

***In vitro* studies of early cardiac remodeling: impact on contraction and calcium handling**

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1. ABSTRACT

Cardiac remodeling, hypertrophy, and alterations in calcium signaling are changes of the heart that often lead to failure. After a hypertrophic stimulus, the heart progresses through a state of compensated hypertrophy which over time leads to decompensated hypertrophy or failure. It is at this point that a cardiac transplant is required for survival making early detection imperative. Current experimental systems used to study the remodeling of the heart include *in vivo* systems (the whole body), isolated organ and sub-organ tissue, and the individual cardiac muscle cells and organelles. During pathological remodeling there is a derangement in the intracellular calcium handling processes. These derangements are thought to lead to a dysregulation of contractile output. Hence, understanding the mechanism between remodeling and dysregulation is of great interest in the cardiac field and will ultimately help in the development of future treatment and early detection. This review will center on changes in contraction and calcium handling in early cardiac remodeling, with a specific focus on findings in two different *in vitro* model systems: multicellular and individual cell preparations.

2. INTRODUCTION

According to the National Heart Blood and Lung Institute, 5 million people in the United States are currently living with heart failure, a disease that claims an estimated 300,000 lives a year. Pathological cardiac hypertrophy is the leading predictor of heart failure. Current studies investigating potential therapies set out to 1) optimize conventional therapies, 2) focus on new drug development and 3) identify novel therapeutic targets (1). A large number of patients that ultimately come to suffer from heart failure first live in a state of compensatory hypertrophy for many years prior to experiencing the phenotypical effects. This lack of physical manifestation early in the disease process limits our ability to detect the anticipatory signs of heart disease, and thus greatly limits preventative therapeutic measures. Early remodeling processes, due to a prolonged exposure to chronic stressors such as hypertension, cause a compensatory remodeling. This remodeling is characterized by a thickening of the myocardial wall leading to an increase the force production (2). This phenomenon is best characterized by LaPlace's law (3), which refers to a hypertrophic response that is initially clinically beneficial to the subject (2). During the

temporal development of cardiac failure, the heart first compensates for a decreased contractile function through several mechanisms including ventricular hypertrophy and increased catecholamine stimulation (4, 5). On a cellular level, besides the addition of sarcomeres, one of the early molecular characteristics of heart failure is altered calcium handling (6, 7). Since calcium signaling is crucially involved in the regulation of hypertrophy, changes in calcium-handling and cardiac remodeling are an interconnected phenomena that occur during the progression to failure. Hypertrophy is regulated by numerous pathways where calcium ions maintain a central signaling role. Where alterations in calcium handling occur, remodeling events are likely to proceed. For instance, it is commonly understood that increases in calcium lead to activation of the calcineurin (CN) pathway. Once activated, CN binds to nuclear factor of activated T cells (NFAT) and translocates to the nucleus where transcription leads to development of hypertrophy (8). Additionally, the increase in intracellular calcium concentration seen in hypertrophy has been shown to lead to the activation of protein kinases that can alter contractile function of the myocyte.

The contractile function of the heart is governed by excitation-contraction coupling (EC-coupling). This is the term used to describe the link between electrical signaling and the initiation of contraction of a muscle. Driven by fluctuations in calcium level, it is pertinent to have a clear understanding of the complex alterations in calcium homeostasis that occur during the progression to failure. One of the first findings in end-stage heart failure was an increased diastolic calcium level, and unchanged systolic calcium (6). Defective EC-coupling is a major marker for cardiac hypertrophy, and is thought to be the major pathological determinate of failure (9). The goal of this review is to compare and contrast the recent findings in alterations in calcium handling during early cardiac remodeling. We will address this through an approach that highlights the individual model systems and then takes a closer look at findings at the separate steps of EC-coupling focusing mainly on observations made in multicellular and individual cell preparations.

3.1. Current experimental model systems

Currently, there are various model systems being utilized to increase our understanding of the workings of the heart under pathological and normal conditions. These models include the mammalian whole body, the heart, multicellular muscles, single cells, and sub-cellular fractions. Over the past two decades it has become commonplace to use smaller animal models, specifically the mouse, to investigate molecular processes underlying hypertrophy and calcium handling. In order to induce hypertrophy, these animals are either subjected to vascular binding surgeries such as trans-aortic constriction (TAC) (10), that are designed to elevate the work-load of the heart, or are genetically modified to produce a model of the pathological condition. In some studies, a combination of genetic manipulation and surgery is used (11). These murine studies have greatly advanced our knowledge about heart physiology, as many processes that occur in the murine heart likewise occur in larger mammals, including

human. Still, there are crucial differences between murine models and humans, and this often poses a problem in the interpretation of results obtained. For this reason, the study of the normal functioning myocardium of larger mammals such as pigs or dogs has been utilized to better elucidate the effects on human cardiac function. Although the extrapolation from work done in whole organisms cannot be replicated in reduced model systems, the whole organism does not lend itself well to in-depth investigations of molecular pathways. In addition, problems that arise with the interpretation of results from experiments on the whole organism are under the influence of autocrine, endocrine, and paracrine factors that regulate the heart on a long term as well as beat-to-beat basis. These confounding factors make it difficult and often impossible to isolate the impact of a single intervention. In order to tease out these effects and elucidate basic processes, researchers have utilized organ, cellular, and sub-cellular preparations.

These *in vitro* model systems now allow for tighter control over external factors, such as hormonal interactions and even calcium fluctuations. Whole heart preparations, such as the Langendorff or working heart models, are often used to determine the direct effects of cardiac specific injury such as ischemia reperfusion injury or drug treatment on the functional changes in different contractile chambers. To further control for endogenous factors, multicellular preparations, which will be discussed in further detail, can also be utilized for the study of functional alterations during remodeling and the effects on calcium transients. Such multicellular preparations possess fibroblasts and endothelial cells in addition to cardiac myocytes, which enable researchers to observe cell-to-cell signaling pathway alterations and calcium fluctuations during mechanical or therapeutic insults while avoiding outside effectors such as neurohumoral stimulates (12). To better control the environment of the myocyte and to remove more, if not all confounding factors, even smaller preparations are utilized.

Single cell preparations are utilized to better determine the effects on pathways and more specifically, excitation-contraction coupling. The individual myocyte, discussed in detail below, has been utilized for a number of years for preliminary studies on drug and toxin effects and calcium handling in the cell. Studies on individual myocytes allow for the most robust control of the endogenous environment, and such isolated cells can be maintained in culture for several days (13). Previous studies have shown an increase in myocyte length and a decrease in myocyte contractile parameters once a heart is in failure (14). Finally, organelle specific studies, such as isolated SR or myofibril preparations, go even further to eliminate external variables such as ion fluxes and further minimize protein-protein interactions. These preparations allow for the direct determination of the specific role and function of these organelles or even single molecules in a highly controlled environment. Through the use of these various *in vitro* models the scientific field has been able to study the progression from normal to early stage remodeling and have discovered alterations in calcium transients that occur.

Many different models are currently in use for the study of hypertrophy, contractility, and calcium handling. In order to increase our understanding of the alterations that occur during hypertrophy, the choice of model system highly depends on which system is most relevant for answering specific questions. Multicellular preparations and individual myocyte preparations are the most basic systems utilized to observe both changes in calcium handling, cardiac remodeling, and mechanical output. Thus far, the studies that have utilized these models have greatly advanced our current understanding of the signal transduction pathways induced through hypertrophic responses. Below, this review article focuses on current findings and technical aspects from individual myocytes and multicellular preparations during EC-coupling alterations in the early stages of cardiac remodeling. We will compare and contrast finding in these different modeling systems to shed light on the potential of both for furthering our knowledge of cardiovascular function.

3.2. Excitation

Dihydropyridine receptors and ryanodine receptors (RyR) are the two major calcium-sensing components of the excitation step in excitation-contraction coupling. The individual myocyte is the most commonly used experimental model for studying excitation, due to the ease of detecting transmembrane activation and calcium transients, and due to the availability of numerous myocytes in one heart. By using patch-clamping and other electrophysiological techniques scientists have been able to uncover changes in calcium influx during EC-coupling. The initial step of EC-coupling is triggered through the propagation of action potentials down the T-tubule portion of the plasma membrane. Once the membrane is depolarized there is an influx of calcium through (mainly) L-type and (partially) T-type channels (15, 16). L-type channels were first identified in cardiac ventricular myocytes where they are ubiquitous. T-type channels, though also seen in ventricular myocytes, are much fewer in number, have lower calcium transient amplitude, and are mainly present in atrial cells where they were first identified. Thus, T-type channels are thought to only play a very minor role in the overall calcium handling of the cardiac myocyte (16). Once the increase in calcium in the junctional sarcoplasmic reticulum (SR) is achieved, the ryanodine receptors (RyR) sense the change in calcium concentration and release additional calcium from the SR (15, 16). This coupling phenomenon is known as calcium induced calcium release, or CICR. Through the use of electrophysiological techniques in conjunction with fluorescent dyes such as fura-2, fluo, and indo-1, or the photoprotein aequorin (17), researchers are able to determine changes that occur in calcium levels and therefore channel function during remodeling events.

The individual myocyte model system allows for the isolation of individual channels and the determination of changes in membrane potential and calcium transients for any number of studies. At the excitation level, because of hypertrophy and/or heart failure, there is an increase in action potential duration (16). This suggests an increase in the amount of time available to allow calcium to enter the

cell via L-type channels (18). It has also been determined through the use of whole-cell voltage clamp techniques that the activation range of the L-type calcium channel is positive from $\sim -40\text{mV}$ on and peaks at 0 to $+10\text{ mV}$ (18). Decreasing the inactivation potential of the myocyte leads to an increase in the open probability of the L-type channel which increases the calcium influx (19, 20). Studies in guinea-pig myocytes have shown that a hypertrophy-induced change in voltage dependency of current inactivation is more positive (21), while studies in human and mammalian failing models have shown little to no changes in the functional activation of the L-type channel (16). The studies that did observe changes did so most likely due to the severity of the disease stage of that particular model, such that some animals may have been closer to or in failure compared to others (22). Many studies have shown increases in the calcium transient amplitude via the L-type channel and a prolongation of calcium current with no increase in the relative density of the channel on the individual myocyte (18, 23). Taken together, these findings suggest that during the hypertrophic response there is an increase in activation of calcium channels resulting in an increase in the overall cytosolic calcium concentration.

An increase in catecholamine production is common during the onset of heart failure (5). As the heart progresses to failure there is an increase in beta-stimulation which leads to an increase in contractile function (4, 5). This modulatory role of b-adrenergic stimulation in heart failure directly affects the L-type calcium receptor. It is currently understood that during failure this increase in beta-stimulation occurs in conjunction with a decrease in receptor density (16, 24). Investigations into the failing myocardium have shown a decrease in the effects of b-stimulation in human hearts (20). Studies in failing human myocytes have also revealed a decrease in the number of L-type calcium channels altered by CaMKII phosphorylation (25). This alteration has been shown to lead to increases in calcium current amplitude and slowed progression to inactivation, which also leads to an increase in cellular calcium levels (25). In models of hypertrophy induced by aortic banding and in studies on human diseased myocytes, L-type calcium current density was increased (23). The number of dihydropyridine receptors revealed an increase in binding sites in hamsters with hereditary cardiomyopathy and in SHR (spontaneous hypertensive rats) models after the development of hypertrophy followed by an increase after the onset of failure (23). Overall, there has yet to be a significant change in calcium channel activation in the myocyte at the early phases of the hypertrophic response suggesting that these changes only play a role in the later stages of heart failure, which can act as a potential marker for level of failure development.

Additional calcium fluctuations can also occur through the activation of the ryanodine receptors. Studies on myocytes from ferrets utilizing BayK (an L-type agonist) have revealed a potential physical link between L-type channels and RyR (15). In rats that underwent mild hypertrophy by suprarenal abdominal aortic constriction treatment, RyR mRNA levels were shown to be increased

(22). Work done in rats treated to induce a volume overload model of cardiac hypertrophy showed increased diastolic function, impaired systolic function, and increased RyR binding protein FKBP12.6 phosphorylation. This suggested that, in this particular model, RyR played an important role in the maintenance of systolic function (26). Fluctuations in the phosphorylation and activation of these channels also lead to changes in calcium flux. Permeabilized myocyte and plasmon resonance techniques have been utilized to determine the effects of this enhanced phosphorylation of key proteins such as FK506 binding proteins FKBP12 and 12.6 by PKA (27, 28). Such studies have shown a correlation between alterations in RyR function during heart failure and the effects of alterations to these binding proteins can lead to an increase in spontaneous calcium release and cardiac arrhythmias further dysregulating calcium homeostasis (27, 28). A potential culprit is the phosphorylation of RyR which occurs through activation of CaMKII, initiated through increases in cytosolic calcium (29). Currently, however, there are significant disagreements on the role and mechanism underlying phosphorylation of RyR, and its functional consequences(30).

Drug treatments are a common mode of studying activator calcium and remodeling based effects in the individual cell and multicellular preparations. In more basic experiments caffeine has been a commonly used drug intervention in the study of SR calcium load and function in myocytes, while rapid cooling contractures (RCCs) are also used in the isolated muscle preparation (31) to semi-quantify SR load. Caffeine removes the gating ability of RyR, leading to the complete emptying of calcium ions from the SR. The effects of caffeine on the emptying of the SR depends on its ability to diffuse equally into each cell, and in a multicellular preparation this leads to an increase in overall time for the muscle to fully contract and relax, making caffeine-based protocols better suited for use in the myocyte (31). On the other hand, RCCs, which utilize sub-physiological temperatures to induce release of SR calcium, provide more direct effects on total calcium in multicellular preparations (31). Isolated muscle studies, which utilize the RCC to quantify the SR calcium levels in aortic banded rats, revealed a decrease in SR calcium concentration after post rest RCC (32). To better control intercellular calcium level, the drug ryanodine is commonly used as an inhibitor of SR function in individual myocytes. In multicellular preparations it is thought that ryanodine inhibits calcium release; however, the exact mechanism of its activation still remains unclear, while the effects are concentration-dependent (31, 33). The use of this inhibitor has enabled researchers to determine the calcium load within the SR in the individual myocyte. Many studies reveal a decrease in SR calcium load that is dependent on the level of hypertrophy or stage of failure. All of these experimental techniques have revealed that as the heart progresses to failure and through the different stages of hypertrophy, there is a significant decline in SR calcium load. This decrease in activator calcium and its effects on the contractile machinery of the heart are of great interest in current studies and the topic of the following sections.

3.3. Myofilaments

Calcium acts as the activator of the myofilaments. After excitation, once the overall concentration of intercellular calcium increases in the myofilament matrix where calcium binds to the C (TnC) component of the troponin complex. The binding of calcium induces a conformational change in proteins located on the thin filament, including troponin I (TnI), Troponin-T (TnT), and Tropomyosin (Tm). This conformational change moves the position of tropomyosin on the thin filament, allowing for myosin to bind to the actin filament leading to the shortening of the myofilament (34) and/or force production. Once calcium is removed from TnC by lowering cytosolic calcium via SR uptake and NCX calcium extrusion, the myosin head is able to detach from the actin filament, initiating relaxation.

Diastolic dysfunction, or impaired relaxation, is a predominant type of dysregulation in heart failure. Studies on the cause and effects of hypertrophy on diastolic function are of current interest in our lab and others (35-37). Data suggests that cardiac remodeling is often marked by changes in the contractile function of the heart in a calcium dependent manner (16). It remains unclear exactly how remodeling affects the thin and thick filaments within the functioning heart. Many studies have revealed clues to aid in determination of the effects of failure and remodeling. Muscles taken from failing human hearts showed that the cross-bridge cycling rate was reduced (38) whereas studies in rodents have shown that through mild ischemia reperfusion injury there is an increase in truncated TnI (39). This truncation has been shown to impair force production and slows relaxation kinetics (40). These data have led many to believe that relaxation is dependent on the re-uptake/removal of calcium from the thin filament and that myofilament calcium sensitivity provides a rate-limiting step for relaxation (17, 41). Studies on intact rat cardiac muscles under near physiological conditions have also suggested that cross-bridge cycling kinetics can be rate-limiting for relaxation (42-44). Studies in failing and non-failing human myocardium have not always shown changes in the responsiveness to intercellular calcium concentration (38), myofilaments from failing hearts have revealed slower cross-bridge cycling rates and have often undergone troponin-T isoform alterations that affect myofilament calcium sensitivity (38).

Not only does protein isoform expression matter, but also post-translational modification of myofilament proteins contribute to their overall function. Multiple phosphorylation targets of PKA, PKC, and CaMKII exist on various myofilament proteins, which likely play a significant role in hypertrophy-induced changes in myofilament responsiveness. The targets of these kinases include, but are not limited to, myosin light chain (MLC), troponin I (TnI), troponin T (TnT), titin, and myosin-binding protein C (MyBPC). Increases in intercellular calcium have been shown to lead to increases in expression of PKC and CaMKII, which lead to increases in the phospho-related changes in myofilament function (8, 45). PKC has been implicated in the targeting of additional phosphorylation sites on myosin-binding protein C and on

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titin (46). Previous studies have found that cardiac remodeling induces changes in the affinity of TnC for calcium through changes in the phosphorylation status of the myofilament at TnI (34). Studies in human isolated myocytes have shown that PKC-mediated phosphorylation of the myofilament on TnI has been shown to lead to a reduction in myofilament calcium sensitivity as the non-failing heart progresses to failure (34). Additionally, studies on myocytes have shown that the induction by PKC on TnI phospho-specific sites can cause an increase in myofilament calcium sensitivity (34). PKA, the main signaling molecule in the β -adrenergic stimulation pathway, has also been implicated in the targeting of TnI during cardiac remodeling (34). It is commonly understood that as hypertrophy and failure occur, *in vivo* catecholamine production is increased and PKA, which is activated by β -adrenergic stimulation, is upregulated (4, 5). The ability of PKA to decrease the calcium sensitivity of force and to enhance diastolic function has been most expressively attributed to TnI phosphorylation leading to a faster dissociation of calcium from TnC (34, 47-49). Additionally, work in transgenic mice with pseudo-phosphorylation sites for PKA and PKC on TnI showed their involvement in the force frequency response (48).

A recent investigation also suggested a correlation between the frequency-dependent calcium sensitivity and hypertrophy (37). Our lab and numerous others have highlighted a decrease in myofilament calcium sensitivity following hypertrophy (37, 38), the direct cause of these possible alterations in myofilament calcium sensitivity remains undetermined. One of the common markers of heart failure following hypertrophy is a blunted or negative force frequency response (FFR) (50-52). Normally when a healthy muscle is paced at a slower and then faster frequency, while these frequencies are within the physiological heart rate range, there is an increase in the force the muscle produces (53). When the heart is in failure, there is often a significant increase in size (hypertrophic response) along with a blunted or negative FFR (51-53). Studies in pulmonary artery banded rabbits reveal a decrease or blunting of frequency-dependent myofilament calcium sensitivity in trabeculae (37). The force-frequency response is a commonly used cardiac muscle excitation test to deduce the effects of frequency on force production. In the compensated state of early cardiac remodeling this blunted effect is not seen (37). The rate of relaxation seems thus dependent on the frequency and is altered during heart failure and through the development of hypertrophy.

3.4. Calcium Transient Decline

To allow for relaxation, the calcium ion concentration in the myofilament matrix has to be reduced. This occurs via reuptake of calcium through the function of the sarcoplasmic-endoplasmic reticulum calcium ATPase (Serca) pump and the extrusion of calcium by the sodium calcium exchanger (NCX). Serca is a calcium-ATPase pump that functions through a co-localization with phospholamban to pump intracellular calcium ions into the SR on a beat-to-beat basis. It has been shown that in humans close to 70% of the free (cytosolic) calcium is

taken back into the SR through the Serca pump while roughly 30% is extruded extracellular via NCX (16). Serca2a is the main isoform present in cardiac ventricular muscle cells and facilitates the storage and reuptake of nearly 70% calcium into the SR in humans and $\geq 95\%$ in small rodents (16). Alterations in calcium concentration reduction is mainly controlled by the function of the Serca-PLB complex formation and activation (through phosphorylation) in the membrane of the SR. Through the use of fluorescent indicators, researchers are able to visualize the decrease in cytosolic calcium in both the individual myocyte and the multicellular preparation for every beat and elucidate relaxation due to calcium removal effects. Calcium extrusion and subsequent relaxation is also controlled by the sodium-calcium exchanger (NCX). NCX is a voltage-dependent, bi-directional, transmembrane channel which regulates the contractile function of the heart through its transference of 1 calcium ion outside of the cell and 3 sodium ions into the cell. (16). Generally the pump functions to extrude calcium, although during the plateau phase of the action potential, it can work under reverse mode and pump calcium into the cell. NCX is mostly regulated by sodium concentration, calcium concentration, and membrane potential, but its function is impacted by ATP, phosphorylation, pH, and lipids as well. During heart failure there is a significant decrease in Serca function with an increase in NCX function (54-57), and as the calcium extrusion process dominates, this results in a net loss of calcium from the cell.

Relaxation is one of the key mechanisms altered during early cardiac remodeling, and due to direct changes in the expression levels of proteins involved in calcium extrusion and reuptake such as Serca, phospholamban (PLB), and NCX, many studies have focused on impaired calcium handling. Within this impaired calcium handling, the role of the SR as a calcium pool during the progression to failure and during the early stages of remodeling has been looked at in vast detail over the past few decades. While during heart failure a decrease in Serca mRNA is seen, during mild/early hypertrophy there is no alteration in Serca gene expression (22, 58). A decrease in Serca mRNA expression level has been shown to be dependent on the degree of hypertrophy occurring in the system and has been seen in hypertensive rat models (22, 32). This change in expression shifted the normal extrusion percentages and decreased the overall SR calcium store load. PLB, commonly thought of as a switch to activate Serca when phosphorylated, exists in two main forms: a monomer and a pentamer, it is commonly thought that as a pentamer PLB is unable to bind to and affect Serca function (59). More widely understood is the effect of the phosphorylation of PLB. Phosphorylation has been shown to occur on three major sites by three major kinases, ser16 mainly through PKA, thr17 mainly by CaMKII, and the lesser known ser10 through PKC (60, 61). PLB acts as a switch for the activation of Serca where upon phosphorylation the inhibitory effects of PLB are relieved and Serca is free to pump calcium back into the SR (62-64).

Phosphorylation of phospholamban by PKA has been shown to increase the velocity of calcium transport

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(45, 49). The ser16 sites seems to be the most vital phosphorylation site with many studies highlighting its necessity to induce any effect on Serca activation (65). However, thr17 has become a target of interest specifically in studies looking at compensatory effects during ischemia reperfusion injury and coronary artery occlusion (66). It has been shown that CaMKII phosphorylation of PLB may play a role in frequency-dependent acceleration of relaxation which is altered in failure (67), although recent evidence argues against a prominent role in this regulation(68). During heart failure there is diminished peak calcium and a prolonged decay in calcium, yet there are no major changes in the PLB protein expression levels. SR calcium uptake in heart failure patients is decreased by 50% (69). This suggests that during remodeling there are changes in calcium handling due to either the phosphorylation status of Serca and/or the interaction of PLB with Serca (70, 71). Studies have modified PLB expression to alter the Serca function in what would be representative models of early cardiac remodeling (72). Through alterations in PLB monomer and pentamer localization to Serca in mouse models, it was determined that the effects of decreased PLB expression was associated with increased SR calcium reuptake in heart failure phenotypes (65, 73).

During hypertrophy it has been shown that there is an increased expression and/or activation of NCX (74). Studies on stretched individual myocytes have revealed increases in NCX mRNA levels (16). Further studies in canine myocytes have revealed decreases in extracellular calcium extrusion following hypertrophic stimulation (75). Interestingly, studies on the partial blockade of NCX in dog and rabbit myocardium has shown no significant effect on calcium extrusion levels (76). Studies on early hypertrophy in rat myocytes extracted during short-term aortic constriction revealed no change in NCX function but significant increases in SR calcium content and extrusion (77). These findings taken together suggest that while there is an increased NCX function in heart failure, remodeling has a minimal effect on the NCX calcium sensitivity and extrusion function as compared to effects on Serca and PLB.

3.5. Recent developments in multicellular tissues for investigation of remodeling and calcium handling

The individual myocyte and isolated muscle techniques have proven to be the catalyst for numerous studies which focus on non-traditional approaches to clinical therapies and basic research. One of the greatest innovations that is currently taking hold is the idea of tissue engineering. Myocardial tissue engineering is a technique utilizing laboratory-grown cell matrices or grafts as a replacement for dysfunctional cardiac tissue (78-80). The overall goal of engineered tissue is "to provide a physical scaffold for cell adherence through the use of biologically acceptable materials (81)." They aim to repair, regenerate and replace myocytes after myocardial infarction (82).

As with all grafts, there are numerous problems that arise with engineered cardiac tissue including the technical feasibility, tissue homogeneity, and myocyte differentiation (79). Earlier studies have run into further

problems with the types of cells used and the inability for the cells to form the proper scaffolding proteins to incorporate into the whole heart (79, 83, 84). More recently, studies have been devised where engineered tissue has been able to successfully convert to functional cardiac tissue producing spontaneous contractions and more exact myofibril organization (79, 83), and can form 3-dimensional structures(85). In addition, transplantation studies of neonatal myocytes on rats have yielded better prospects for engineered heart tissue by producing well organized heart muscle structure as indicated by actinin, connexin 43, and cadherins up to fourteen days after implantation (84). Such tissues will likely prove of increasing value in the study of remodeling and calcium handling.

One of the unique benefits of multicellular preparations is the ability to gain insight into functional changes that occur in cell-to-cell signaling and to determine the pathological effects of hormonal interventions on the functioning muscle. Myocytes are mechanically, electrically, and chemically connected to each other via gap junctions. The collagen matrix maintains the scaffold and the junctions provide a communication link between myocytes to help control muscle synchronization. With the onset of hypertrophy and remodeling, there is a potential for signaling alterations to occur between cells due to increased fibrosis. Fibrosis is one of the main causes of ventricular stiffness on the post-infarcted heart and is a marker of hypertrophic events in the progression to failure. In experiments on rat papillary muscle where GSSG was used to induce collagen degradation it was found that active stiffness was increased as compared to controls (86). This suggests that decreased collagen content is associated with the phenotype of hypertrophy, where an increase in collagen content leads to diastolic dysfunction, otherwise characterized as increased stiffness, with no direct effect of the systolic function (86). Previous studies in our own lab have shown that connexin 43 (a key gap junctional protein) expression gradually decreased during the progression to heart failure (87). The latter study was done in a multicellular preparation that can be kept contracting for several days (88), and can be obtained from rat (12, 89), rabbit (90), humans (91), and even be transfected with virus (92) to study the development of altered function. This multicellular culture approach and similar to studies on engineered tissue, allow multi-day investigation of cardiac multicellular tissue while alterations in calcium transients can be assessed via various methods in these tissues. Such cultured muscles and engineered tissues will likely provide future approaches that may provide further insight into calcium handling and changes in calcium handling during remodeling, hypertrophy, and heart failure.

4. CONCLUSIONS

In vitro studies and model-based systems have played a significant role in the advancement of our knowledge of calcium handling in the normal and failing heart. Through the isolation of individual cells or muscles, the field has been able to refine approaches to study and understand specific cause and effect issues in early

hypertrophy. Unfortunately, there are limitations to every model system and in the study of calcium regulation these limitations apply. Because the myocyte must undergo the hypertrophic development *in vivo*, there are limitations to the use of the individual cell. Regrettably, because we cannot provide control on whole-body neurohumoral stimulation that the animal undergoes post-operation, we are unable to fully determine the exact cause of observed results. Also, the inability to define the level of hypertrophy occurring within the animal prevents the proper elucidation of the effects of certain stimuli. This is often the case which confounds literature when determining the extent of the effects of hypertrophy. The use of stretch and drug treatment on the individual myocyte are often used to extrapolate the direct effects of specific alterations, yet, because the myocyte is removed from its normal environmental, contact to other myocytes as well as endothelial cells and fibroblasts, results remain ambiguous. The lack of cell-to-cell connections that appear to be crucial in the functional phenotype of the cell make it difficult, if often not impossible to relate them to the *in vivo* pathophysiological condition.

The multicellular preparation or engineered tissue is potentially more suited for studying the functional effects of different mechanisms in the heart. Unfortunately, unlike isolated cells, these are significantly limited by the inability to study the isolated channels. Both myocytes and muscle preparations allow for the assessment of intracellular calcium at various stages during the hypertrophic remodeling process, and with advancements in drug technologies we are better able to manipulate channel function to determine such effects. In summary, when studying calcium handling in early hypertrophy, all *in vitro* models have specific benefits and all have compromises which must be taken into consideration when analyzing and extrapolating data. As long as these considerations are made taken into account, findings from *in vitro* based model systems will remain an important tool to enhance and initiate future scientific investigations.

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