a severe form of skeletal muscle dystrophy, which was not apparent in human DCM patients as well as cardiac lesions (249). Essentially, the hamster model of DCM shows similar symptoms to human DCM that no other animal model can, and also provides a proper healthy control hamster that is necessary for efficient comparison (22).

As a naturally occurring model of dilated cardiomyopathy from autosomal recessive inheritance, the hamster strain BIO14.6 has been extensively studied for the past 30 years (70). The major factor in contributing to cardiomyopathy is the mutation of the δ-sarcoglycan gene (71,72), and more specifically the 30-kb deletion in the first two exons (73). As a result of this fascinating discovery, other strains have been successively derived from BIO 14.6. They include UMX7.1, CHF147, and T0-2, which are all being investigated in terms of pathophysiology and potential therapeutic treatements for heart failure (74). These hamster models are all unique in that they possess single gene mutations, but lead to various forms of cardiomyopathy, depending upon their genetic makeup (19). Hence, compensatory hypertrophy with gradual progression into dilated cardiomyopathy is characterstic of BIO14.6, yet the TO-2 model is known to develop dilated cardiomyopathy without the initial stages of the cardiac hypertrophic response (72). Moreover, one of the most fascinating hamster models created for the study of dilated

cardiomyopathy comes from Japan and is known as the J2N-k cardiomyopathic hamster.

d. J2N-k Cardiomyopathic Hamster Model

The male J2N-k hamster has an average lifespan of 243 ± 18 days (51), and during this life expectancy displays the following estimated time course: myocardial necrosis at 4-5 weeks, cardiac dilatation followed by cardiac dysfunction at 20 weeks, and finally death due to congestive heart failure at approximately 1 year of age (75,76). This model is of particular interest, as it displays similar symptoms of human dilated cardiomyopathy the way no other animal model previously has, and it has a proper healthy match as a control (73,77).The J2N-k cardiomyopathic hamster is unique in the fact that it lacks the δ-sarcoglycan (δ-SG) gene, which is thought to be involved in the proper function of dystrophin (22). As a consequence of the deletion of the δ-SG gene, the corresponding members of the sarcoglycan family (α, β, and γ) are induced to disappear completely in both the heart and skeletal muscles (73). The multisubunit dystroglycan complex that includes the SG family extends across the sarcolemma to form a structural link between the actin cytoskeleton and the extracellular matrix to protect the cell from mechanical damage due to contraction and relaxation (78). Disruption of this very elegant array could potentially result in the compromise of membrane stability and integrity (79).

3. Cardiac Function

a. Calcium Cycling

Ca^{2+} is of paramount significance in cardiac physiology, as this ion is involved in processes as diverse as cell growth, metabolism, hormone secretion, motility, gene expression, protein trafficking, regulation and finally apoptosis (80). The concentration gradient of Ca^{2+} in

and out of the cell is criticial to its survival, where the intracellular cytoplasmic free Ca^{2+} concentration is 10^3-10^4 times less than that of the extracellular space (81). This great Ca^{2+} gradient is sustained through the use of Ca^{2+} channel situated in the membrane networks of the cell's outer surface and inner sarcoplasmic reticulum (81)

Upon electrical stimulus of an action potential, the L-type voltage-gated Ca^{2+} channels in the sarcolemma membrane open to allow an influx of Ca^{2+} into the cytoplasmic space (82). This introduction of Ca^{2+} ions into intracellular space, gives rise to the rapid release of Ca^{2+} from the SR through its ryanodine-sensitive Ca^{2+}-release channels, which are conveniently located in the area adjacent to the L-type Ca^{2+} channels (83-85). This entire process is referred to as calcium-induced-calcium release (86). Immediately following the release of Ca^{2+} into the cytosol, inactivation of the L-type Ca^{2+} channel occurs, as Ca^{2+} ions bind to it, thereby contributing to a process called Ca^{2+} -dependent inactivation of Ca^{2+} influx (87,88). This intricate cycle is maintained in the cell to promote survival based on a local negative feedback effect, which starts with a large influx of Ca^{2+} that leads to release from the SR to counteract more Ca^{2+} influx by binding to the L-type channel, thereby inactivating the whole process (89).

In cardiac muscle, Ca^{2+} is the ubiquitous second messenger, which once released into the cytosol is the key component for initiating the transition of the resting state to the contractile state via binding of contractile proteins (86,90). To achieve this, Ca^{2+} binds to the myofilament, troponin C (89). Troponin is a hetero-trimer that is composed of three distinct proteins: the calcium receptor (Tn-C), the inhibitor of the actin-myosin binding site (Tn-I) and the binding portion of troponin that effectively relays the Ca^{2+}-binding signal from Tn-C to the thin filament through the interaction of Tn-C and tropomyosin (Tn-T) (90). The contractile machinery of the cell is turned on as a result of the association of Ca^{2+} with troponin C, but is quickly turned off

for the relaxation process. The $[Ca^{2+}]_i$ is lowered as Ca^{2+} dissociates from troponin C and is transported out of the cytosol (89).

Essentially, intracellular Ca^{2+} is lowered mainly by the uptake of Ca^{2+} back into the SR via a Ca^{2+}-ATPase, the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA2a) (91,92). Ijn addition, intracellular Ca^{2+} is lowered by leaving the cytosol via: (a) internal Ca^{2+} exchange for external Na^+ via the sarcolemmal Na^+/Ca^{2+} exchanger (91,93) (b) and SL Ca^{2+} pump that expels Ca^{2+} using ATP as an energy source (93), and (c) uptake of Ca^{2+} via the mitochondria (94). Though the SL Ca^{2+} pump and mitochondria participate in the uptake of Ca^{2+} during the contraction-relaxation cycle, the amount is minimal. The mechanism of mtochondrial uptake is not clearly understood (43). The Na^+/Ca^{2+} on the other hand, is driven by an electrochemical gradient, and essentially extrudes 1 ion of Ca^{2+} for every 3 ions of Na^+ that is brought into the cell (95). It is interesting to note that the removal of Ca^{2+} from the cell during diastole is species specific in terms of what machinery is employed. Studies have shown that rat ventricular myocardium utilizes SERCA2a to sequester 92% of the Ca^{2+} into the SR, while only a mere 7% of Ca^{2+} is expelled by the Na^+/Ca^{2+} exchanger (96,97). However, in the human, rabbit, ferret, guinea pig and cat it is observed that SERCA2a takes in 70-75% of the Ca^{2+} into the SR, leaving the remainder 25-30% to be removed by the Na^+/Ca^{2+} exchanger. The rat shows an increase in SERCA2a pump activity in comparison to the rabbit, as it contains a greater concentration of protein pumps (98). Additionally, analysis of mouse models produces results that are quantitatively similar to those of the rat (99).

b. Sarcoplasmic Reticulum Function

As the major storage of the cell's calcium, the sarcoplasmic reticulum (SR) is of crucial importance to the performance of the heart (25). Through prominent features of its ability to

regulate the myoplasmic free Ca^{2+} concentration, the SR is the vital link in cardiac performance on a beat-to-beat basis (100). The SR includes both longitudinal and junctional sections, as well as the corbular SR found in the I-band of the sarcomere (101,102), which are all home to many regulatory proteins that help the SR function as a whole (103). Included in the longitudinal SR is phospholamban (PLB) and SERCA2a, whereas the ryanodine receptor (RyR) is located in the junctional SR (104). The sequence of events in cardiac contractility is regulated by interaction amongst these specific proteins. Of primary interest, is the interaction between the SR protein PLB and SERCA2a.

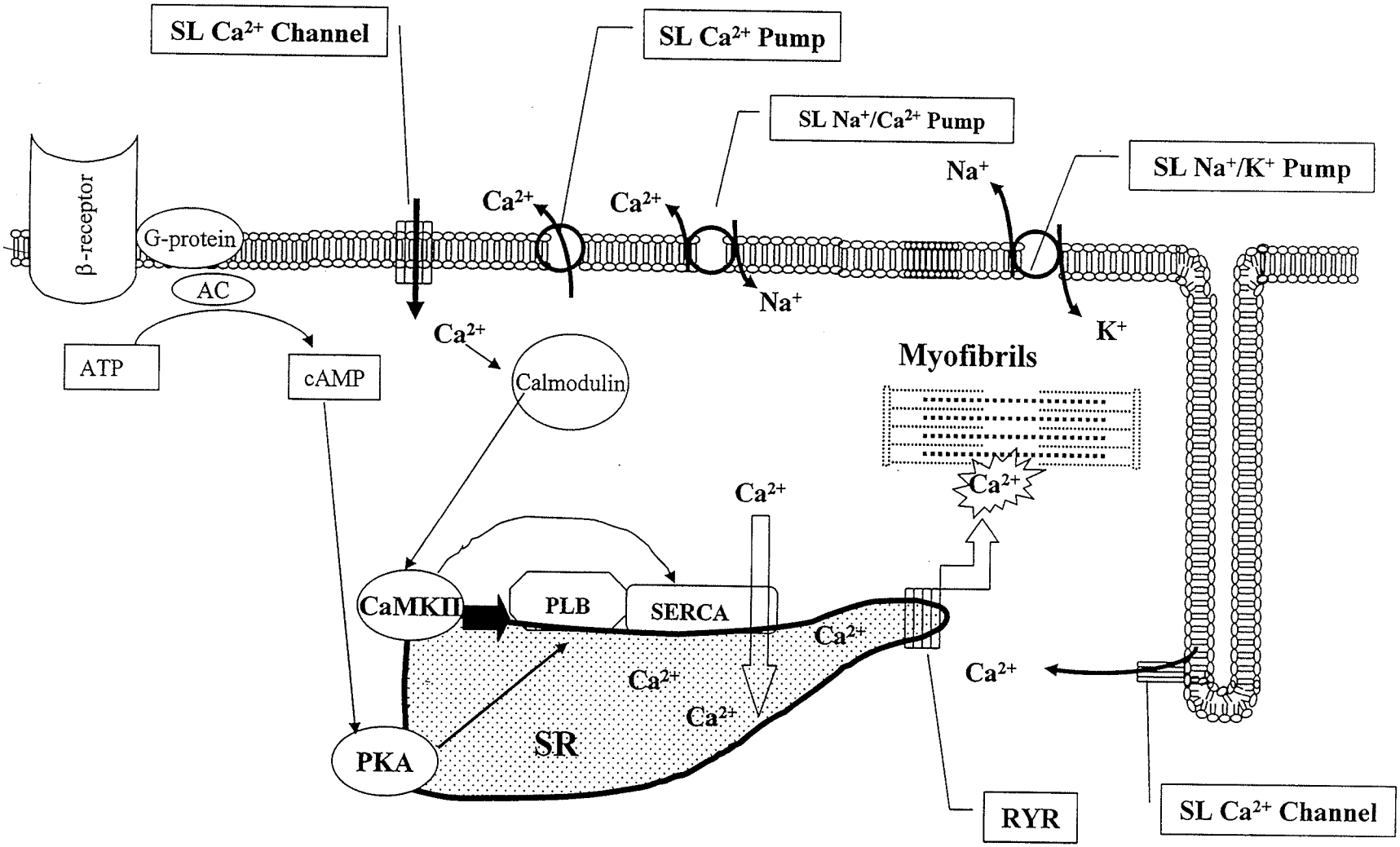

Figure 1. Schematic representation of the regulation of SR Ca^{2+} flux by Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) and cAMP-dependent protein kinase (PKA) pathways in the normal cardiomyocyte. SL, sarcolemma; SR, sarcoplasmic reticulum; AC, adenylyl cyclase; SERCA2a, sarcoendoplasmic reticulum Ca^{2+} ATPase; PLB, phospholamban; RyR, ryanodine receptor.

i. Phospholamban (PLB)

Comprised of 52 amino acids, PLB is described as an intrinsic membrane protein of the SR that intimately interacts with SERCA2a by exerting a negative influence to maintain an inactive state during diastole in the cardiomyocyte (105,106). PLB is a 27 kDa pentamer composed of 5 identical subunits that can be further separated into monomers of 6 kDa when subjected to boiling in sodium dodecyl sulfate buffer (107-109). The regulation of intracellular Ca^{2+} is maintained in part by phosphorylation of this inhibitory protein, PLB (110). During diastole when the cardiac muscle is at rest PLB negatively regulates the activity of SERCA2a through direct protein-protein interactions on the SR membrane (26,91). The inhibition of SERCA2a is alleviated by the activity of the endogenous cAMP-dependent protein kinase (PKA) and the Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), which allows SERCA2a to sequester Ca^{2+} into the SR to begin the relaxation stage of diastole (11,26). Specifically, PLB physically dissociates from SERCA2a when phosphorylated at the Ser^{16} site by PKA (111) and at the Thr^{17} site by CaMKII (112-113) to allow influx of Ca^{2+} transport into the SR (114). From this reaction, it is believed that PLB is the key phosphoprotein that is involved in the control of SERCA2a, and ultimately, in the transfer of Ca^{2+} from the cytosol into the SR (115).

ii. Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)

The SR is home to SERCA2a, which has the primary role of maintaining a 1000-fold Ca^{2+} gradient across the SR membrane (116). Composed of three different genes that can be spliced in several isoforms (116), SERCA1a is found in fast-twitch skeletal muscle, whereas SERCA1b is distributed in neonates (117). SERCA2a is dominant in slow-twitch skeletal and cardiac tissue, while SERCA2b prevails in nonmuscle and neuronal cells (118). The SERCA3 isoform is found exclusively in the epithelial and endothelial cells (119). Through recent studies, it has now been well established that the SERCA2a isoform is exclusively expressed in the

normal and diseased myocardium (120). With a molecular weight of 105 kDa, it is Ca^{2+}-stimulated, Mg^{2+}-dependent, and constitutes 35-40% of the total SR protein content (121,122). The SERCA2a pump functions to transport two Ca^{2+} ions into the SR against a high ionic gradient at the expense of the hydrolysis of one molecule of ATP (97). The activity of SERCA2a is the rate-determining step of sequestering Ca^{2+} into the SR (123), and a decrease in this Ca^{2+}-uptake activity is the central feature of the failing heart in humans and animals (124).

iii. Ryanodine receptor/Ca^{2+} release channel (RyR)

In contrast to the calcium uptake activity of the SR, the SR contains calcium release proteins such as the RyR and the inositol 1,4,5-triphosphate receptor (94). Intracellular Ca^{2+} release is accomplished through the activation of these Ca^{2+} release channels (125). RyR is located on the SR, in the surrounding area of the sarcolemmal L-type calcium channel, and upon stimulation is responsible for the release of Ca^{2+} into the cytoplasm (11,24). The ryanodine receptor received its name primarily by the fact that it has the ability to bind the chemical ryanodine, which is a highly toxic plant alkaloid (126). RyR is a homotetramer of ~564 kDa (104). In the mammalian muscle, there exist three unique isoforms of RyR (127). RyR1 resides mainly in skeletal muscle, RyR2 is the dominant form in cardiac muscle, and RyR3 is widely distributed throughout many tissues, including muscle (128). Particularly, RyR2 acts as a multimembered signalling complex, whereby specific communicating proteins, such as kinases and phosphatases are able to bind to certain domains on the cytoplasmic side of the receptor (129,130). This receptor is mainly distributed in the section of the SR that lies in close proximity to the invaginations of the T-tubules of the SL (131). This close association with the L-type voltage-dependent Ca^{2+} channel provides optimal conditions for the signal amplification progression that is the key to excitation-contraction coupling (132). RyR is also in close association with the luminal SR proteins such as triadin, junctin and calsequestrin, which all

participate in the buffering of intracellular SR Ca^{2+} and the modulation of Ca^{2+} release (133). Upon activation, RyR generates a global increase in cytoplasmic Ca^{2+} that initiates the binding of free Ca^{2+} to contractile myofilaments to achieve contraction of the cardiac muscle (134).

iv. FK-Binding Protein (FKBP) 12.6

In the SR of normal hearts, Ca^{2+}-induced-Ca^{2+} release via RyR releases the Ca^{2+} that is needed for contraction during systole (135). However, during the resting phase of the contractile cycle when no Ca^{2+} is needed in the cytoplasmic space, RyR must be tightly closed so that Ca^{2+} ions cannot leak uncontrollably into the cytoplasm and contribute to cardiac arrhythmias by causing an imbalance in the electrical properties of the heart (136). This is accomplished by the molecule FKBP 12.6 (12 kDa), which binds exclusively to each of the four subunits that compose the RyR, to create a seal and prevent accidental Ca^{2+} leak from the SR (137).

v. Calsequestrin (CQS)

Calsequestrin is a Ca^{2+} binding protein located inside the SR lumen (138) that serves to make Ca^{2+} available for release into the cytosol upon stimulus via a wave of depolarization. CQS is characterized as having a high-capacity binding and moderate affinity for the Ca^{2+} ion (93). At 55 kDa, CQS is situated on the junctional SR in very close proximity to RyR (139). Certain ideas have also been proposed that suggest that CQS, along with RyR and various other SR proteins such as FKBP, junctin and triadin, form unique complexes that function to assist in the release of Ca^{2+} from the SR during systole (133).

c. Sarcoplasmic Reticulum Regulation

Protein kinases and protein phosphatases work in a harmonized equilibrium to provide stability and integrity to the functioning cell. The actions of these enzymes can determine whether a cellular process is short-term or long-term, and can also dictate the behaviour of the cell based on the demands set forth by its surroundings (140). Cardiac protein kinases function to phosphorylate cardiac regulatory proteins on a beat-to-beat basis to achieve proper cardiac performance (141). Since approximately one-third of these proteins are reversibly phosphorylated (142), it is the job of the protein phosphatases to complete this cellular cycle by removing the targeted phosphate, and permiting the opposite reaction to occur. Together, this sequence of events forms the basis of one of the fundamental signal transduction pathways involved in the regulation of the cell.

i. cAMP-dependent protein kinase (PKA)

The intricate and complicated network of the signal transduction pathway for cardiac contractility uses cAMP as a second messenger to trigger a cascade of events involved in the β-adrenergic system (143). Its effects include increased heart rate (144-146), increased force of contraction of the heart muscle (145-147), and a change in the rate of cardiac relaxation resulting from the various effects of the sympathetic neurotransmitters (145,147). The events that occur in response to β-adrenergic stimulation begin with the binding of the β-adrenergic agonists to their receptors in the cell membrane, which in turn interact with G-proteins, continue with the activation of the second messenger adenylyl cyclase to convert ATP to cAMP, and finish with increasing the amount of intracellular cAMP that is required in the process of phosphorylation of proteins via cAMP-dependent protein kinase (PKA) (148).

PKA is a very popular kinase that has been studied in cardiomyocyte protein phosphorylation (149). In cardiac myocytes, PKA activity is vital to the regulation of metabolism, muscle contraction, gene expression and ion fluxes (150). The PKA family of proteins is typically composed of four regulatory (R) subunits that are involved in regulation (Riα, Riβ, RIIα, and RIIβ), as well as three subunits that have catalytic activity (Cα, Cβ, and Cγ)(151). At the molecular level, the R subunits form dimers through the N-terminal sequences to result in a molecule in which a single C subunit can bind to, thereby giving an overall tetrameric holoenzyme (152). Through external stimulation, cAMP initially binds to the R subunit and releases the C-catalytic subunit results in activation of PKA (153). Having a wide variety of targets, PKA phosphorylates the SR membrane protein PLB at the Ser^{16} residue (154). This in turn, relieves the inhibitory effect on SERCA2a, thereby allowing Ca^{2+} to be sequestered into the SR to enhance relaxation of the cardiac muscle (116). PKA is also involved in the phosphorylation of troponin I, and through β-adrenergic stimulation, troponin I releases its binding with Ca^{2+} so that it can be taken up by the SR (93). The third major target of PKA is the L-type voltage-gate Ca^{2+} channel located in the sarcolemma (93). Upon increased levels of cAMP, PKA is stimulated to phosphorylate this channel to cause an influx of Ca^{2+} into the cell to further trigger a major release of Ca^{2+} from the SR (93,155). This effect is two-fold, as the newly introduced intracellular Ca^{2+} also activates the Ca^{2+}/calmodulin-dependent protein kinase (CaMK), which is a second major cardiac kinase involved in the regulation of the SR (156).

ii. <u>Ca^{2+}/calmodulin-dependent protein kinase (CaMK)</u>

The CaMK family is involved in phosphorylating a number of essential molecules that ultimately control cardiac contractility, and rates of cellular transcription (157). This family has several members that include CaMKI, CaMKII and CaMKIV (158). Of all the reports to date, the most thoroughly studied member is CaMKII (159). CaMKII is predominantly seen in the cardiac SR

membranes as the CaMKII-δ isozyme (160). It is characterized as an oligomer that is made up of 10-12 separately active α- and β- catalytic domains (152). Activation of CaMKII is achieved through conformational changes upon binding of Ca^{2+} and calmodulin (a calcium binding protein), binding to Mg^{2+}ATPase, as well as autophosphorylation at the threonine 286 site (141,161). Calmodulin is a 16-17 kDa protein that acts as a transducer to regulate the enzyme activity through Ca^{2+} signalling (162). It has the ability to bind a maximum of four Ca^{2+} ions (163,164) and in doing so, changes its conformational state to become active (165).

CaMKII is chiefly responsible for phosphorylating various cardiac proteins in response to Ca^{2+} signals (166). The frequency of stimulation of this kinase is determined by both the duration and overall amplitude of the Ca^{2+} spikes present in the cell (134). CaMKII specifically phosphorylates PLB at the threonine-17 residue and SERCA2a at the Ser^{38} residue to promote SR Ca^{2+} uptake from the cytosol (167). This process occurs via a membrane-association interaction (168), although this kinase predominantly resides in the cytosol (169). CaMKII is also known to phosphorylate the Ca^{2+} release channel on the SR to promote SR Ca^{2+} release and increase cardiac contraction (168). Another function of CaMKII includes the inactivation of the $Ca_v1.2$ channels (170). In essence, cytoplasmic Ca^{2+} facilitates the entry of Ca^{2+} through the L-type channel via activation by CaMKII to induce a modal gating shift that promotes extended opening of this voltage-gated membrane channel (171).

v. Protein Phosphatase 1 (PP1)

Due to the fact that most cells possess the characteristic of reversible phosphorylation, the past ten years have been a whirlwind of progress in learning the specific roles of phosphatases (140). Dephosphorylation of cellular proteins is accomplished by protein phosphatases (172). Though the physiological role of kinases in cardiac contractility has been relatively well

established, the role of phosphatases is yet to be fully understood (173). According to enzymatic studies and biochemical classifications, mammalian phosphatases have been neatly divided into two major groups: protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2) (174,175). Together, these two groups of phosphatases occupy more than 90% of the mammalian heart phosphatases (176,177). The PP1 enzymes are composed of oligomers that include one of four possible homologous catalytic subunits in addition to the necessary regulatory subunits (178). PP1 is unique, as it is inhibited by two cytosolic heat-stable and acid-stable proteins, inhibitors 1 and 2 (I1 and I2) (140,167,179), where I1 inhibits PP1 only when specifically phosphorylated by PKA (180). Moreoever, molecular characterization has revealed that the catalytic regions of PP1 can be further subdivided into three categories: PP1α, PP1δ, and PP1γ (181). By dephosphorylating PLB, PP1 inhibits the activity of SERCA2a (182) and ultimately prolongs the duration of contraction, as Ca^{2+}-uptake into the SR is reduced (172).

vi. Protein Phosphatase 2A (PP2A)

PP2A is described as a heterotrimer that includes three subunits: PP2A-C (a catalytic subunit), one of many regulatory subunit regions, and a single structural subunit (183). Although the mechanisms of the regulation of PP2A have not been fully elucidated (184), this phosphatase shows *in vitro* regulation from methylation (185) and phosphorylation (186,187). PP2 preferentially dephosphorylates the α-subunit of phosphorylase kinase, and unlike PP1, is insensitive to I1 and I2 (188). PP2 can further be categorized into three different classes: PP2A that is okadaic sensitive, PP2B that is Ca^{2+}-dependent, and PP2C that is Mg^{2+}-dependent (189).

vii. Protein Phosphatase 2B (PP2B)

Also referred to as calcineurin (CN), PP2B is a Ca^{2+} activated CaMKII phosphatase that is located in various tissues and has maintained a conserved form throughout evolution (190).

CN is a serine/threonine protein phosphatase that is a heterodimer and is comprised of a catalytic region and regulatory region of 61 kDa and 19 kDa, respectively (22,140). These two subunits show extreme specificity for substrates, as they target the following: a region of the PKA regulatory subunit type II, an inhibitor of PP1 (I1) subunit, the phosphorylase kinase subunit, and site 2 in the glycogen binding domain of PP1 (140,191). Calcineurin is exclusively stimulated by prolonged levels of increased intracellular Ca^{2+} (192-194) and is regulated by intracellular Ca^{2+} and calmodulin (22). New evidence has revealed that there is a Ca^{2+}-dependent association of calcineurin with the SR RyR that concurs with the idea that calcineurin becomes activated and further interacts with the Ca^{2+} release channel in the presence of an increased cytosolic level of Ca^{2+} (195). The study further supports the concept that calcineurin interaction with the ryanodine receptor could provide strong evidence for possible regulation of SR Ca^{2+} by calcineurin (195). Based on the homeostatic balance of Ca^{2+} ions, it has been thought that CN is involved in cardiac hypertrophy and myocyte apoptosis through the activation of transcription factors, namely the nuclear factor of activated T cells (NFAT), in association with the increased levels of intracellular Ca^{2+} (22). Once NFAT comes in contact with CN, it translocates to the nucleus where is associates with other transcription factors to ultimately activate the transcription of genes that are involved in the remodelling of the myocyte (250).

4. The Effect of Cardiomyopathy on the Sarcoplasmic Reticulum Proteins

Dilated cardiomyopathy is often associated with a drastic decrease in the calcium transport properties of the sarcoplasmic reticulum (47). Ample evidence has shown that the function of the sarcoplasmic reticulum was depressed (196,197), thereby contributing to abnormal Ca^{2+} regulation and ultimate contractile dysfunction.

a. Sarcoplasmic reticulum Ca^{2+}-ATPase pump in Cardiomyopathy

In mammalian cardiac myocytes, relaxation is primarily achieved through the sequestration of Ca^{2+} into the SR via SERCA2a, and a reduction of this activity could potentially result in abnormalities that lead to severe cardiac contractile complications (198). A common such complication can be seen in the case of diabetic cardiomyopathy. Diabetic cardiomyopathy stems from the disease of diabetes, and progresses into heart failure, which eventually goes on to claim the lives of over 65% of all diabetic patients (199). This form of cardiomyopathy displays a dysfunctional and uncoordinated SR that leads to abnormal intracellular Ca^{2+} handling (200). Alteration of this structured transport of Ca^{2+} within the cell is the hallmark of cardiomyopathy and its successor, heart failure (201). As a result, this reduction in sequestration of Ca^{2+} can essentially explain the delay in cardiac relaxation observed in the development of diabetic cardiomyopathy (202). In turn, the lower amount of Ca^{2+} found in the SR gives rise to less Ca^{2+} released into the cytosol and ultimately a weaker contraction during systole is formed (203,204). The main player involved in this process is SERCA, which is the major determinant of the beat-to-beat regulation of cardiac contraction (200). In diabetic cardiomyopathic rat myocytes, the function and rate of Ca^{2+}-uptake into the SR is significantly reduced (204). Further studies that focus on the level of SERCA2a, show that overexpression of SERCA2a in mice has been observed to enhance the performance of cardiac contractility (205). However, mice suffering from diabetic cardiomyopathy exhibit a decrease in SERCA2a protein levels that contribute to reduced cardiac function (200). Yet, when these diabetic mice overexpress SERCA2a, their hearts are protected from severe cardiac dysfunction, possibly due to the improvement of Ca^{2+} sequestration of the SR (200).

Another common form of heart disease is hypertrophic cardiomyopathy, in which diastolic dysfunction is the most characteristic pathophysiologic abnormality (206). Studies have gone on

to show that in the most severe cases of hypertrophic cardiomyopathy, SERCA2a is involved in the abnormal Ca^{2+}-handling that contributes to impaired cardiac contractile performance (207). Specifically, decreased levels of the gene expression of SERCA2a have been observed in patients who suffer from left ventricular hypertrophy (206). Somura *et al.*(206) have also reported a decrease in the expression of SERCA2a protein levels, as well as a reduced ratio of SERCA2a to PLB. They further propose that this reduction in the ratio of SERCA2a/PLB can potentially be responsible in contractile dysfunction in hypertrophic cardiomyopathy.

Though cardiomyopathic-induced heart failure is manifested by factors such as diastolic and systolic dysfunction, there are various forms of cardiomyopathy that exist and are characterized by unique features. Almost all cardiac dysfunctions described thus far, focus on the alteration in Ca^{2+}-handling of the SR. These next two cardiomyopathies are based upon the same elements. Sen *et al.* (45) have recently described the difference in mechanisms underlying ischemic and idiopathic dilated cardiomyopathy. In their study, they have characterized the irregularities in contractile dysfunction in association with Ca^{2+} homeostasis in the myocardium of end-stage heart failure due to ischemic and dilated cardiomyopathy. This has truly been of unique significance, as this was the first time that isolated ventricular myocytes have been analyzed for contractile properties and Ca^{2+} transients of SR Ca^{2+}-uptake and release activities, all in the same heart. Sen *et al.* (45) have therefore, proposed that dysfunction of the Ca^{2+}-uptake activity via SERCA2a is the primary factor contributing to cardiac dysfunction in ischemic cardiomyopathy, wherein the opposite action of Ca^{2+}-release is an important pathogenetic role in the disruption of Ca^{2+} homeostasis involved in idiopathic dilated cardiomyopathy.

Studies have also been done comparing animal models of both dilated and hypertrophic cardiomyopathy, using Syrian cardiomyopathic hamsters. Studies conducted by Whitmer *et*

al.(47) have measured the Ca^{2+}-uptake activity in homogenates and microsomal preparations. At 3 months of age there was no significant change in Ca^{2+}-uptake in homogenates from control or myopathic hearts, and at 9 months, although there was no change in homogenates from the control and hypertrophic hearts, there was a significant reduction in initial rate and capacity of Ca^{2+}-uptake activity in the homogenates from the dilated cardiomyopathic model. Similar trends were observed in the microsomal fractions, but the dilated cardiomyopathic heart showed a significant decrease in activity by 3 months of age.

Recent studies done at the molecular level show that hamsters suffering from cardiomyopathy experience a premature death from fibrosis and calcifications (197), have displayed less than normal levels of SERCA mRNA levels. Of particular interest, is a study that compared the SERCA activity between hypertrophic and dilated cardiomyopathic hamster hearts. This study concluded that the dilated cardiomyopathic hamster hearts (BIO 53.58) exhibited a decrease in the level of Ca^{2+} uptake into the sarcoplasmic reticulum, whereas the hypertrophic cardiomyopathic hearts indeed matched the Ca^{2+} uptake levels of the control hearts (47).

b. Phospholamban in Cardiomyopathy

A major regulator of cardiac activity that plays the prime role in cardiac contraction and relaxation, is the SR protein PLB (115). Due to its paramount significance, PLB may be a potential candidate gene responsible for the onset of cardiomyopathy (208). Recent genetic studies have shown that inhibition of PLB can prevent the development of cardiomyopathy (123,209), and mutations of PLB result in gain of function (210), whereas overexpression of PLB in mice exhibits a phenotype greatly characteristic of cardiomyopathy (211). PLB-null mice display superior cardiac function that does not in any way compromise the exercise

endurance of the heart, or its life span (212,213). Though PLB-deficient mice show enhanced myocardial inotropy and lusitropy (diastolic relaxation), which is not accompanied with any damaging effects, human PLB is essential for maintaining cardiac health and its absence indefinitely leads to heart failure (214).

As different alterations in the SR proteins contribute to different forms of cardiomyopathy, Minamisawa *et al.*(123) have postulated that mutations in PLB can ultimately contribute to both dilated and hypertrophic cardiomyopathies. Human dilated cardiomyopathy is the result of increased interaction of PLB with SERCA2a, causing impaired Ca^{2+} uptake (215,216) and leading to chronic hypocontractility of the heart (217,218). However, an increase in the expression of PLB from genetic mutations modulates cardiac function for an extended period of time and eventually leads to the remodelling of the cardiac muscle (208). Changes at the molecular level, which involve missense mutations in humans, are shown to cause human dilated cardiomyopathy (214,219). On the basis of SR function in cardiomyopathy, Flesch *et al.* (220) have reported that a reduction in the Ca^{2+}-cycling activities of the SR contributes to an altered intracellular Ca^{2+} handling and results in dilated and ischemic cardiomyopathic hearts.

Further studies of human dilated cardiomyopathy that focused on PLB and SERCA2a, were conducted by Fentzke *et al.* (221). Ca^{2+}-uptake and –release activities were measured, as well as the determination of mRNA and protein levels in nonfailing and terminally failing hearts. The Ca^{2+}-cycling activities involving sequestering Ca^{2+} into the SR and release back into the cytosol were shown to be significantly reduced in the crude membrane preparations of the dilated cardiomyopathic hearts. At the molecular level, the protein concentrations of both PLB and SERCA2a were unchanged in the failing tissues. Yet, mRNA transcript levels of these two prominent SR proteins were significantly reduced. Schwinger *et al.*(221) have provided evidence

to deduce that dilated cardiomyopathy affected SERCA2a function in Ca^{2+}-transport without affecting the protein levels.

c. Ryanodine Receptor (Ca^{2+} release channel) in Cardiomyopathy

Alteration in the Ca^{2+}-cycling process may occur at the level of Ca^{2+}-uptake by SERCA2a, or may occur at the level of Ca^{2+}-release through the Ca^{2+}-sensitive ryanodine receptor (RyR) (215). As a result, a lot of energy is being focused on both mRNA and protein analysis, as both mRNA and protein synthesis and degradation may be affected in the cardiomyopathic heart. Molecular studies have shown that steady-state mRNA levels of RyR are unaltered in human dilated cardiomyopathy (222), yet mRNA levels of SERCA2a and PLB are clearly decreased in this same disease (223). Meyer *et al.* (215) have analyzed SR proteins in human dilated cardiomyopathy and have observed a decrease in the protein level of SERCA, yet saw no change in RyR, PLB, CQS or calreticulin. They have further supported their findings by comparing their study with one of Brillantes *et al.* (224) who have reported no significant alteration in the mRNA expression of RyR in human heart failure caused by dilated cardiomyopathy (224)

The UM-X7.1 cardiomyopathic hamster has been extensively studied, including the area of deficient Ca^{2+}-cycling in cardiac contractility. This particular hamster model has provided an opportunity to study the alterations that could occur in muscle disorders of the human, which frequently include that of the myocardium (68). Most studies that have been performed on UM-X7.1 have focused on SR function and Ca^{2+}-cycling during the end-stage of congestive heart failure (43,47). In doing so, these studies have lacked the presence of a serial physiological correlate for the changes in the biochemical aspect detected in the early stages of the development of heart failure (92). Ueyama *et al.*(92) have recently investigated the SR Ca^{2+}-release properties of this animal model that exhibits hypertrophic cardiomyopathy. They have

attempted to investigate changes in the SR function by determining the cellular content of the cardiac SR protein RyR, as well as ryanodine binding that occurs in the myopathic hamster hearts during the onset of heart failure. At 18 weeks of age, they found that the hypertrophic heart had a significant reduction in the Ca^{2+}-release activity, and at 28 weeks of age, the maximum number of [^{3}H] ryanodine binding sites had greatly declined. Interestingly, it was also reported that the protein content of RyR in the hypertrophic heart was initially increased, possibly as a compensatory mechanism, but the expression of RyR quickly declined below normal as heart failure advanced. Ueyama *et al.*(92) also noted that the amount of RyR protein in the cardiomyopathic hamster was preserved during hypertrophic cardiomyopathy and early heart failure, and then declined with the development of heart failure.

Schillinger *et al.*(225) have focused on RyR in human ischemic cardiomyopathy, and have reported a decrease in the gene expression of RyR mRNA. In addition, they observed no change in the protein levels of RyR when normalized to total protein. In contrast to the proteins responsible for Ca^{2+}-release, the proteins involved in Ca^{2+}-uptake, namely SERCA2a, showed a significant reduction in ischemic cardiomyopathy, when normalized to total protein. They further proposed two ideas regarding the progression of ischemic cardiomyopathy: 1) the sites of Ca^{2+}-uptake activity of the SR are decreased relative to the Ca^{2+}-release sites, and 2) the changes in the proteins of the SR were similar in both ischemic and dilated cardiomyopathy.

In view of comparing dilated and ischemic cardiomyopathy in terms of SR Ca^{2+} release properties, Sainte Beuve *et al.*(226) have analyzed the expression of RyR in the failing heart, by examining both mRNA and protein levels of RyR2 of the same heart samples. Particularly, they observed a decrease in the mRNA transcripts of RyR in dilated cardiomyopathy, with no change in ischemic cardiomyopathy. They reported a two-fold increase in the number of high affinity

ryanodine receptors in both ischemic and dilated cardiomyopathy. The authors then attempted to explain this discrepancy by suggesting that it was the change in the RyR properties instead of changes in the gene expression that altered the channel activity in the failing myocardium, which ultimately contributed to the abnormalities of intracellular Ca^{2+}-transport. They supported this by commenting on the fact that the dissociation between the increased number of high affinity receptors, with the levels of mRNA and protein, were due to the phenomenon of posttranscriptional regulatory factors that ultimately contributed to changes in the activity of Ca^{2+}-release in the failing heart of the human.

Another study that was conducted on the cirrhotic cardiomyopathic rat by Ward *et al.* (227) showed that RyR, along with SERCA2a and CQS did not illustrate any changes in the protein levels upon Western blot analysis. Further examination of the mRNA transcripts of these SR proteins revealed a similar trend of unaltered expression. This study further examined the binding characteristics of ^{3}H-ryanodine, in addition to measuring the Ca^{2+}-uptake activity to ultimately give the result of no quantitative change. In another unique study involving doxorubicin, Dodd *et al.* (228) inspected the association between doxorubicin cardiomyopathy and SR Ca^{2+} release in the rabbit. In comparing the doxorubicin-treated hearts with normal control heart, they observed no changes in the amount or the activity of the Ryr protein in the SR.

Another study by Sarpp *et al.* (50) measured the density of [^{3}H]-ryanodine receptors in the sarcoplasmic reticulum of cardiomyopathic Golden Syrian hamsters, and detected an increase in the density and affinity of these receptors as compared to control hamsters (50). It was then further suggested that an elevation of [^{3}H]-ryanodine binding in cardiomyopathic hearts occured before the emergence of cardiomyopathy. Interestingly, other studies that focused on the effects

of ryanodine release channels in cardiomyopathy, stated that as heart failure advanced, the levels of the ryanodine protein decreased, giving rise to overall cardiac contractile dysfunction (92).

SYNTHESIS OF DATA

The cardiomyopathic hamster experiments presented in this literature review have provided a background into the research done by previous researchers. Investigations into ischemic, idiopathic, diabetic, dilated and hypertrophic cardiomyopathies involving the SR function have revealed possible mechanisms that are implicated in the progession of heart disease. Starting at the molecular level, PLB, SERCA and RYR mRNA levels have been quantified amongst the various cardiomyopathies in the animals of mice, rats and hamsters. Their corresponding protein levels have also been measured, and have been further associated with experiments that overexpress and knock-out SR proteins to observe response of the heart in a variety of simulated conditions. Moreover, Ca^{2+} handling in diseased cardiomyocyte via Ca^{2+}-uptake and Ca^{2+} -release actions has also been highlighted and its results have benn linked to the process of cardiac contractile dysfunction. Inclusively, these experiments represent the accomplishment achieved by investigators world-wide.

To stimulate and engage in novel research, it is crucial to explore new avenues in search for higher answers. In doing so, this study focuses on the function and regulation of the SR in the cardiomyopathic hamster model. The cardiac contractile dysfunction that occured the J2N-k hamster has been hypothesized to originate at the level of the SR. As the SR is the main organelle responsible for cardiac contraction and relaxation, a study that focuses on its protein members will provide key information that is needed to elucidate the mechanisms involved in cardiomyopathy. From Ca^{2+} uptake via SERCA, to inhibition from PLB, the cardiac contractile cycle is dependent upon the actions of the SR. By identifying the SR proteins as a source linked to mechanisms associated with cardiac contractile dysfunction, this study fundamentally recognizes potential therapeutic targets to be used as a stepping stone for further scientific studies.

STATEMENTS OF THE PROBLEM AND HYPOTHESES TO BE TESTED

Dilated cardiomyopathy is a disease of the heart, which progresses into heart failure as a result of various pathophysiological changes that contribute to cardiac contractile dysfunction. The abnormalities that give rise to dysfunctional cardiac performance are closely associated with changes that occur in the SR. The cardiac SR is the key regulator of intracellular Ca^{2+} movements in the cell during the cardiac contractile-relaxation cycle, serving as both a Ca^{2+} pump and Ca^{2+} sink. Any alterations in this elegant orchestration of Ca^{2+} ion movements will ultimately result in changes in contractility of the heart muscle. Though many studies have tried to explain how the cardiac contractile cycle becomes disrupted, the subcellular mechanisms that contribute to the imbalance of Ca^{2+} handling still remain unclear. This study was therefore designed to enhance the understanding of the underlying mechanisms involved in the alterations of the SR function and its regulation that lead to contractile dysfunction in dilated cardiomyopathy of the J2N-k hamster. It is hypothesized that defects in the sarcoplasmic reticulum function and its regulation contribute to cardiac contractile dysfunction in dilated cardiomyopathy.

Previous reports have shown that the heart in the cardiomyopathic state experiences chronic SR dysfunction due to a depression in SR Ca^{2+} uptake and release activities. A central area of this study therefore focused on the Ca^{2+} transport activities of the SR in both the cardiac and skeletal muscle. This phenomenon of a dysfunctional SR could possibly be due to a reduction in SR protein levels, as well as a change in the phosphorylation or dephosphorylation of the SR Ca^{2+}-cycling proteins. To further elucidate the disruption of Ca^{2+} homeostasis in the dilated cardiomyopathic hamster heart, this study investigated the protein and mRNA levels of the Ca^{2+}-cycling SR members, as well as the alterations of the SR-associated kinase and

phosphatase activites. In addition, since both cardiac and skeletal entities were genetically affected in the J2N-k cardiomyopathic hamster, analysis of SR Ca^{2+} transport during contraction and relaxation of both muscles was also examined. It is anticipated that this study will provide comprehensive insight into the pathophysiology of contractile cardiac dysfunction in dilated cardiomyopathy. It is hoped that by identifying potential therapeutic targets of the SR, this information may be useful for the advancement of clinical therapeutic agents to work primarily to attenuate the dyfunctional cardiac contractile cycle that gives rise to eventual heart failure.

MATERIALS AND METHODS

1. Experimental model

The animal model used in this study was the cardiomyopathic J2N-k male hamster along with its age-matched healthy control, the J2N-n male hamster. Male hamsters were used, as they provide less physiological variables than would their female partners. These animals were provided from Dr. Nobuakira Takeda, in association with the Jikei University Aoto Hospital in Japan. The hamsters were housed in humidity- and temperature-controlled rooms and were allowed free access to water and standard chow. The hamsters were kept until the age of 36 weeks, at which time they were assessed for cardiac function via echocardiography, and then promptly sacrificed.

2. Echocardiographic Assessment

Cardiac ultrasound studies were carried out using the SONOS 5500 ultrasonograph (Agilent Technologies). On the day of the study, the hamsters were anesthetized with isoflurane gas on 2L oxygen, and were allowed to breathe spontaneously. The chest was shaved with electric clippers and echodcardiographic readings were recorded with the hamsters lying on their left side. A 12-MHz annular array ultrasound transducer was gently positioned on the coupling gel over the hemithorax, allowing appropriate contact without excessive pressure on the chest. Transthoracic short axis measurements were performed in the left lateral decubitus position, while the transducer was gently rotated to achieve the best position. In the parasternal short axis orientation, the probe recorded left ventricular diastolic and systolic measurements. The M-mode echocardiograms, at the papillary muscle level, measured the following parameters: interventricular septum diastole/systole (IVSd, IVSs), left ventricular internal dimension diastole/systole (LVIDd, LVIDs), left ventricular posterior wall diastole/systole (LVPWd,

LVPWd), ejection fraction (EF), percent fractional shortening (FS), cardiac output (CO), and heart rate (HR).

3. Sacrifce of Control and Cardiomyopathic Hamsters

Cardiomyopathic hamsters (J2N-k) and control hamsters (J2N-n) were anaesthetised with isoflurane on 2L oxygen, which was administered via head mask. Blood collection was obtained by inserting a syringe directly into the left ventricle of the intact beating heart. Blood was collected for measuring creatine kinase levels using the VET TEST CK kit (manufactured by Ortho-Diagnostics Inc. for IDEXX Laboratories, Inc., Westbrook, Maine, USA). Hearts were then quickly excised, washed in cold 0.9% saline, weighed and placed directly into liquid nitrogen. Skeletal muscle from both the right and left hind legs were obtained and placed immediately into vials, and submersed in liquid nitrogen. The heart and skeletal muscle were then stored at -70^{0}C until further use. Wet weights of the lungs, liver, right and left kidneys were measured and placed in dishes to completely air dry. Several weeks later, dry weights of these organs were measured and compared against the initial wet weights.

4. Isolation of Sarcoplasmic Reticulum Vesicles

SR vesicles from the cardiac muscle were isolated in accordance with a previously described method (149,229). Briefly, the heart tissue was minced, and immediately homogenized twice for a period of 20 seconds with a Polytron homogenizer (Brinkman, Westbury, NY) set at half the maximal setting. The buffer used for homogenisation consisted of the following (in mM): 10 $NaHCO_3$, 5 NaN_3, 15 Tris-HCl pH 6.8 and protease inhibitors (in μM): 1 leupeptin, 1 pepstatin and 100 phenylmethul-sulfonylflouride. Shortly after the homogenization process, the resulting homogenate was promptly centrifuged for 20 min at 9,500 rpm (Beckman, JA 20.0) to

remove cellular debris. The remaining supernatant was centrifuged for 45 min at 19,000 rpm (Beckman, JA 20.0). The supernatant, which contained the cytosolic fraction, was aliquoted and immediately stored at – 70° C. The pellet was suspended in a buffer containing 0.6M KCl, 20 mM Tris-HCl pH 6.8, and was further centrifuged for 45 min at 19,000 (Beckman, JA 20.0). The final pellet that contained the SR fraction was then suspended in 250 mM sucrose and 10 mM histidine pH 7.0, and aliquoted. The entire procedure of the isolation the SR vesicles, was carried out at 4° C.

5. Protein Estimation

Determination of the protein concentration of the SR vesicles was done using Lowry's method. Different concentrations of bovine serum albumin were used as standards. 2ml of working solution that contained 2% potassium sodium tartarate, 1% $CuSO_4$ and 2% Na_2CO_3 (in 0.1N NaOH) in a ratio of 1:1:100 was added to the standards, blank and samples followed by immediate vortexing. After a period of 10 min, each tube received 0.2ml of 1N phenol reagent (Folin and Ciocalteau reagent). 20 minutes later, the absorbance of each tube at 623nm was measured using the Ultrospec 2100 pro spectrophotometer (Biochrom). The protein concentration was thus determined using the standard curve of BSA, using a custom- made computer software program in Microsoft Excel.

6. Determination of Ca^{2+}-Uptake Activity

The SR Ca^{2+}-uptake activity was measured using a previously described procedure (230). For a total volume of 250µl, the reaction mixture contained (in mM): 50 Tris-maleate (pH 6.8), 5 NaN_3, 5 ATP, 5 $MgCl_2$, 120 KCl, 5 K-oxalate, 0.1 EGTA, 0.1 $^{45}CaCl_2$ (12,000 cpm/nmol) and 0.25 ruthenium red. Using the computer program of Fabiato (231) the concentration of free Ca^{2+}

in this medium was 8.2μM. The reactions were all performed at 37^0 C. The Ca^{2+}-release channel was primarily inhibited by the addition of ruthenium red to the mixture. The Ca^{2+}-uptake reaction was initiated by the addition of SR membranes (20μg) and terminated by filtering 200μl aliquots through 0.45μm Millipore filters after 1 minute. Upon filtration, the filters were washed twice with 3 ml washing buffer and then promptly dried for 1 hour at 60^0 C. The filters were then placed into scintillation vials, 10 ml of scintillation fluid was added to each vial and all vials were counted in a beta liquid scintillation counter. During the 2 min incubation period in the counter, the Ca^{2+}-uptake reaction was linear.

7. Determination of Ca^{2+}-induced-Ca^{2+} Release Activity

Ca^{2+}-release activity of the isolated SR vesicles was measured by a modified procedure as reported previously (106,232). SR vesicles were suspended in a reaction mixture of total volume 625μl that included (in mM): 62.5 μl of 0.5mg/ml SR, 100 KCl, 5 $MgCl_2$, 5 potassium oxalate, 5 NaN_3 and 20 Tris-HCl (pH 6.8). The SR fraction was then incubated for 45 minutes at room temperature with 10 μM $^{45}CaCl_2$ (20 mCi/L) and 5 mM ATP. Ca^{2+}-induced-Ca^{2+}-release was then carried out through the addition of 1 mM EGTA plus 1 mM $CaCl_2$. The reaction was promptly terminated 15 seconds later using the Millipore filtration technique. The filters were then put in vials and 10ml of scintillation fluid was added. The vials were then counted in a beta-scintillation counter. The Ca^{2+} -induced Ca^{2+} release was completely prevented (95% to 97%) by the treatment of 20μM ryanodine to the SR preparations.

8. Measurement of CaMK and PKA Activities

For the following phosphorylation experiments, the SR preparations were initially isolated in the presence of a phosphatase inhibitor, 1mmol/l Na pyrophosphate, to prevent any

occurance of dephosphorylation. Using assay kits from Upstate Biotechnology (Lake Placid, NY), the activities of both CaMK and PKA from cytosolic and SR preparations were determined. The assay kit for CaMK is based upon the theory that the specific substrate peptide KKALRRQETVDAL) is phosphorylated via the transfer of the γ-phosphate of [$^{32}\gamma$ P] ATP by CaMK II. Due to the fact that CaMK from both the SR and the cytosol phosphorylated the exogenous substrate, the activity of CaMK was calculated as the difference of the values in the presence and absence of the exogenous substrate. Initially, the assay dilution buffer I for CaMK was mixed with the substrate and inhibitor cocktail (from the assay kit) along with the sample and DDW. Next, the radioactive mixture was prepared by adding $^{32}\gamma$-P to Mg-ATP (from the assay kit) in a concentration ratio of 1:9. The reaction was then initiated by adding 10 µl of the radioactive mixture to the reaction mixture and then incubated at 30 °C for 10 min. To stop the reaction, 25 µl the radioactive-sample cocktail was then spotted on numbered phosphocellulose filter papers. Immediately after, the phosphocellulose squares were washed 3 times for a period of 5 min each with 0.75% phosphoric acid, and then one last period of 5 min with acetone. The phosphocellulose squares were then placed in scintillation vials, 10ml of scintillation fluid was added, and then all vials were placed in the beta-liquid scintillation counting machine.

The assay kit for the PKA activity measurement is based on the theory of phosphorylation of a specific substrate, kemptide, by cAMP-dependent protein kinase via the transfer of γ-phosphate of [$^{32}\gamma$P] ATP. The procedure for the measurement of PKA activity for both SR and cytosolic fractions were identical to the above descried for CaMK.

9. Measurement of Phosphatase Activity

The phosphatase activity of the SR was based upon the technique previously established (149). Briefly, phosphatase activity was determined using the Ser/Thr assay kit obtained from Upstate Biotechnology (Lake Placid, NY). This assay was based upon the principle of the

dephosphorylation of the synthetic phosphopeptide K*Rp*TIRR. The reaction was promptly initiated by the addition of 30 μg of SR to the microtiter well that contained the presence or absence of the synthetic substrate (200 μm) and was incubated for a period of 30 min, giving a total assay volume of 25 μl. Termination of the reaction was achieved by the addition of 100 μl of Malachite Green solution. The reaction mixture then sat for 15 minutes to allow proper time for color development, and then the absorbance was read at 660 nm to determine the amount of inorganic phosphate released. This assay was performed using the conditions that contained both the presence and the absence of the exogenous substrate. The phosphatase activity was therefore calculated by subtracting the values that included the absence of the substrate from the values including the exogenous substrate.

10. Northern Blot Analysis and Molecular Probes

Total RNA was extracted from heart tissue by the TRIzol ® Reagent method (GIBCO BRL, Burlington, ON, Canada). Samples of total RNA in sterile distilled water with 0.2% DEPC were denatured at 65^{0}C for 10 min, followed by electrophoresis in a 1.2% agarose/formaldehyde gel containing 1 M formaldehyde. 20 μg of total RNA were used in the rest of the study. Gel electrophoresis was immediately followed by transfer of the fractionated mRNA transcripts to a charge-modified nylon filter for a period of 24 hours (NYTRAN Maximum Strength Plus, Schleicher and Schuell, Keene, NH, USA). The nylon membrane was then promptly UV cross-linked (UV Stratalinker 2400 Stratagene). Hybridzation of the blots took place at 42°C overnight in the INNOVA 4080 incubator that oscillated at a rate of 65 rpm. Labeled random primed cDNA or oligonucleotide probes were added to the prehybridization solution and incubated overnight under the same conditions. Washing of the membranes with 1X standard saline citrate and 0.1% SDS occurred at room temperature. The membranes were then exposed to Kodak X-Omat-AR film using intensifying screens at –70° C. The radiolabeled mRNA bands were then

scanned using a densitometer GS-800 (BIO-RAD, California, USA) and quantified with the Quantity One 4.5.0 software (BIO-RAD, USA). The optical density of each band was then divided by that of the 18S band for normalization as an internal standard and the relative levels were calculated as percentage of the mean value of the corresponding control. The inserts were separated from recombinant plasmids and used as probes. RyR was probed with a 2.2 kb cDNA fragment from rabbit cardiac ryanodine receptor (courtesy of Dr. D.H, MacLennan, University of Toronto, Toronto, Canada,). SERCA2a was probed with a 0.762 kb cDNA fragment from the rabbit heart Ca^{2+}-pump ATPase (courtesy of Dr. A.K. Grover, McMaster University, Hamilton, Canada). PLB was probed with a 0.52 kb cDNA fragment from the rabbit heart (courtesy of Dr. D.H. MacLennan, University of Toronto, Toronto, Canada). CQS was probed with a 2.5 kb cDNA fragment from the rabbit heart (courtesy of Dr. A. Zilverman, University of Cincinnati, Cincinnati, OH, USA). The 18S probe was a 24 base oligonucleotide probe (5'ACGGTATCTGATCGTCTTCGAACC –3') of the rat ribosomal RNA and was used as an internal standard to account for differences in nucleic acid loading as well as for the transfer. The cDNA used to hybridize specific mRNA transcripts were prepared and autoradiographed using a Random Primer DNA labelling system (New England Nuclear, Boston, MA) radiolabelled with α -^{32}P-dCTP.

11. Western Blot Analysis

The relative protein contents of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a), phospholamban (PLB), phosphoserine phospholamban (P-Ser16-PLB), phosphothreonine phospholamban (P-Thr17-PLB), calsequestrin (CQS), and c-AMP-dependent protein kinase (PKA isoforms α, β, and γ) were measured in accordance with a previously described method (229). Protein samples were suspended in a 1:1 ratio with Laemmlli buffer that contained: 0.1 M Tris-HCl (pH 6.8), 15% (w/v) sodium dodecyl sulphate (SDS), 15% glycerol,

8% β-mercaptoethanol and 0.002% bromophenol blue. The solution was then denatured by boiling in water for 3 min. SR protein samples (13.8 μg protein) were then separated by SDS-PAGE on a 15% gel for PLB, Ser16-PLB, Thr17-PLB, 12% gel for CQS, PKA α, β, and γ, and 10% gel for SERCA. The protein bands were transferred to polyvinylidene diflouride membranes (Millipore Corporation, Bedford, MA, USA) at 200 V for 2 hours. The transfer buffer included 25 mM Tris-HCl, 192 mM glycerine and 4% methanol (v/v). After the transfer procedure, the membranes were incubated overnight at 4° C in blocking buffer (TBS: 10 mM Tris and 150 mM NaCl, combined with 5% fat-free powered milk). The next day, the membranes were incubated at room temperature for 1 hour in monoclonal anti-SERCA2a (*1:10,000*) (Affinity Bioreagents, Inc., Golden, CO, USA), monoclonal anti-phospholamban (*1:5000*) (Upstate Biotechnology, Lake Placid NY, USA), monoclonal anti-calsequestrin (*1:1000*) (Upstate Biotechnology, Lake Placid, NY, USA), polyclonal anti-p-phospholamban (Ser 16) (*1:5000*) (Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA), polyclonal anti-p-phospholamban (Thr 17) (*1:5000*) (Badrilla, Leeds, UK), polyclonal anti-PKAα (*1:1000*) (Santa Cruz Biotechnology, Inc,. Santa Cruz California, USA), polyclonal anti-PKAβ (*1:200*) (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), and polyclonal anti-PKAγ (*1:1000*) (Santa Cruz Biotechnology Inc., Santa Cruz California, USA). The membranes were then incubated at room temperature for 45 min with a secondary antibody. For SERCA2a, PLB, CQS, P-Ser16-PLB and P-Thr17-PLB, a biotinylated anti-mouse IgG antibody (*1:5000*, Amersham Life Science, Oakville, ON, Canada) was used. For PKA α, β, and γ, a biotinylated anti-rabbit IgG antibody (*1:10,000*, Amersham Life Science, Oakville, ON, Canada) was used. This was followed by incubation of membranes for 30 min at room temperature with a streptavidin-conjugated horseradish peroxidase (*1:4500*, Amersham Life Science, Oakville, ON, Canda) in TBST. In between the incubation with antibodies, the membranes were washed 3 times with TBST for a period of 15 min. The antigen-antibody complexes incorporated in each membrane

were then detected using the chemiluminesence ECL kit (Amersham Corporation, Arlington Heights, IL, USA). The protein bands were then visualized on Hyperfilm-ECL (Amersham Corporation, Arlington Heights, IL, USA). For analysis of density of the protein bands, an Imaging Densitometer model GS-800 (Bio-Rad Ltd., Hercles, CA, USA) was used. These values were quantified using the Quantity one 4.4.0 software from Bio Rad. Equal protein loading was verified by staining the membrane with Ponseau S before immunoblotting and with Coomassie Brilliant Blue at the end of the experiment.

12. Statistical Analysis

All the results were expressed as mean ± SE and were evaluated by Analysis of Variance (ANOVA) test as well as the student t-test. A level of $P > 0.05$ was considered the threshold for statistical significance between the control and cardiomyopathic hamster groups.

RESULTS

1. General Characteristics of the Hamster Model

The array of general hamster characteristics in Table 1 depicted no change in heart mass, body weight or heart to body weight ratio in these 36-week cardiomyopathic hamster experimental animals. Table 1 also showed the wet and dry weights of the lungs, liver and kidneys, with no significant changes between the two models. However, the cardiomyopathic hamster exhibited a major increase in its blood creatine kinase levels (units/L).

2. Cardiac Performance

Figure 2 has outlined the *in vivo* parameters that were measured in both the J2N-k and J2N-n models. The echocardiographic assessment ultimately revealed a significant decrease in ejection fraction (EF), percent fractional shortening (%FS) (Figure 2A,B), cardiac output (CO) and heart rate (HR) (Figure 2C,D) in the cardiomyopathic model, suggesting a decline in cardiac performance. The dimensions of the heart chamber and walls were also measured, showing no change in the interventricular septum (IVS), left ventricular posterior wall-diastole (LVPWd), or left ventricular posterior wall-diastole (LVPWd), yet a slight increase in the left ventricular internal dimension – diastole (LVIDd) and left ventricular posterior wall-systole (LVPWs) was detected (Figure 3).

Table 1: General Characteristics of Control and Cardiomyopathic Hamsters

Parameters	Control	Cardiomyopathic
Heart Weight (x10-1)(g)	**0.38 ± 0.04**	**0.44± 0.05**
Body Weight (g)	**118 ± 3.50**	**121 ± 2.30**
Heart/Body Weight Ratio ($x10^{-3}$)	**4.3 ± 0.42**	**4.7 ± 0.35**
Blood Creatine Kinase (units/L)	**811 ± 239**	**3636 ± 705 ***
Wet Lung Weight (g)	**0.68 ± 0.01**	**0.72 ± 0.03**
Dry Lung Weight (g)	**0.16 ± 0.002**	**0.17 ± 0.004**
Wet / Dry Lung Weight Ratio	**4.23 ± 0.07**	**4.36 ± 0.14**
Wet Liver Weight (g)	**4.31 ± 0.14**	**4.72 ± 0.25**
Dry Liver Weight (g)	**1.53 ± 0.06**	**1.61 ± 0.08**
Wet / Dry Liver Weight Ratio	**2.81 ± 0.03**	**2.92 ± 0.03 ***
Wet Right Kidney Weight (g)	**0.49 ± 0.01**	**0.44 ± 0.01**
Dry Right Kidney Weight (g)	**0.12 ± 0.002**	**0.12 ± 0.003**
Wet / Dry Right Kidney Weight Ratio	**4.08 ± 0.07**	**3.89 ± 0.12**
Wet Left Kidney Weight (g)	**0.49 ± 0.01**	**0.47 ± 0.02**
Dry Left Kidney Weight (g)	**0.12 ± 0.002**	**0.12 ± 0.003**
Wet / Dry Left Kidney Weight Ratio	**4.01 ± 0.08**	**3.86 ± 0.08**

*$P < 0.05$ vs. control; (n=8 for each group).

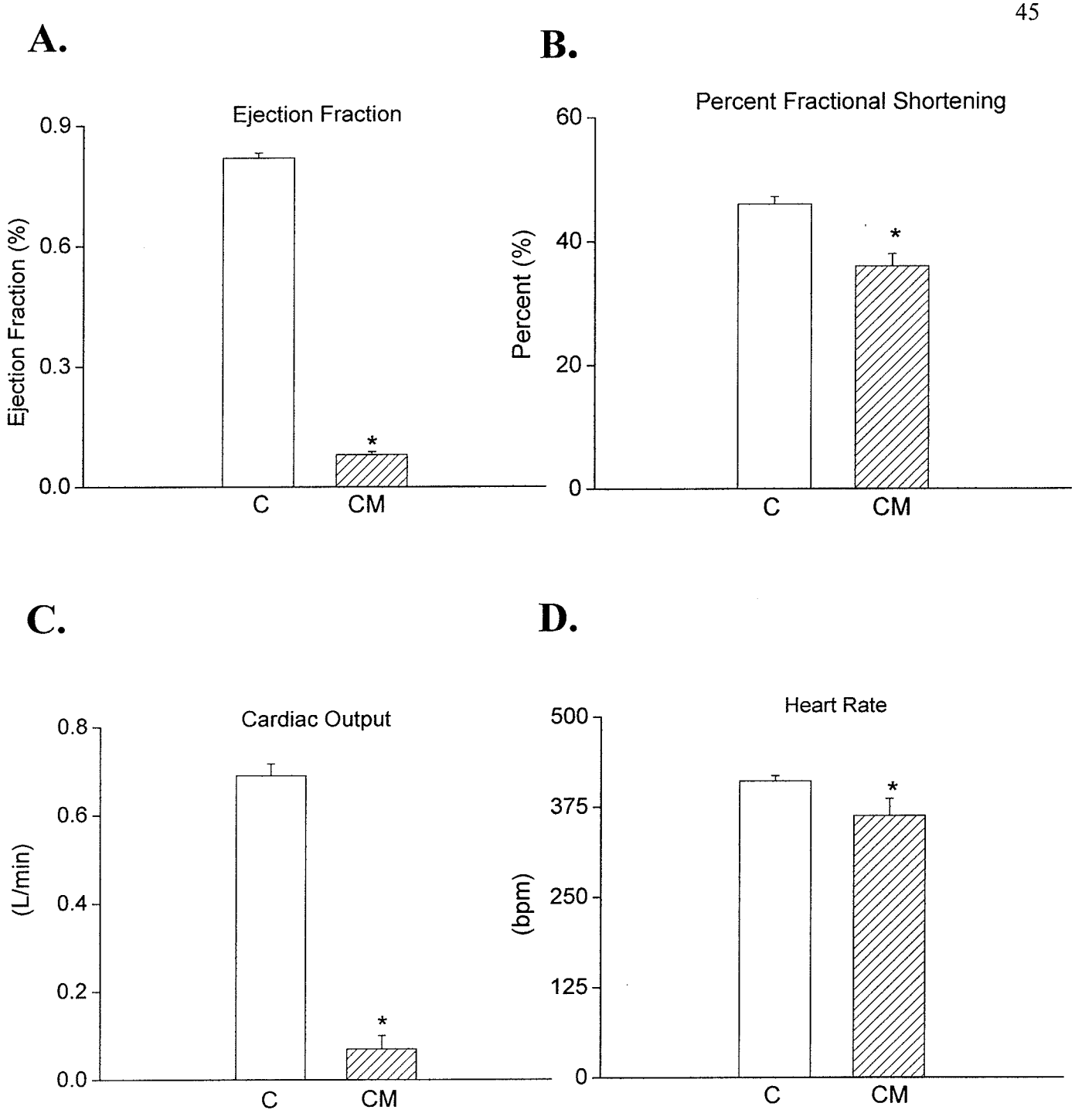

Figure 2: Echocardiographic assessment of cardiac function in control (C) and cardiomyopathic (CM) hamsters. *P < 0.05 vs. control; (n=8 for each group); bpm, beats per minute; L/min, litres per minute.

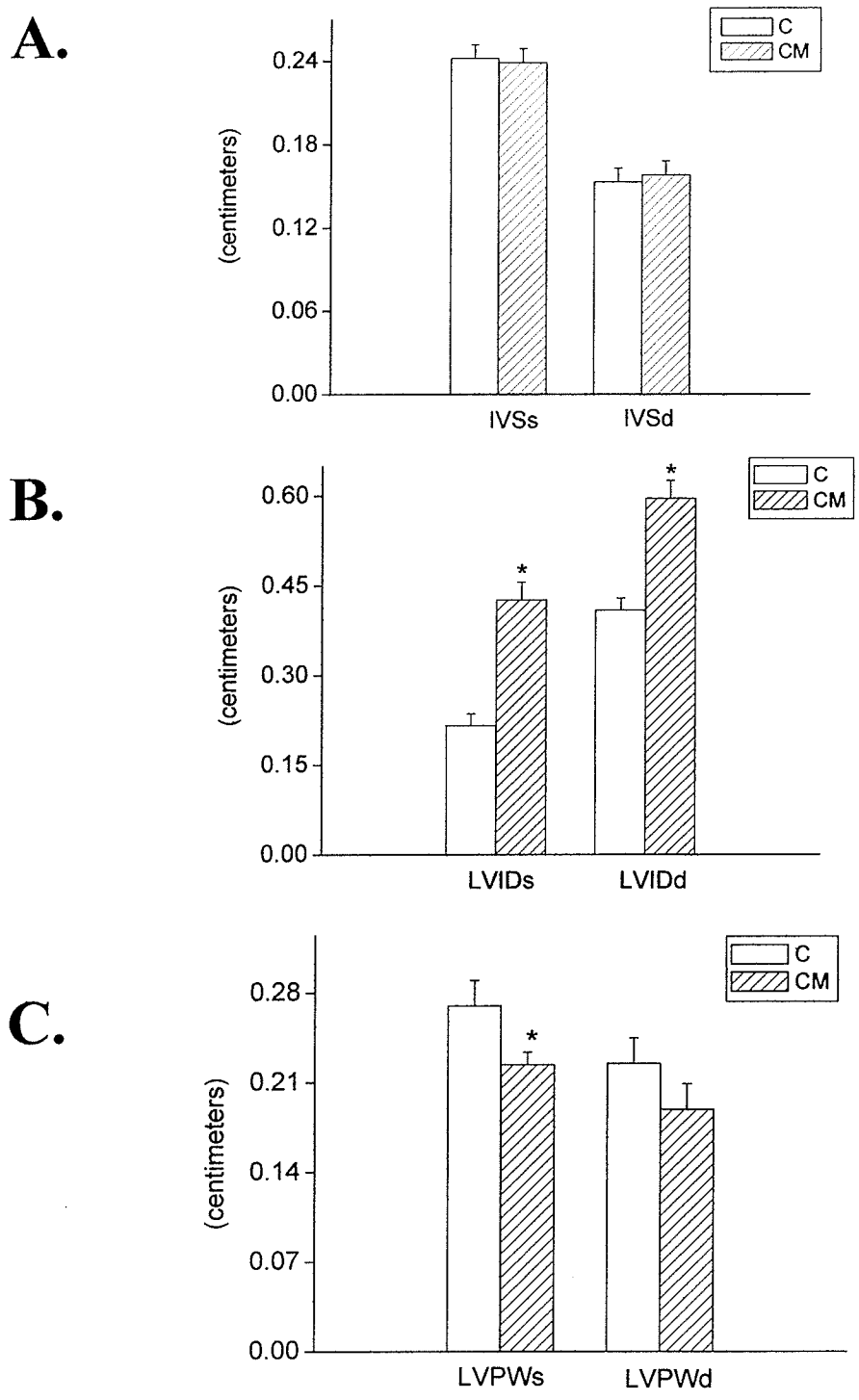

Figure 3: Echocardiographic assessment of internal heart dimensions of control (C) and cardiomyopathic (CM) hamster hearts. IVS (internal ventricular septum), LVID (left ventricular internal dimension), LVPW (left ventricular posterior wall), s/d (systole/diastole). *$P < 0.05$ vs. control; (n=8 for each group).

3. Ca^{2+} Uptake and Ca^{2+} Release Determination

Any alterations in cardiac contractile function could be associated with abnormalities in SR function. To examine this possibility cardiac muscle SR function of both the control and cardiomyopathic hearts were analysed by examining SR Ca^{2+} uptake and Ca^{2+} release. Figure 4A has depicted a decrease in Ca^{2+} uptake in the J2N-k model, while Figure 4B has shown a similar trend of reduction in the Ca^{2+} release in the cardiomyopathic heart. The cardiomyopathic hearts showed a 50.5% decrease in the SR Ca^{2+}-uptake and a 57.2% decrease in the SR Ca^{2+}-release.

4. PLB Phosphorylation by CaMK and PKA

a. PLB Phosphorylation and - SR Associated CaMK- and PKA Activities

Due to the fact that the abnormalities in phosphorylation of PLB by PKA and CaMK would influence the SR Ca^{2+} uptake, we studied the status of phosphorylation of PLB at ser16 by PKA. In addition, we also examined the status of phosphorylation of PLB at thr17 by CaMK. Furthermore, to correlate with the changes in the phosphorylation status of PLB, we examined the enzymatic activites of PKA and CaMK in control and cardiomyopathic hamster hearts. Figure 6A showed no changes in PLB phosphorylation at Thr-17 by CaMK. Figure 6B has shown no relative change of SR associated CaMK activity in cardiomyopathic hearts in comparison to control. PKA phosphorylation of PLB was also investigated in control and cardiomyopathic hamster hearts. A significant reduction in PKA-SR associated kinase activity of 77.8% in the cardiomyopathic heart (Figure 7).

b. CaMK- and PKA- Cytosol Associated Activity

To determine if the effects of CaMK and PKA phosphorylation in cardiomyopathy was soley restricted to the SR, we expanded our studies to examine the activity of these kinases in the cytosolic fraction.

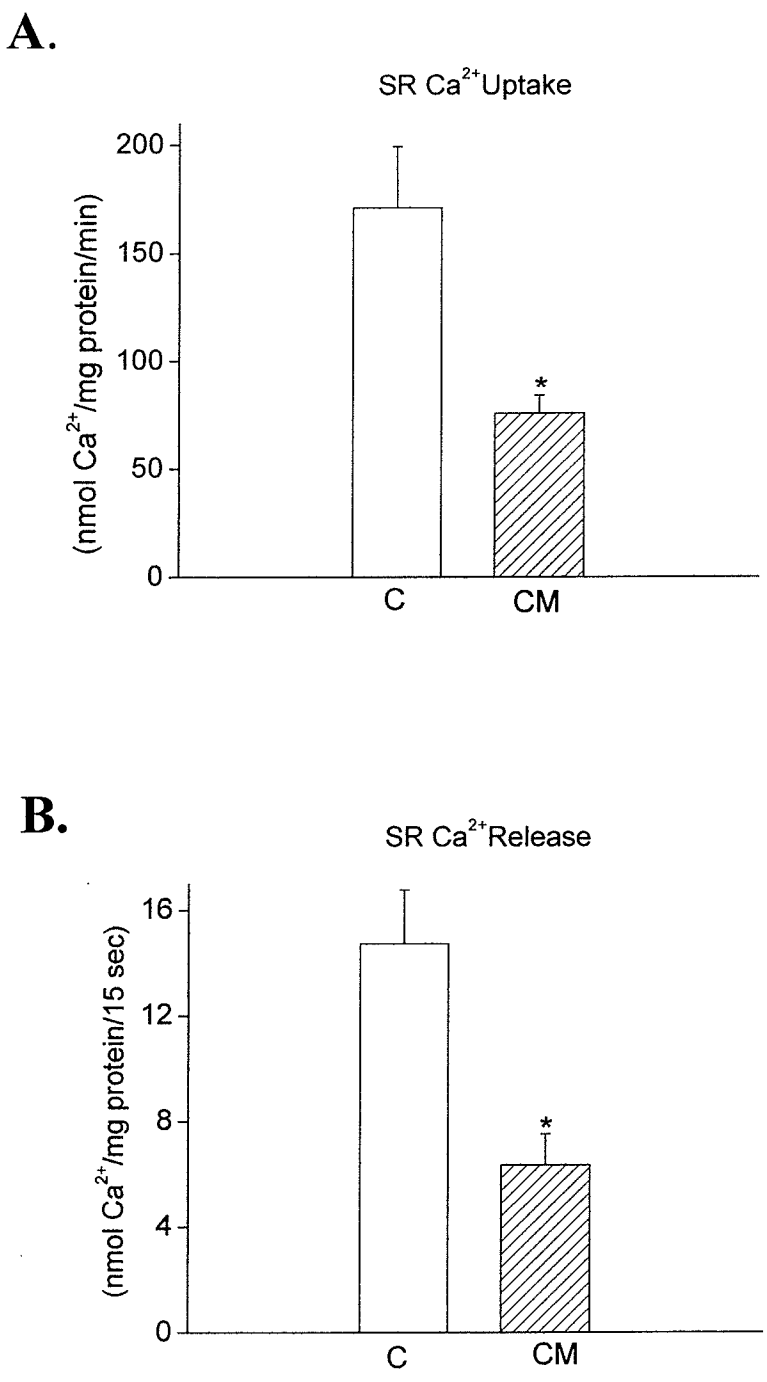

Figure 4: Analysis of sarcoplasmic reticulum (SR) Ca^{2+} -uptake and –release in control (C) and cardiomyopathic (CM) hamsters. *P < 0.05 vs. control; (n=7 for each group).

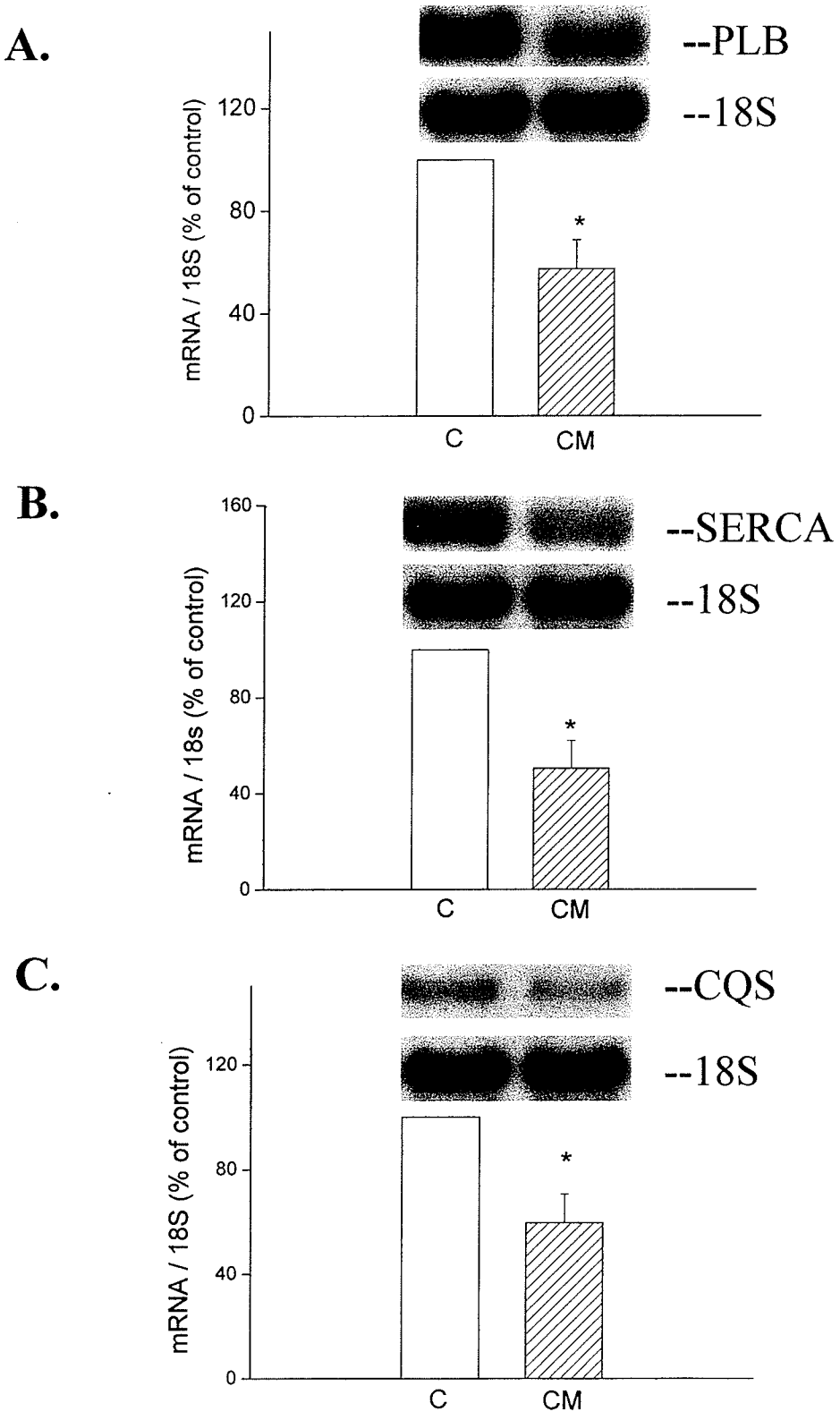

Figure 5: Phospholamban (PLB), sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA2a) and calsequestrin (CQS) gene expression in control (C) and cardiomyopathic (CM) hamster hearts. 18S was used as a loading control. *P < 0.05 vs. control; (n=8 for each group).

As shown in Figure 6C, there was no change in CaMK phosphorylation in the cytosol of cardiomyopathic hearts, whereas Figure 7C clearly indicated a significant reduction in the PKA cytosolic activity of 17.4% in the cardiomyopathic state.

5. Protein Content Analysis

As changes in the SR function and its regulation are observed, it can be postulated that this was a result of the alterations in the expression of the SR Ca^{2+}-cycling and SR regulatory proteins. Consequently, we examined the total protein content of the following proteins: SERCA2a, PLB, CQS, α-PKA, β-PKA, and γ-PKA. The J2N-k cardiomyopathic hamsters showed a decrease in expression of SERCA2a by 67.8%, with no significant change in the expression of PLB and CQS, followed by an increase in the ratio of SERCA/PLB by 7 fold (Figure 8). Figure 9 has shown an increase in the protein content of PKAα by 1.5 fold, a decrease in the protein level of PKAβ by 50.2%, and an increase in the PKAγ isoform of 1.01 fold in the J2N-k cardiomyopathic hamster.

6. Determination of mRNA expression

To further understand the reasons for changes in the protein levels of the SR Ca^{2+} proteins responsible for Ca^{2+} uptake, the mRNA levels of SERCA2a, PLB were examined in control and cardiomyopathic hearts. Northern blot analysis showed that the J2N-k hamster had reduced mRNA levels of PLB to 57 % (Figure 5A), SERCA2a to 50.5% (Figure 5B), and CQS to 59.5% as compared to control (Figure 5C).

7. Protein Phosphatase Activity

As changes were observed in SR-associated PKA phosphorylation of PLB in the cardiomyopathic state, the SR-associated protein phosphatase activity in the SR was measured. Our results revealed a significant increase in the protein phosphatase activity of 2.3 fold in the cardiomyopathic hearts with more than double the amount observed in the control hearts (Figure 10).

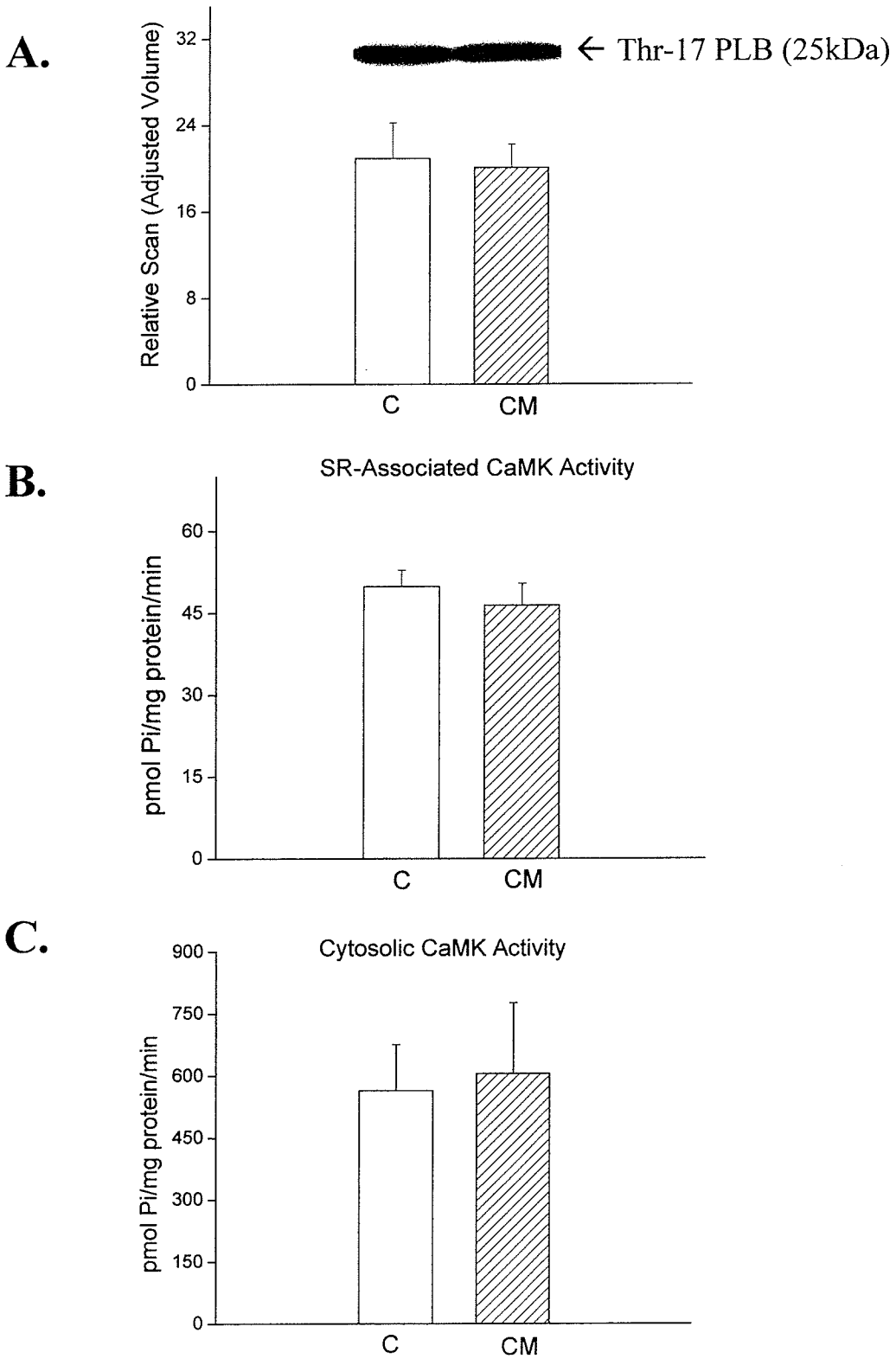

Figure 6: Phosphothreonine phospholamban (thr-17 PLB) levels (A) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) activity (panel B,C) in control (C) and cardiomyopathic (CM) hamster hearts. *P < 0.05 vs. control; (n=8 for each group).

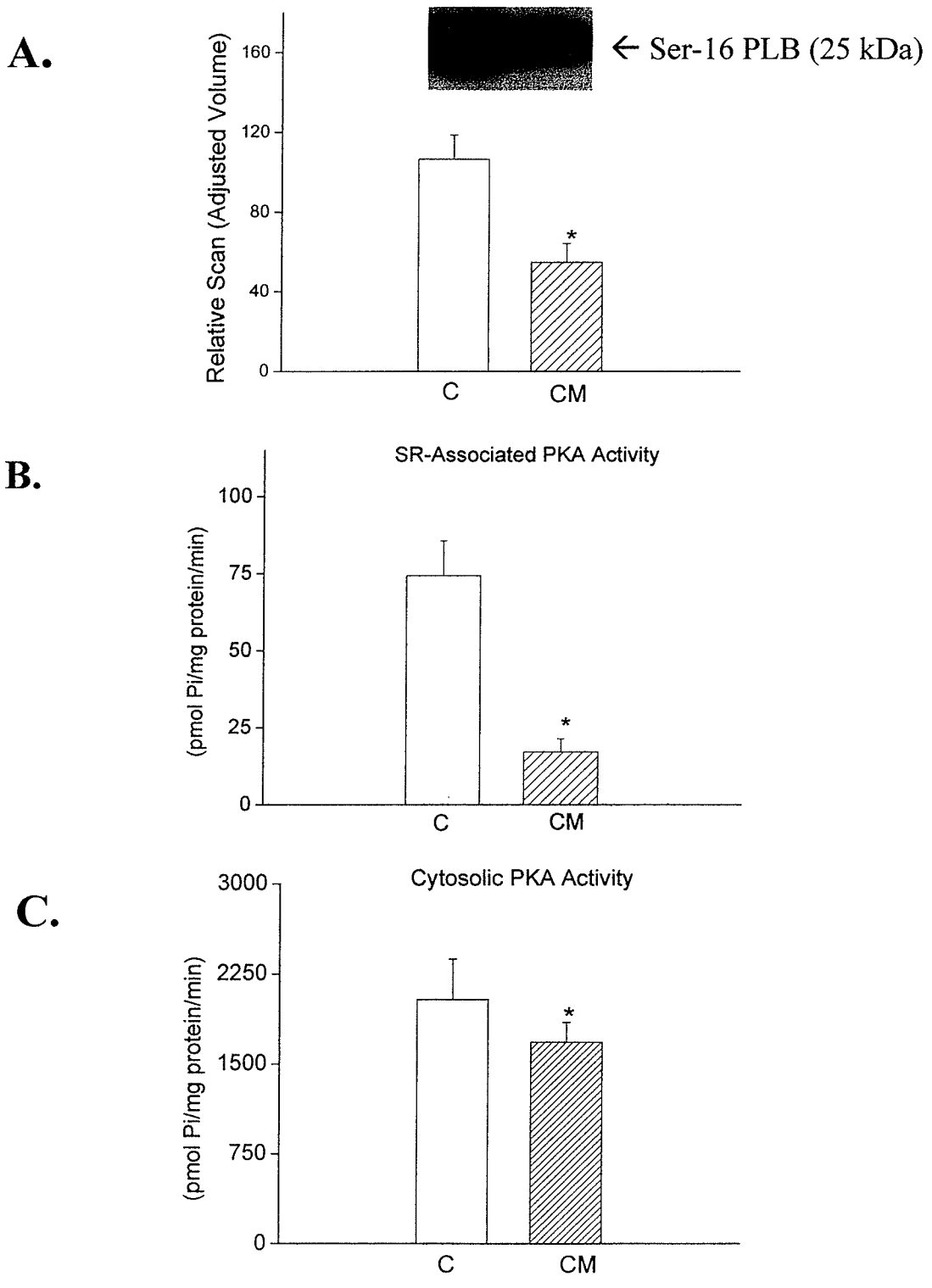

Figure 7: Phosphoserine phospholamban (ser-16 PLB) levels (A) and cAMP-dependent protein kinase A (PKA) activity (panel B,C) in control (C) and cardiomyopathic (CM) hamster hearts. *P < 0.05 vs. control; (n=8 for each group).

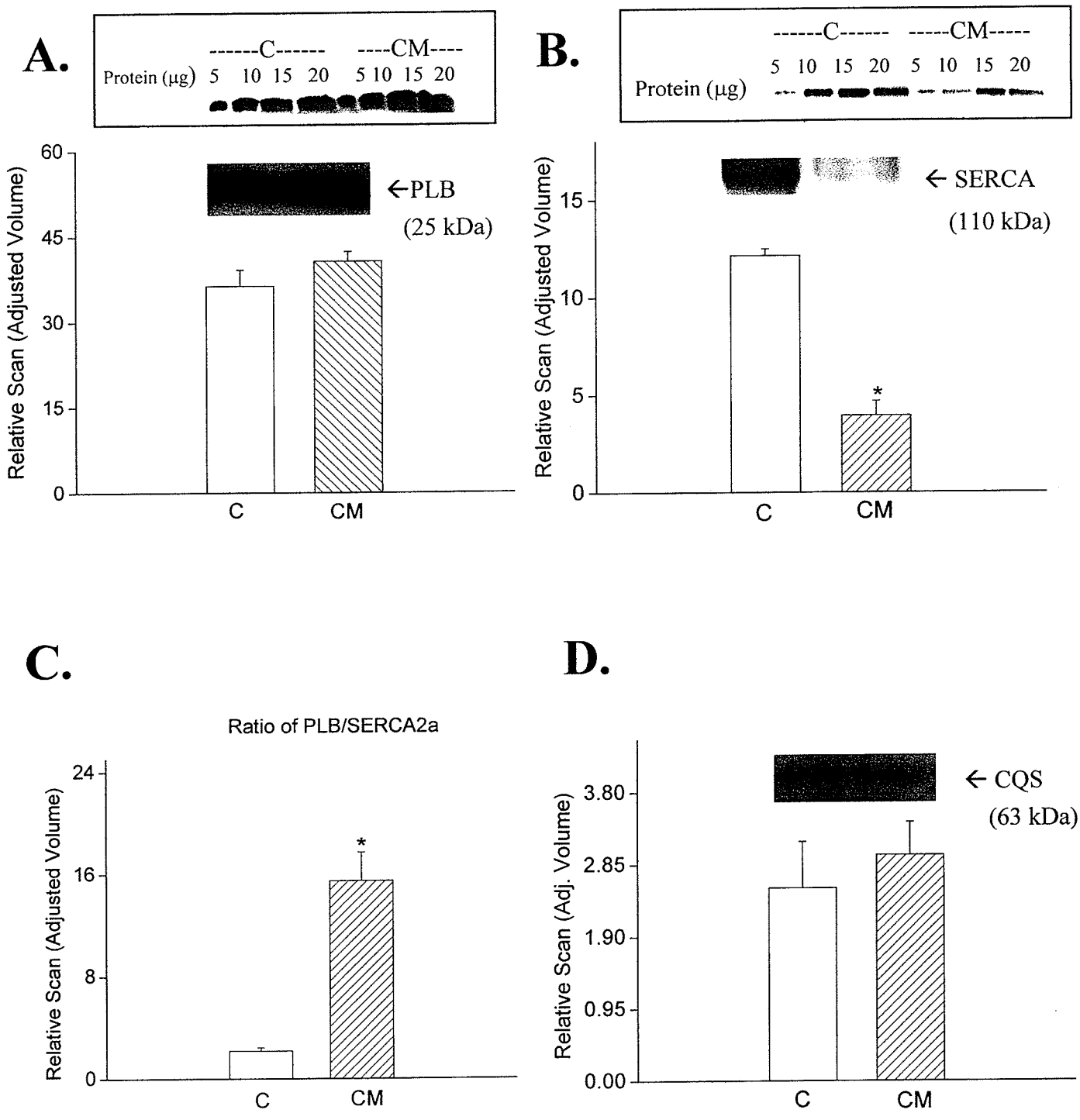

Figure 8: Western blot analysis of phosholamban (PLB), sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA2a) and calsequestrin (CQS) in control (C) and cardiomyopathic (CM) hamster hearts. *P < 0.05 vs. control; (n=6 for each group).

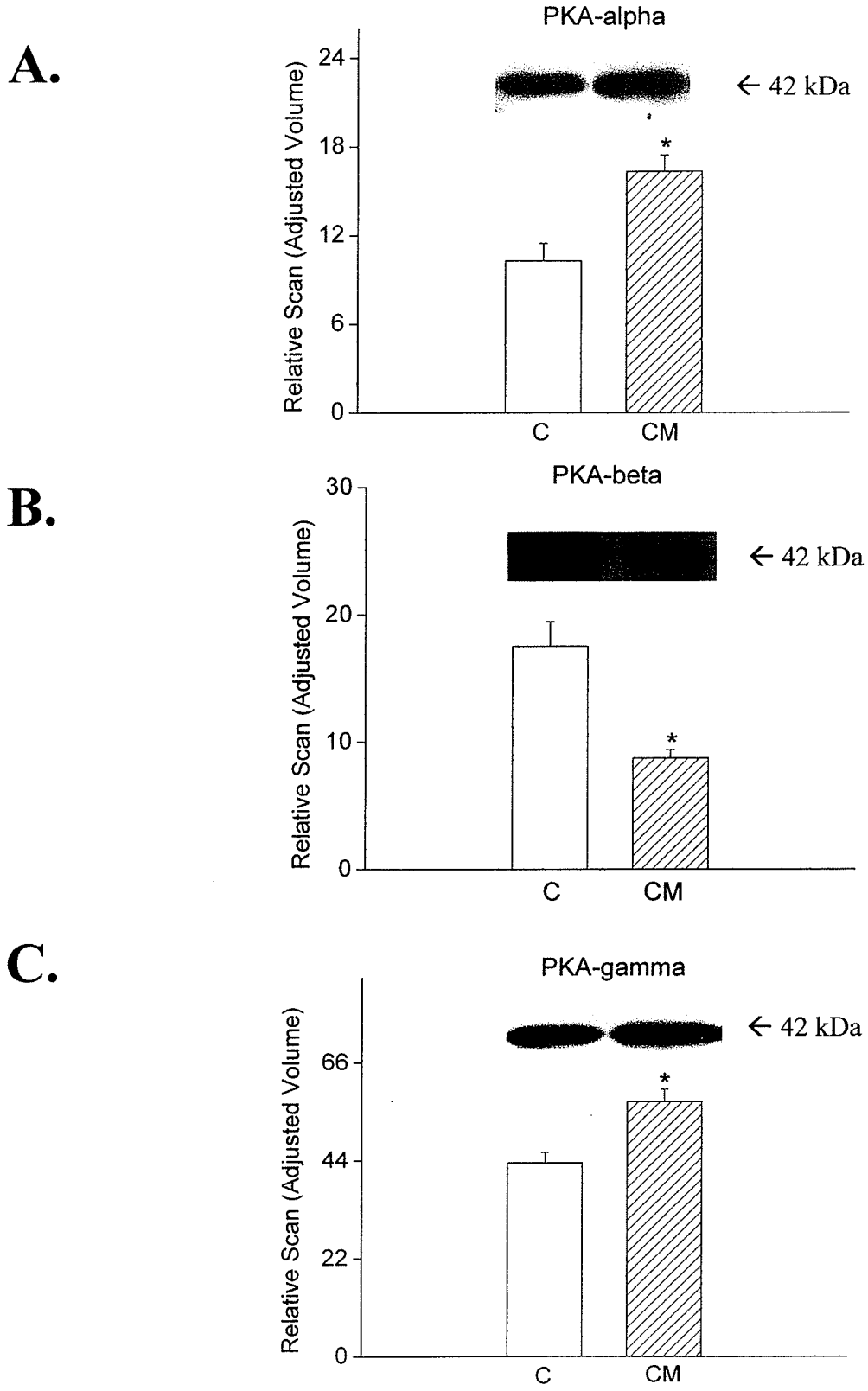

Figure 9: Western blot analysis of cAMP-dependent protein kinase A (PKA) isoforms α, β, and γ in control (C) and cardiomyopathic (CM) hamster hearts. *P < 0.05 vs. control; (n=8 for each group).

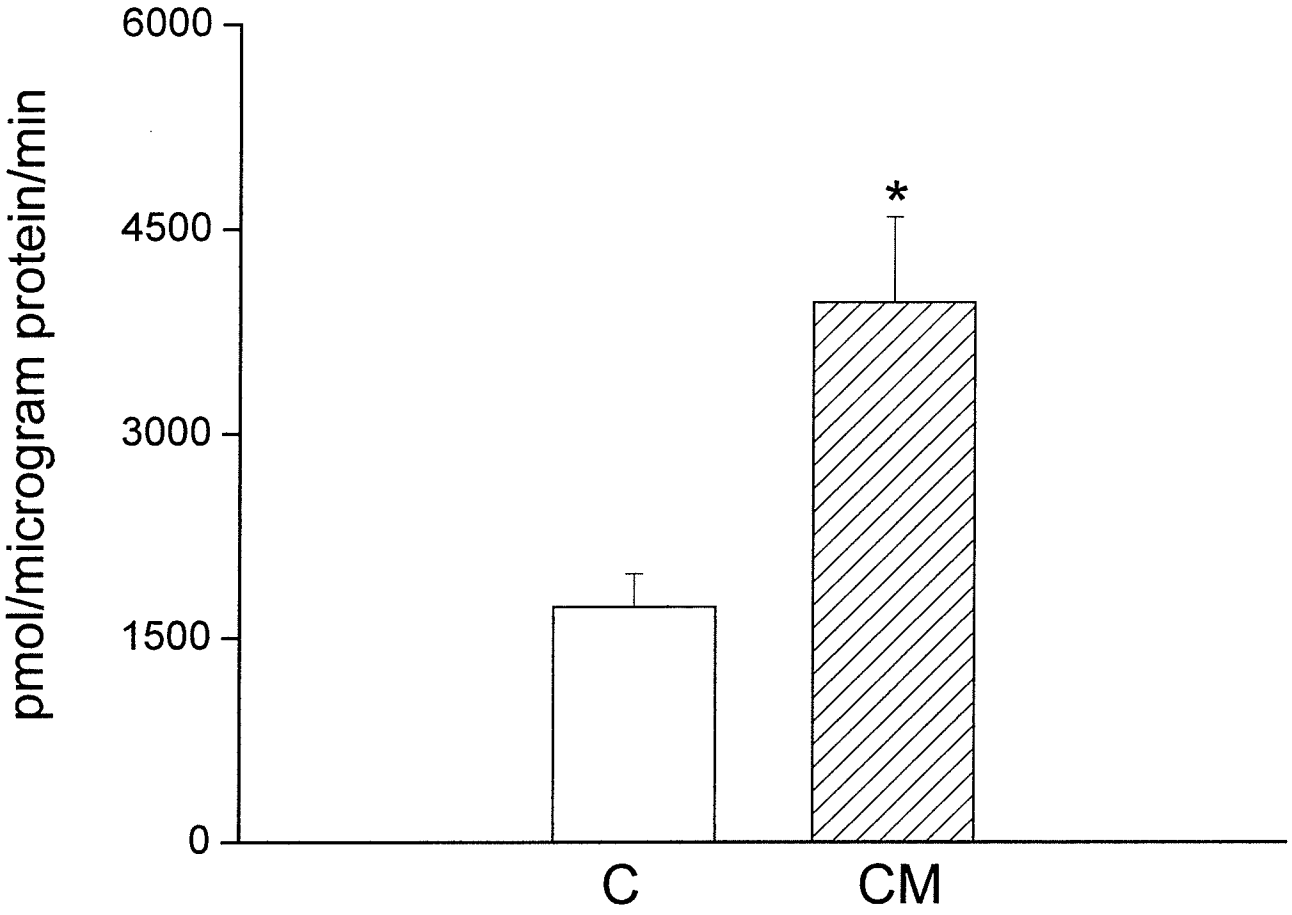

Figure 10: SR associated phosphatase activity in control (C) and cardiomyopathic (CM) hamster hearts. *P < 0.05 vs. control; (n=8 for each group).

DISCUSSION

1. General Characteristics and Cardiac Performance

The results in this study revealed impaired cardiac function in the cardiomyopathic hamster heart. This was evident from the decrease in the following parameters that were examined via echocardiography: ejection fraction, percent fractional shortening, cardiac output and heart rate. General characteristics of the control and cardiomyopathic hamster models showed no increase in body weight and no increase in the heart/body weight ratio, suggesting the absence of hypertrophy. However, there was an increase in the dimension of the ventricular chamber (LVIDd), which correlated with the fact that there was dilatation present, thereby representing the key characteristic of dilated cardiomyopathy. As previously supported by Kato *et al.*(233) dilation of the ventricular chambers is the hallmark feature that characterizes dilated cardiomyopathy, which has been observed in our studies of the J2N-k hamster. There was no increase in the lung wet weight of the cardiomyopathic hamster, not was there the presence of ascites, which serve as clinical manifestations that ultimately lead to congestive heart failure (4). Even though Takagi *et al.*(51) have reported that the life span of the J2N-k hamster was 248 ± 18 days, and Mitsuhashi *et al.* (22) have reported that this animal model died of congestive heart failure at approximately one year of age, our studies done at 36 weeks showed that the hamsters were not in congestive heart failure. The results obtained from this study have maintained that the cardiomyopathic hamster clearly has exhibited signs of dilated cardiomyopathy and therefore, are in agreement with Mitsuhashi *et al*, (22) who have proposed that cardiac dilatation and dysfunction begins at approximately 20 weeks.

In another study conducted by Kato *et al.* (233), serum creatine kinase levels of the J2N-k hamster were foundto be greatly elevated, which greatly paralleled our findings. The increase in

the creatine kinase levels have given rise to the idea of cardiac tissue damage. The J2N-k hamster has been reported to experience a slow progression of interstitial fibrosis throughout its course that parallels the focal necrosis experienced by its parent, the BIO 14.6 cardiomyopathic hamster (233). Moreover, most studies to date on cardiomyopathic hamsters have generally reported on the occurrence of cardiac hypertrophy in the BIO 14.6 cardiomyopathic hamster (73). This is the first study to focus on the combined comparison of general characteristics, cardiac performance, SR function and its regulation in the J2N-k hamster.

2. Ca^{2+} Cycling Activities of the Sarcoplasmic Reticulum of the J2N-k Hamster

In this experimental model of cardiomyopathy, the J2N-k hamster has been reported to display dystrophic features of the striated muscle (22), and concomitantly, SR Ca^{2+}-transport in cardiac muscle was examined. The cardiomyopathic J2N-k hamster showed defects in SR Ca^{2+} uptake exclusively to the cardiac muscle, even though this animal contained a mutation of the δ-sarcoglycan gene that affected the entire sarcoglycan complex of all distributed striated muscle throughout its body. This deleterious mutation in one of the chief members of the sarcoglycan complex of the cardiomyopathic hamster is considered of great significance since dusruption of the δ-sarcoglycan gene in humans induces dilated cardiomyopathy (73).

Interestingly, the results of this particular study have confirmed that there is indeed an alteration in the muscle physiology on the basis of Ca^{2+} homeostasis, yet these J2N-k cardiomyopathic hamsters, which were characterized by mutations in cytoskeletal genes of multimeric complexes specific to muscle cells (235), only showed alterations in the Ca^{2+} transport activity that were evident solely in the cardiac muscle. This defect, in which only the

heart is affected, has been formerly supported by an earlier study that has shown that hamsters lacking the δ-sarcoglycan gene show only a slight reduction in the force of skeletal muscle function, as also exhibited by humans patients (236).

Correlating with the decrease in heart function, SR function showed a decrease in Ca^{2+}-uptake activity of the cardiomyopathic heart. Though previous cardiomyopathic hamster studies have suggested a dysfunctional SR in terms of the balance of intracellular Ca^{2+}, the mechanisms underlying the changes of SR function are not fully understood (47,68,234). The alterations in SR function may be a result of an impairment of key SR Ca^{2+} proteins or may be due to a depression in the SR regulatory mechanisms, such as protein phosphorylation or dephosphorylation. Accordingly, analysis of the protein content of these SR Ca^{2+}-cycling proteins revealed a decrease in SERCA2a, with no change in PLB. The ratio of PLB to SERCA2a protein levels was increased and presumably resulted in an increased inhibition of SERCA2a by PLB in the cardiomyopathic hamster heart. The reduction in SERCA2a protein content is likely a consequence of decreased mRNA levels of SERCA2a. These results could possibly used to explain the reduction of SR Ca^{2+} uptake in the cardiomyopathic hamster heart. Further western blotting analysis showed no change in the protein content of CQS in the cardiomyopathic hamster, suggesting no abnormalities in SR Ca^{2+} storage. Due to the fact that the SR is so intricately involved in cardiac contraction and relaxation as observed through the cycling of the Ca^{2+} ions during systole and diastole, the reduction in the expression of a key protein SERCA2a, would be expected to lead to the depressed cardiac performance in the cardiomyopathic heart.

3. Regulation of the Sarcoplasmic Reticulum via Phosphorylation

Regulatory mechanisms involved in the beat-to-beat basis of the heart include the precisely balanced protein phosphorylation/dephosphorylation reactions that occur in synchrony to ensure efficiency of the cellular function (149). In particular, the two major signalling pathways of CaMK and PKA that regulate SR function were examined in this study. Under normal physiological conditions, enhanced Ca^{2+}-uptake is achieved primarily by the phosphorylation of PLB by CaMK and PKA (115,149,237). The underlying inhibitory effect of PLB on SERCA2a is removed as a result of the phosphorylation by PKA and CaMK on PLB (112,115,237-240), which in turn SR Ca^{2+} transport (241). It is also interesting to note that phosphorylation of PLB by both CaMK and PKA result in an increased affinity of SERCA2a for Ca^{2+} (238,239), whereas phosphorylation of PLB by PKA shows an increased velocity of Ca^{2+} uptake (243) (240). The opposite action of Ca^{2+} -release through the RyR protein, is regulated by direct phosphorylation of RyR by CaMK and PKA, as reported by Strand *et al.* (242). This study has therefore shown a significant decrease in the SR-associated activities of PKA phosphorylation of PLB at the serine 16 site in the cardiomyopathic heart. This alteration was of a specific nature, because PLB phosphorylation at threonine 17 by CaMK was unaffected in the cardiomyopathic heart. Since PLB protein content was unaltered in the cardiomyopathic hearts, reduced PLB phosphorylation by PKA could be directly due to a significant decrease in the SR-associated PKA activity. The alteration in PKA activity was not exclusively restricted to the SR-associated PKA pool, as the cytosolic PKA activity was also significantly reduced in the cardiomyopathic heart. The unaltered status of PLB phosphorylation by CaMK in cardiomyopathic hearts was consistent with no changes in the SR-associated CaMK activity. Thus, a reduction in PLB phosphorylation by PKA could also contribute to the impairment of SR Ca^{2+} transport in cardiomyopathic hearts by increased inhibition of PLB on SERCA2a.

This study focused on the protein levels of the three catalytic subunits of PKA (α,β and γ). Although α and γ subunits of PKA were increased, there was a significant reduction in the β-subunit of PKA. This reduction may correlate with the reduction in the SR-associated actitivity of PKA and phosphorylation of PLB at the ser16 site. Reduced phosphorylation would result in inhibition of SERCA2a by PLB.

4. Regulation of the Sarcoplasmic Reticulum via Desphosphorylation

Due to the fact that phosphorylation and dephosphorylation are complementary regulatory mechanisms in metabolic and cellular processes, the decreased PLB phosphorylation observed in the cardiomyopathic state can be partly explained by the increase in phosphatase activity. As phosphatases work in harmony with kinases under normal physiological conditions, any alteration in diseased states can greatly contribute to a lethal imbalance. It has been previously observed that the SR-associated phosphatase, characterized as a type I phosphatase (244), functions to dephosphorylate PLB at both the CaMK and PKA sites (245). This increase of SR–associated phosphatase activity that has been observed in the cardiomyopathic state, combined with the decrease in PKA activity is expected to have contributed to decreased PLB phosphorylation and reduction in Ca^{2+}-uptake by the SR during cardiac relaxation. PKA reduced phosphorylation of PLB would result in further inhibition on SERCA2a, giving rise to SR dysfunction and would contributed to impairment of cardiac contractility in cardiomyopathic hamsters.

5. Comparison with other Cardiomyopathic Studies

Former studies have reported mechanisms of SR dysfunction in dilated cardiomyopathic human hearts. These include a reduction in expression of SERCA2a (215), reduced PLB phosphorylation at serine-16 (246) and increased phosphatase activity (247). Our results on SR dysfunction in cardiomyopathic hamster hearts were consistent with those of cardiomyopathic human hearts. Nonetheless, it must be noted that in the human heart studies dilated cardiomyopathy was of unknown nature (idiopathic). Incidentally, our results may be relevant to mechanisms underlying cardiac contractile dysfunction in inherited genetic disorders such as X-linked DCM, as well as Duchenne and Becker muscular dystrophies, ie. Disorders caused by the deletion of the delta- sarcoglycan gene. To our knowledge, this is the first study to report potential mechanisms underlying SR dysfunction in cardiomyopathic hamster hearts.

In summary, our results indicate that cardiac contractile dysfunction occurred in the J2N-k cardiomyopathic hamster and this phenomenon could be associated with impairment of SR function. SR dysfunction presumably occurred due to marked inhibition of SERCA2, caused by the increase in PLB/SERCA2a ratio, as well as reduced levels of phosphorylation of PLB by PKA (due to decreased PKA activity and increased phosphatase activity) in the cardiomyopathic hamster heart.

CONCLUSIONS

This study focused on the SR function and its regulation in correlation with cardiac contractile dysfunction in the cardiomyopathic hamster. The parameters of this study measured cardiac performance, SR Ca^{2+}-uptake and –release, protein phosphorylation and dephosphorylation, gene expression and total protein expression. The results suggest the following:

1. Overall cardiac function involving ejection fraction, fraction shortening, heart rate and cardiac output were depressed in the cardiomyopathic hamster.
2. The reduction in SR function in cardiomyopathy was a result of decreased cardiac SR Ca^{2+}-uptake, which was consistent with a decrease in SR protein expression of SERCA2a. SR dysfunction occurred due to marked inhibition of SERCA2a likely caused by the increase in the PLB/SERCA2a protein ratio. Reduced levels of SERCA2a protein was in accordance with reduced SERCA2a mRNA levels in the cardiomyopathic hamster.
3. Decreased cardiac SR Ca^{2+}-uptake was paralleled with a reduction in cardiac SR Ca^{2+}-release in cardiomyopathic hamster hearts.
4. Regulation of SR function was depressed due to a reduction in PLB phosphorylation at ser-16 by PKA. PLB phosphorylation at thr-17 by CaMK was unaffected in the cardiomyopathic hamster hearts.
5. The reduction in regulation of PLB phosphorylation by PKA was in accordance with a reduction in SR-associated PKA activity.
6. The decrease in PLB phosphorylation may also be attributable to enhanced dephosphorylation of PLB via increased SR-associated protein phosphatase activity in cardiomyopathic hearts.

FUTURE EXPERIMENTS

Future goals in studying the defects of the sarcoplasmic reticulum function and regulation of the J2N-k cardiomyopathic hamster include a time course of various intervals that compares different stages of dilated cardiomyopathy as this disease progresses onward. During these diverse time stages, experiments that measure overall cardiac performance can be broadened to include not only ejection fraction, fractional shortening, cardiac output and heart rate, but also can encompass the Doppler technique and possible hemodynamical studies of left venticular systolic and diastolic pressures. At the molecular level, mRNA and protein levels of the ryanodine receptor can be determined and potentially correlated with experiments of protein determination of the FKBP12.6 protein. The results of these experiments can be correlated with the SR protein function that relate to the Ca^{2+} handling of the cell. Moreover, the phosphorylation of the ryanodine receptor itself can be measured using cardiac protein phosphorylation assay kits. Essentially then, these experiments can supplement the research already completed in the J2N-k cardiomyopathic hamster model, and will help build on the information already gained in the quest for elucidating the mechanisms involved in the development of dilated cardiomyopathy.

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