Biological and Medical Physics, Biomedical Engineering

R. John Solaro
Jil C. Tardiff *Editors*

Biophysics of the Failing Heart

Physics and Biology of Heart Muscle

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Editors
R. John Solaro
Department of Physiology
and Biophysics
Center for Cardiovascular Research
University of Illinois at Chicago
Chicago, IL, USA

Jil C. Tardiff
University of Arizona
Tucson, AZ, USA

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Introduction to the Biophysics of the Failing Heart

R. John Solaro and Jil C. Tardiff

Introduction

Perusal of the contents of any basic textbook in physics gives a list of the topics that are of relevance to biological processes and in the examples given in this book to the heart in health and in failure. The breakdown falls along the lines of mechanics, electricity, magnetism, thermodynamics, sound, and optics. In one way or another, each of these topics in physics comes into play in the biophysics of the heart. Chapters in this volume provide strong evidence of the significance of biophysical principles in our understanding of the normal mechanical, energetic, and electrical aspects of how the heart functions normally and how it fails in this function in response to diverse forms of stress. The physical principles of sound and optics are, of course, inextricably tied to the use of biophysical principles to determine functional, structural, and mechanistic aspects of the heart. Here we provide an overview of the topics covered and how each of the topics represents underpinning biophysical principles. The emphasis and focus is on working cardiac myocytes in the ventricle, although we wish to point out that other cell types are present in the heart and contribute substantially to homeostatic mechanisms. The working cardiac myocyte provides an excellent example of the marriage of biology, chemistry, and physics in a cell that undergoes lifelong changes in electrical state, force generation, energy turnover, and length with every beat of the heart. The homeostasis of this continually active cell involves maintenance of a range of electrical, mechanical, and energetic activities, which are regulated to meet to the demands of exercise and

R.J. Solaro (✉)
Department of Physiology and Biophysics, Center for Cardiovascular Research,
University of Illinois at Chicago, Chicago, IL 60611, USA
e-mail: solarorj@uic.edu

J.C. Tardiff
University of Arizona, 1656 East Mabel Street, MRB 312, MS# 245217,
Tucson, AZ 85724-5217, USA

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external stressors of normal life. What is apparent from the studies presented here is that when these systems operate outside this range of homeostatic activity, the system enters into a disordered state leading in some cases to a slow process toward death and in others to sudden death.

Cytoskeletal, Electrical, Metabolic, and Mechanical Elements in the Initiation of Progression to Heart Failure

What distinguishes physics from biophysics? A major difference between the two is that springs, levers, fulcrums, and screws are in principle inanimate, and this is not the case with the proteins that make up the electrical and mechanical elements of the cardiac myocytes. In proteins, lever lengths, the points about which they pivot (fulcrum), and their point of contact with neighbors are variables and the variability of these physical properties may be adaptive to the stresses of normal life, or may be maladaptive as the stresses exceed the capability of the system to cope. An excellent example is the chapter by Anderson and Granzier "Biophysics of Titin in Cardiac Health and Disease." Titin, the biggest protein in our bodies, has all the mechanical properties described above. It has spring elements that determine resting tension in the cardiac myocyte and are therefore a major determinant of diastolic tension and pressure. Titin is a lever with evidence that when it stretches it moves cross-bridges away from the thick filament proper. It is also likely that rotational movements of elements in titin act like a screw. What's fascinating is that all these properties are variables subject to adaptation important in maintaining normal cardiac function by post-translation modifications. Moreover, unlike physical machines, biophysical machines can change parts by dispensing with motifs and replacing them to form isoforms with different properties. This happens with the development of the heart and in response to the variations in hemodynamic load. The fine-tuning required in maintaining function is remarkable in that when mutated genes induce inappropriate substitutions of amino acids in titin, there is a progression toward various states of maladaptive cardiac growth, often ending in sudden death. The linkage of mutations in a biological spring to cardiac growth indicates that the mechanical state of the cell is monitored by sensors, which set into motion effectors that adjust to the altered mechanical state, and remodel the heart. This may occur physiologically as with chronic exercise, or pathologically as with inherited mutations in proteins such as titin or with stresses on the heart as occurs with hypertension and myocardial infarction.

The conceptualization of a mechanical system as a regulator of cell growth is considered in the chapter by Samarel et al. "Biophysical Forces Modulate the Costamere and Z-Disc for Sarcomere Remodeling in Heart Failure." They emphasize the idea that "mechanical forces drive the growth of heart cells." They review key cellular elements in the sensing of mechanical strain in the form of the costameres (the "rib cage" of the cell) and the Z-disc, which not only contain an array of mechano-sensors but also an array of proteins, which influence transcription, translation, and post-translational modification of cells as well as assembly of sarcomeres in the remodeling process. Although not fully understood, it is evident that the physics of the system is modified as a mechanism to convert a mechanical state to an altered biochemical state. The versatility of the system and the long range communications from the extracellular matrix to the nucleus are amazing examples of how biology employs physics to accomplish regulation. As with every mechanism discussed in the book, there is a range of homeostatic adaptive modifications and a range of maladaptive modifications.

Electrophysiology provides another example of the application of physical principles to biological processes. The physics of electricity has been a mainstay in biology beginning most significantly with the work of Luigi Galvani, who around 1780 came to the realization that a frog's leg is able to conduct electricity to metal. Galvani was a practicing physician with an interest in "medical electricity" and certainly could be considered one of the first biophysicists. The concepts of voltage, current, resistance, and capacitance retain their significance and applicability in ion flows. Yet, the proteins that form the channels through which the ions move demonstrate remarkable abilities to respond to signals that alter their physical properties so that conductance and voltage changes dramatically via biological processes and the voltage changes serve as signals for propagation of activation among cells. In the chapter "Biophysics of Membrane Currents in Heart Failure" Liu et al. focus on active and passive processes that maintain gradients of ions across the cell membrane and establish the transmembrane potential, which depends on the conductance of an array of channels to various ions including Na, K, Cl, and Ca. Biophysical measurements provide high fidelity read-outs of channel activity; patch clamping, in fact, permitted the accurate measurement of current through single channels. Again channel pores are conceptualized in terms of levers and gates, which are modified by biological processes. "Ball and chain" models of the gating of ion channels provide a graphic example of this conceptualization.

The passage of the ions through these channels, in particular Ca-channels, serves to signal multiple and diverse events in the myocytes. Most prominent is the ability of Ca currents to trigger contraction by promoting the release of Ca from stores in the sarcoplasmic reticulum. The mechanisms are considered in the chapter by Zima and Terentyev "Sarcoplasmic Reticulum Ca Homeostasis and Heart Failure," and the chapter by Banach "Ca-homeostasis and Heart Failure: Focus on the Biophysics of Surface Membrane Ca-Fluxes." A thread running through these and other chapters is the critical dependence of control of intracellular Ca with regard to the electrical stability of the cardiac cells. Minor modifications in the fluxes promote inappropriate changes in membrane potential that trigger and promote arrhythmias. The chapter by Vatta and Solaro "Arrhythmogenesis, Heart Failure, and the Biophysics of Z-band Protein Networks" explicitly relates the activity of K and Ca-channels to the strains in the network of proteins in the Z-disc cytoskeletal network. Maladaptive mutations in these proteins that lead to sudden death are thought to alter the biophysical properties of the networks.

Mechanisms controlling Ca-fluxes via the channels, exchangers, and transporters also regulate Ca-binding to proteins that modify kinases and phosphatase activities. These enzymes not only control Ca delivery and removal from the myofilaments, but also transcriptional events at the nucleus. Myocyte hypertrophic signaling to the nucleus via Ca-calmodulin and calcineurin is explored in the chapter by Cheng et al. "Heart Failure-the Final Frontier for Biophysics in Cardiovascular Medicine." Moreover, the chapter, "Cytoskeletal Nuclear Interactions" by McNally, deals explicitly with the filaments that connect the extracellular matrix to pores in the nucleus and how modifications in these proteins in the form of mutations linked to muscular dystrophies alter cardiac structure, excitation–contraction coupling and lead to aggregates of proteins that induce failure.

Ultimately all the electrical and mechanical-related signaling processes are geared to cardiac output and the efficient coupling of energy supply and energy transduction to the promotion force (pressure) and shortening (ejection), and relaxation of the cardiac myocytes. The chapter by Lewandowski "Biophysical Mechanisms for the Metabolic Component of Impaired Cardiac Function" provides an excellent example of the application of biophysical principles of magnetic resonance imaging (MRI) for the assessment of in situ cardiac continuum mechanics and the determination of regional contractility via determination of strains and torsion. Lewandowski also describes the power of high resolution magnetic resonance spectroscopy in noninvasive determination of metabolic fluxes in the heart. Ultimately the ATP generated by these metabolic processes is hydrolyzed in a process coupled to a mechanical event in the form of the actin-cross-bridge reaction. Simon et al. in their chapter "Sarcomeres and the Biophysics of Heart Failure" summarize evidence that altered biophysical properties of the contractile machine of the myocardium may lead to altered growth, remodeling, and eventually sudden death. A profound example is their consideration that altered flexibility of tropomyosin, a key protein in switching chemo-mechanical activation on and off, is likely to induce a progression toward hypertrophy and sudden death. Taken together all the chapters emphasize how biophysical principles form a vital aspect of the quest to prevent, halt, and diagnose the progression to heart failure.

Sarcoplasmic Reticulum Ca Homeostasis and Heart Failure

Aleksey V. Zima and Dmitry Terentyev

SR Ca Regulation in Heart

Heart function vitally relies on precisely controlled intracellular Ca homeostasis during each cardiac cycle. Abnormalities in Ca regulation cause contractile dysfunction and arrhythmias under different pathological conditions including heart failure (HF). Playing a particularly important role in heart contraction is the sarcoplasmic reticulum (SR). In adult ventricular myocytes, the SR forms a highly interconnected network of tubules (free SR) and cisterns (junctional SR). Although the SR occupies only 2–4 % of the total cell volume [1], it provides the major portion of Ca that initiates contraction. The ability of the SR to accumulate large amounts of Ca is ensured by the SR-specific low affinity and high capacity Ca binding protein calsequestrin (CASQ) [2].

Ca is released from the SR as a result of activation of specialized Ca channels—ryanodine receptors (RyRs; type 2 isoform). These Ca channels are activated by a relatively small inward Ca current via L-type Ca channels (LTCCs) during an action potential (AP). This mechanism which mediates cardiac excitation–contraction coupling (ECC) is known as Ca-induced Ca release (CICR) [3]. In ventricular myocytes CICR occurs at specialized subcellular microdomains where a T-tubule of the sarcolemma is proximal to a junction of the SR forming a dyad (Fig. 1a). Dyadic junctional SR membrane contains clusters of ~100 RyRs [4] that are strategically aligned with LTCCs by junctophilins [5] which ensure that the dyadic cleft is narrow enough (~10–30 nm) to promote efficient signaling from the T-tubule network to the SR. Each of these microdomains constitutes a SR Ca

A.V. Zima (✉)
Stritch School of Medicine, Loyola University Chicago, Maywood, IL, USA
e-mail: azima@lumc.edu

D. Terentyev
Rhode Island Hospital and The Warren Alpert Medical
School of Brown University, Providence, RI, USA

R.J. Solaro and J.C. Tardiff (eds.), *Biophysics of the Failing Heart: Physics and Biology of Heart Muscle*, Biological and Medical Physics, Biomedical Engineering,
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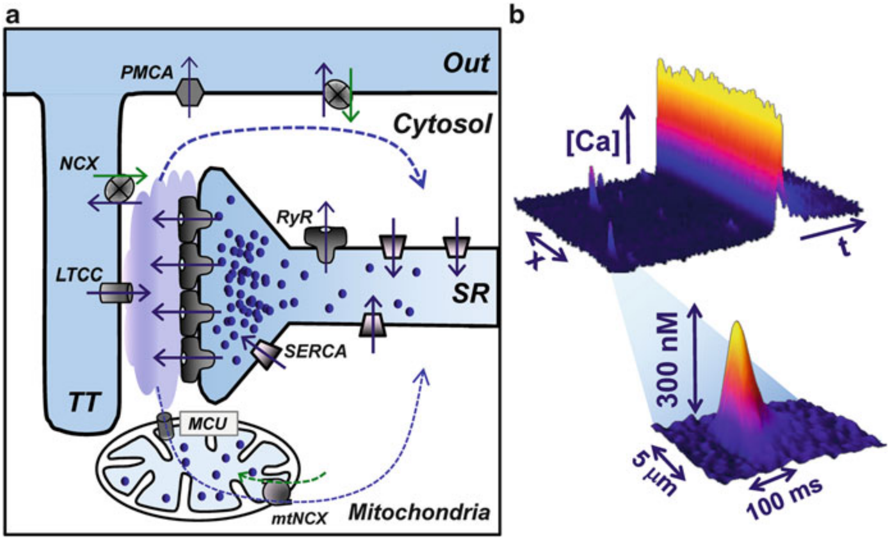

Fig. 1 Structure and function of the SR Ca release unit in ventricular myocytes. (**a**) The organization of the main components of Ca regulation in ventricular myocytes at the subcellular level. The diagram illustrates L-type Ca channels (LTCCs) in the T-tubule (TT) and ryanodine receptor (RyR) clusters in the junctional sarcoplasmic reticulum (SR) form a Ca release unit. Ca-ATPase (SERCA) pumps cytosolic Ca back into the SR and the Na–Ca exchanger (NCX) removes Ca from the cell. The plasmalemmal Ca-ATPase (PMCA) and mitochondria play a minor role in cardiac relaxation. The mitochondrial Ca uniporter (MCU) and the mitochondrial Na–Ca exchanger (mtNCX) regulate mitochondrial Ca homeostasis. (**b**) A confocal image of diastolic Ca sparks and an action potential-induced Ca transient recorded from rabbit ventricular myocyte. Activation of a single Ca release unit generates a Ca spark (*bottom inset*), whereas simultaneous activation of thousands of these individual release units generates a global Ca transient

release unit [4, 6] or couplon [7]. The simultaneous activation of RyRs within the release unit generates a locally restricted increase in cytosolic [Ca] ($[Ca]_i$), or Ca spark [8, 9]. Ca release during a spark causes partial depletion of intra-SR [Ca] ($[Ca]_{SR}$) limited to a single SR junction, also known as a Ca blink [10–13]. During ECC, the global SR Ca release that initiates contraction is the result of the spatio-temporal summation of Ca release from thousands of these individual release sites (Fig. 1b). Thus, the amplitude of the Ca transient is largely controlled at the local subcellular level by gradual recruitment of these Ca release units [14]. In addition to the RyRs organized in clusters, it has been proposed the existence of isolated "rogue" RyRs localized in free SR [15].

Because CICR by definition is a self-regenerative process, it would be expected to continue until SR Ca is fully exhausted. However, convincing results from different laboratories have shown that during a physiological stimulus (action potential or Ca current) $[Ca]_{SR}$ only partially depletes [10, 12, 16, 17]. Thus, a robust termination mechanism must exist to counteract the positive feedback of CICR and avoid "all-or-nothing" SR Ca release events [18]. Several possible mechanisms that control Ca release termination have been suggested including

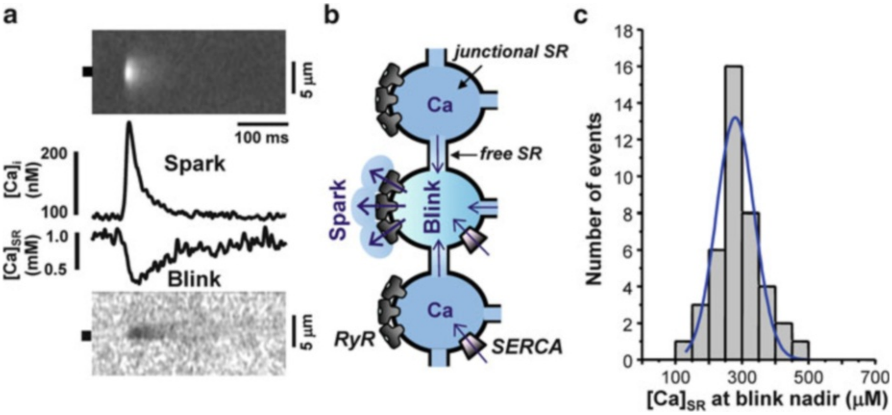

Fig. 2 Elementary SR Ca release events. (**a**) Averaged images of Ca sparks (*top*) and Ca blinks (*bottom*) recorded in permeabilized ventricular myocytes. Ca sparks were measured using the high-affinity Ca indicator Rhod-2 loaded into the cytosol. Ca blinks were measured with the low-affinity Ca indicator Fluo-5N entrapper within the SR. For details see [11, 12]. Ca spark and blink profiles were obtained by averaging fluorescence from the 0.8 μm wide regions marked by *black* boxes. (**b**) Cartoon showing the relationship between the SR network and local SR Ca release. Elementary SR Ca release events (spark and blink) arise from the simultaneous opening of clustered RyRs in a single SR Ca release site. Ca blink recovery depended mainly on intra-SR Ca diffusion rather than SR Ca uptake [12]. (**c**) Distribution of the $[Ca]_{SR}$ termination level of Ca sparks. The spark termination level was measured as $[Ca]_{SR}$ at the nadir of blink

stochastic attrition, Ca-dependent inactivation, adaptation, and use-dependent inactivation of the RyR [14, 19–21]. However, none of these mechanisms can fully explain robust termination of Ca sparks in the intact cellular environment.

RyR gating is determined through complex regulation by both $[Ca]_i$ and $[Ca]_{SR}$ [22]. During Ca release, the [Ca] in the cytosol increases from ~100 nM to 1 μM reaching concentrations as high as 300 μM in the vicinity of the RyR [23], while free $[Ca]_{SR}$ reciprocally drops from 1–1.5 mM to 300–400 μM [24, 25]. While an increase of $[Ca]_i$ in the dyadic cleft is critical for triggering of CICR, partial depletion of $[Ca]_{SR}$ seems to be more important for termination of CICR. Direct experimental evidence demonstrates that Ca sparks cease when $[Ca]_{SR}$ falls to a certain critical level (Fig. 2 and also see [10, 12, 26]) supporting the functional link between partial $[Ca]_{SR}$ depletion and CICR termination [27, 28]. It has been proposed that luminal Ca can regulate RyR by passing through an opened channel and acting on the cytosolic Ca activation site of neighboring channels—a "feed-through" mechanism [29, 30]. Therefore, local $[Ca]_{SR}$ depletion could terminate Ca spark by reducing the unitary current of the RyR and thus breaking the positive feedback of local CICR within a cluster [31]. In addition, several independent groups found that the RyR can be directly activated by luminal [Ca] independent of Ca release flux [32, 33]. These findings suggest that the RyR complex contains low-affinity Ca binding site(s) accessible from the luminal side of the channel. Dissociation of Ca from these sites during $[Ca]_{SR}$ decline leads to RyR deactivation

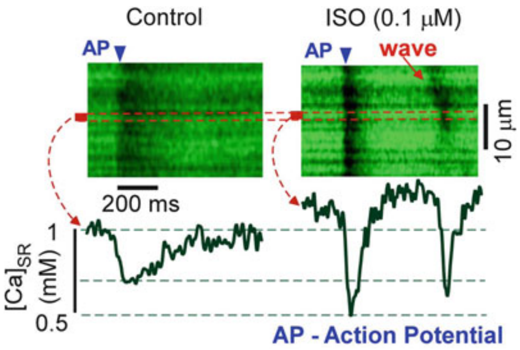

Fig. 3 β-Adrenergic receptor (β-AR) stimulation increases SR Ca release amplitude by decreasing the $[Ca]_{SR}$ termination level. Confocal images of $[Ca]_{SR}$ recorded in control conditions and during β-AR stimulation with isoproterenol (ISO). $[Ca]_{SR}$ profiles were obtained by averaging fluorescence from an individual SR Ca release junction (denoted by *red bars* to *left* of images). $[Ca]_{SR}$ depletions were induced by action potentials. ISO decreased $[Ca]_{SR}$ at which individual SR Ca release junctions terminate

and termination of CICR [27]. It has been suggested that the SR Ca buffering protein CASQ together with auxiliary proteins triadin 1 and junctin form the luminal Ca sensor of RyR [34]. Additionally, purified and recombinant RyRs remain sensitive to $[Ca]_{SR}$ to some extent [35] suggesting that the RyR protein itself contains additional luminal Ca sensor(s). Thus, the sensitivity of the RyR to $[Ca]_{SR}$ is multifaceted, involving several Ca binding sites from both sides of the channel.

Besides safety mechanisms to control inherently unstable CICR, efficient regulatory mechanisms exist to allow CICR substantial flexibility, so that the heart can adequately respond to changes in the metabolic demands of the body during exercise or stress. The strength of cardiac contraction can be adjusted by at least two different mechanisms—by changing sensitivity of the myofilaments to Ca or by changing cytosolic Ca transient amplitude. β-Adrenergic receptor (β-AR) stimulation produces the most important positive inotropic effect on the heart. This effect is mainly mediated via activation of protein kinase A (PKA) [1, 36] which subsequently phosphorylates several key Ca regulatory systems, such as a SERCA regulatory protein phospholamban (PLB), LTCC, and RyR. This results in an increase of Ca flux through LTCC, elevation of SR Ca load, and greater synchronization of SR Ca release during systole [37–40]. These modifications of the Ca transport systems play an important role in increasing Ca transient amplitude and thereby cardiac contraction. We have recently described a novel inotropic mechanism which involves alteration of the CICR termination process [41]. During β-AR stimulation with isoproterenol SR Ca release can be increased at individual release junctions by lowering the $[Ca]_{SR}$ termination level (Fig. 3). Further studies are required to determine which post-translation modifications of RyR are involved in this effect. β-AR stimulation is also associated with increased heart rate (positive chronotropic effect) which by itself can increase Ca transient amplitude [42]. Among the many factors that contribute to the positive force–frequency relationship (FFR) [1], Ca/calmodulin-dependent kinase type II (CaMKII) seems to play a particularly important role by phosphorylation of PLB [43] and RyR [44]. Thus, the increased SR Ca release during β-AR stimulation is likely mediated by activation of two major protein kinases, PKA and CaMKII [25, 45–47].

During diastole, the heart relaxes in preparation for refilling with circulating blood. For relaxation to occur, CICR must terminate allowing the Ca-ATPase (SERCA) to pump cytosolic Ca back into the SR and the Na–Ca exchanger (NCX) to extrude Ca from the cell. Other Ca transport systems such as the plasmalemmal Ca-ATPase (PMCA) and mitochondria play only a minor role in cardiac relaxation [48] (Fig. 1a). Among many proteins (including histidine-rich Ca binding proteins (HRC), calreticulin, S100A, sarcolipin) that regulate SERCA activity, the small transmembrane protein PLB is particularly important [49]. Unphosphorylated PLB inhibits SERCA activity, whereas phosphorylated PLB (by PKA and CaMKII) relieves SERCA inhibition allowing Ca pumping into the SR. PLB phosphorylation by PKA plays a major role in acceleration of SR Ca uptake and myocyte relaxation (lusitropic effect) during β-AR stimulation.

After termination of systolic Ca release RyRs do not enter an absolute refractory state. Instead, the decrease in luminal [Ca] reduces the sensitivity of the RyR to CICR and, therefore, to the cytosolic Ca trigger [50]. This mechanism has been suggested to play a protective role in the healthy heart by limiting extrasystolic after contractions and preventing the occurrence of Ca-dependent arrhythmias [51]. Therefore, RyRs are not completely closed during diastole and spontaneous openings of RyRs can generate a substantial SR Ca efflux commonly termed SR Ca leak [52, 53]. The SERCA-mediated Ca uptake and diastolic Ca leak together determine SR Ca load and, therefore, the amplitude of Ca transients that initiate contraction. Because the fractional SR Ca release steeply depends on SR Ca load [54–56], a small shift in this balance can change SR Ca load and, therefore, Ca transient amplitude. At normal physiological conditions, SR Ca leak may also serve as an important protective mechanism against SR Ca overload [25]. However, in certain pathological conditions associated with Ca overload, SR Ca leak becomes exacerbated. The Ca released during a spontaneous spark diffuses to neighboring release units, triggers CICR, and generates diastolic Ca waves [8, 57]. Spontaneous Ca waves can generate delayed afterdepolarizations (DADs), an effective trigger of cardiac arrhythmias [58]. After the discovery of Ca sparks, it was proposed that the entire diastolic SR Ca leak can be mediated by spontaneous Ca sparks [8, 59]. A Ca spark was considered a release event when a cluster of RyRs is activated in "all-or-nothing" mode because Ca release flux through spontaneous opening of RyR is enough to simultaneously activate all neighboring channels ("cluster bomb effect"). However, this perception has been recently changed. It has been shown that in ventricular myocytes a significant portion of SR Ca leak occurs as undetectable openings of single RyRs or spark-independent Ca leak [53, 60]. The spark-independent Ca leak is particularly significant at low $[Ca]_{SR}$ [53]. The spark-independent leak can arise from the same RyR clusters responsible for Ca spark generation because at low $[Ca]_{SR}$ the RyR openings are insufficient to recruit neighboring RyRs to form a spark [61]. In addition, openings of unclustered RyRs can also contribute to SR Ca leak [15].

Although some important steps of SR Ca regulation are not well understood, it becomes increasingly clear that heart function highly relies on synchronized SR Ca release with robust termination, well-balanced diastolic SR Ca leak, and effective SR Ca uptake. In the following chapter, we will present an overview of the structural and functional alterations of SR Ca transport systems in the failing heart. We will also discuss how dysfunction of different components of the SR Ca release machinery (mainly RyR and SERCA) causes abnormalities in SR Ca regulation in failing heart.

Alteration of SR Ca Homeostasis in HF

Heart failure is a complex clinical syndrome which can be characterized in general as a decreased ability of the heart to provide sufficient cardiac output to meet the energy demand of the body. In addition to decreased pump function, HF is also associated with an increased rate of sudden cardiac death due to ventricular tachyarrhythmias [62]. The pioneering studies of intracellular Ca dynamics in multicellular myocardial preparations from patients with HF exhibited significant alteration of Ca homeostasis [63, 64]. Single cell studies of cardiomyocytes isolated from human HF revealed elevated diastolic [Ca] and reduced Ca transients with a slower decay kinetic [65, 66]. Henceforth, abnormal Ca handling has been considered a hallmark feature of myocytes from failing hearts that leads to contractile dysfunction and arrhythmias.

To maintain proper Ca homeostasis during periodic electrical pacing, the amount of Ca that enters the cell via LTCC during each cycle must be extruded by NCX and Ca released during CICR must be pumped back into the SR by SERCA [67, 68]. In the healthy human heart, the main portion of cytosolic Ca (~70 %) that activates contraction is released from the SR, whereas the rest of Ca (~30 %) enters the cell via LTCC [1]. During development of HF, however, this balance gradually changes and cardiomyocytes become more dependent on Ca influx from the extracellular space [1]. Although factors that cause this shift in the balance may vary (myocardial infarction, energetic stress, changes in neurohumoral tone and pacing rate), decreased ability of the SR to retain Ca seems to be a more consistent finding that can explain depressed contraction of the failing heart. This SR Ca mishandling occurs as a result of an alteration in expression and/or activity of several Ca transporters, including SERCA and RyR. Furthermore, the dependence of myocytes on the trans-sarcolemmal Ca fluxes has another important implication for HF pathology—increased propensity to ventricular arrhythmias. Compelling experimental evidence indicates that ventricular arrhythmias can be triggered by spontaneous SR Ca release events during diastole [58]. It has been suggested that in

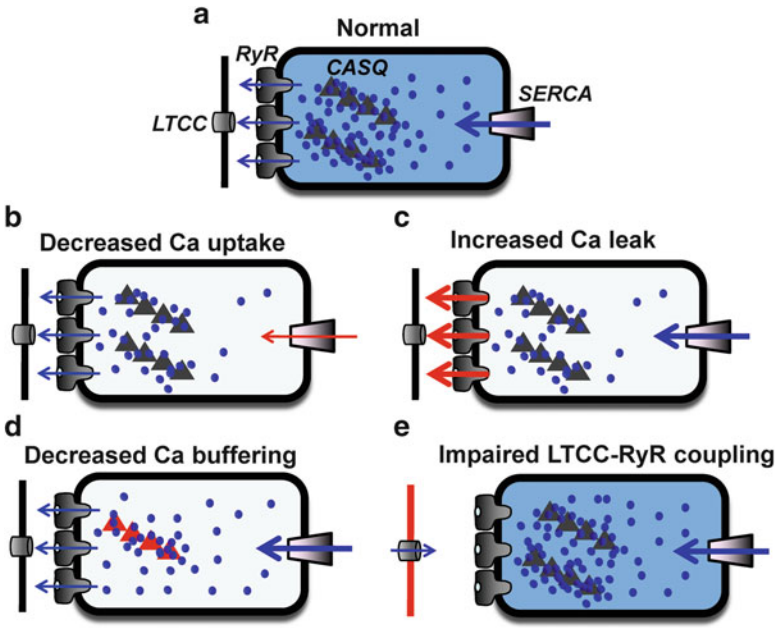

Fig. 4 Alteration of SR Ca regulation in failing heart. (**a**) In the healthy heart, SR Ca load is maintained by SERCA-mediated Ca uptake which effectively counterbalances a small RyR-mediated Ca leak. In failing heart, Ca transient amplitude can be decreased; (**b**) by diminishing SERCA pump function; (**c**) by increasing RyR-mediated Ca leak; (**d**) by decreasing intra-SR Ca buffer capacity; and (**e**) by decreasing the fidelity of the LTCC–RyR coupling resulting in asynchronous activation of SR Ca release units

myocytes from failing hearts excessive SR Ca leak (particularly in the form of Ca waves) increases diastolic Ca extrusion via the electrogenic NCX causing the generation of DADs [69, 70].

Most studies showed that systolic SR Ca release is significantly depressed in HF. From the perspective of SR Ca regulation, Ca transient amplitude can be decreased in several ways (Fig. 4): (1) by diminishing SERCA pump function; (2) by increasing RyR-mediated Ca leak; (3) by decreasing intra-SR Ca buffer capacity; and (4) by decreasing the fidelity of the LTCC–RyR coupling resulting in asynchronous activation of SR Ca release units. Results from different HF models revealed that, with exception of the SR Ca buffer capacity, all these aforementioned mechanisms can contribute to abnormal Ca regulation. The total level of CASQ did not significantly change in HF [11, 24, 71]. However, abnormal distribution of CASQ within the endoplasmic reticulum (ER) of the failing heart has been reported previously [72]. Regarding the other explanations it is ambiguous as to which holds the most central role. Some studies indicate that downregulation of SERCA plays the essential role in depressing SR Ca release in HF [73–77], while other studies explain these abnormalities by increased activity of the RyR [11, 17, 70, 78]. There are also conflicting results about molecular mechanisms that lead to RyR overactivity in HF. Phosphorylation of RyR either by PKA [78] or by CaMKII [79] has been suggested to be critical in increasing the channel's activity in HF. Furthermore, redox modification of the RyR is also an important factor during progression of HF [80, 81]. Other studies showed that HF is associated with remodeling of the T-tubular system leading to functional uncoupling of plasmalemmal LTCCs and RyRs on junctional SR [82–86]. Accordingly, the alteration of cell architecture has been suggested to play a critical role in the abnormality of SR Ca release in failing myocytes [84, 87]. The relative contributions of these different mechanisms may vary in different models of HF and with different degrees of severity of failure. Also, differences in Ca regulation between different species have to be taken into account [1].

Structure and Function of the RyR in HF

Properties of cardiac RyR (type 2) have been documented in several reviews [22, 88–90]. The RyR complex (~2,300 kDa) has an oligomeric structure formed by four identical subunits. The channel exhibits high conductance and low selectivity for Ca. The RyR is not only a Ca release channel but also a giant scaffolding protein on which several regulatory proteins and enzymes are concentrated [78]. On the cytosolic side, RyR interacts with calmodulin (CaM), FK-506 binding proteins (FKBP), sorcin, and Homer-1. It has been shown that FKBP12.6 can affect RyR by stabilizing the interaction between the channel subunits [91], whereas CaM and sorcin affect the channel activity via Ca-dependent mechanisms [92, 93]. The RyR also forms complex with two major protein kinases (PKA and CaMKII) and three phosphatases (PP1 and PP2A and PP2B) indicating the importance of RyR regulation by phosphorylation [88, 94]. The SR-membrane proteins, junctin and triadin, are believed to be crucial for the RyR's ability to sense changes in luminal [Ca] via interactions with the major SR Ca-chelating protein CASQ [34] and HRC [95]. In addition, RyR has multiple sites for regulation by ions and small molecules, including Ca, Mg, and ATP. With so many associated proteins and regulators, a proper arrangement and stoichiometry of the RyR complex is critical for normal SR Ca regulation.

Structural Alteration of the RyR in HF

RyR expression. Numerous publications demonstrated a reduction of RyR expression on mRNA and protein level in human and animal HF preparations [11, 79, 96–101]. It has been shown that HF is associated with decreased binding of ryanodine to the receptor [102, 103] suggesting either reduction in the RyR expression level, open probability, or structural changes in the region of the protein responsible for ryanodine binding. Decrease in RyR expression level may be involved in the development of contractile dysfunction of the failing heart. The reduction in RyR level by itself would decrease SR Ca release. However, this effect can be partially compensated by increased RyR activity as it was shown by several studies (see below). Conversely, there are also several publications that did not find any significant difference in the RyR level between normal and HF [75, 87, 104].

Sub-domain and domain–domain interactions. Emerging evidence demonstrates significant rearrangements within the RyR macromolecular complex in HF [94]. Defective interactions between regulatory domains within the RyR or dissociation of key components from the RyR complex have been suggested to play a critical role in dysfunction of the channel contributing to abnormal SR Ca regulation. FKBP12.6 is one such component believed to be necessary for regulation of RyR at many levels. It has been proposed that FKBP12.6 binds to the cytosolic domain of RyR to stabilize the interaction among RyR subunits. This interaction functionally translates into coupled gating, meaning that all subunits of the RyR open and close simultaneously [96, 105]. FKBP12.6 may also be involved in physical and functional coupling between neighboring tetramers [91, 105]. It has been shown that during HF, chronic RyR hyperphosphorylation by PKA (Ser-2808) causes dissociation of FKBP12.6 from the channel. This alteration in RyR structure leads to increased SR Ca leak [99] as a result of prolonged subconductance openings of the channel [78]. A similar alteration in the RyR complex structure (but not in function) was reported by another group [79]. Furthermore, it has been suggested that FKBP12.6 is also responsible for stabilizing intra-domain interactions within the RyR subunit. In healthy hearts N-terminal and central domains of the RyR interact [106]. This zipping between domains keeps the RyR in closed state and prevents diastolic SR Ca leak. During HF, dissociation of FKBP12.6 from the channel (due to PKA phosphorylation or oxidative stress) causes domain unzipping and increases SR Ca leak [107, 108]. However, despite many years of research, there is still controversy about the functional role of FKBP12.6 and RyR phosphorylation. The role of FKBP12.6 in augmentation of SR Ca leak has been challenged by several different laboratories. It has been shown that level of FKBP12.6 associated with the RyR did not significantly change during RyR phosphorylation by PKA [109, 110] or during HF [75].

RyR phosphorylation. The processes of phosphorylation and dephosphorylation are posttranslational mechanisms utilized to rapidly change the function of many proteins including ion channels. Rapid and robust control of phosphorylation state of the SR Ca release channel is ensured by kinases PKA and CaMKII, phosphatases PP1, PP2A, and PP2B, and phosphodiesterase 4D3, all of which are bound to the RyR [88, 94, 111, 112]. In general, increased RyR phosphorylation in failing hearts can be explained either by increased localized activity of kinases or by diminished activity of phosphatases. In many animal HF models and also human HF, activity and expression of CaMKII are increased [79, 113]. However, this does not always translate into increased levels of CaMKII in the complex with the RyR [78, 114]. On the other hand, several independent groups have suggested that an increased level of RyR phosphorylation in HF can be explained by decreased levels of phosphatases PP1 and PP2A [78, 79, 114] and phosphodiesterase 4D3 [111] associated with the RyR.

It has been demonstrated that the RyR can be phosphorylated by serine–threonine kinases at multiple sites [115]. Thus far only three sites on cardiac RyR were suggested to be of functional relevance, namely PKA-specific sites Ser-2808 and Ser-2030 [78, 116, 117], and CaMKII-specific site Ser-2814 [118]. Functional consequences of RyR phosphorylation have been recently summarized in several reviews [119, 120]. Despite a major collective effort, the role of PKA-dependent phosphorylation in modulation of RyR function in normal and failing hearts remains a subject of heated debate [121, 122]. Studies from different laboratories have yielded conflicting results regarding the role of RyR phosphorylation at Ser-2808 in the progression of HF, which ranged from having an essential role [78, 107, 123, 124] to having only a very limited function [114, 125, 126]. On the contrary, increased RyR activity due to enhanced phosphorylation of RyR at CaMKII site Ser-2814 appears to be a more consistent finding. Hyperphosphorylation of RyR at the CaMKII site has been demonstrated in numerous (but not all [44]) HF models, and CaMKII inhibition was shown to attenuate abnormalities in Ca handling in myocytes from failing hearts [70, 79, 113, 127, 128].

RyR redox modifications. Alteration of RyR phosphorylation status is not the only type of posttranslational modification that could affect RyR function during development of HF. Changes in cellular metabolism that occur during the progression of HF can lead to depletion of the antioxidant defense and to an increase in reactive oxygen species (ROS) production [129]. Oxidation of the RyR by ROS can enhance the channel activity [130]. Accordingly, modulation of RyR activity by ROS in HF has been suggested as a major cause of the observed abnormality in Ca regulation [80, 81]. Cardiac RyR contains 89 reactive cysteine residues [131] and the number of free thiols has been shown to be dramatically decreased in HF. Incubation of myocytes isolated from canine failing hearts with ROS scavengers and antioxidants was able, to a large degree, to restore Ca transient amplitude and reduce RyR-mediated SR Ca leak [70]. ROS scavengers also normalized RyR sensitivity to luminal Ca in lipid bilayer experiments [81]. It has also been suggested that normalization of redox status of RyRs from failing hearts reverses inter-domain unzipping, thereby stabilizing RyR function [80]. The major sources of ROS in ventricular myocytes include NADPH oxidase (NOX), the mitochondria electron transport chain, xanthine oxidase/reductase (XOR), and uncoupled nitric oxide synthase (NOS). NOS1 and XOR were demonstrated to co-immunoprecipitate with the RyR, and their levels in HF are generally increased (for review, see [132, 133]). Activation of NOX2 residing in T-tubules was shown to increase Ca spark activity [134], while attenuation of ROS production by mitochondria stabilized SR Ca release during digitalis treatment [135] or β-AR stimulation [136].

Notably, no clinical studies have demonstrated beneficial effects of long-term treatment with antioxidants (for review see [129]), as well as treatment with a XOR inhibitor allopurinol [132, 133]. The failure of clinical trials can be a result of low scavenging efficacy of the antioxidant, but not the antioxidant therapy per se.

Because ROS have very limited diffusion distance, they predominantly affect closely opposed targets. Therefore, it seems that a more beneficial strategy would be to design antioxidants that can prevent ROS production locally.

RyR luminal Ca sensor. Abnormally high activity of the RyR in HF has been attributed to increased sensitivity to luminal Ca [11]. This defect can be caused by chronic dissociation of several auxiliary proteins (junctin, triadin, and CASQ) that form the luminal Ca sensor of the channel [34, 137]. Although the total level of CASQ does not change in HF [11, 24, 71], it has been suggested that abnormal posttranslational processing of CASQ can cause its defective trafficking [72]. This can potentially lead to a local depletion of CASQ in the junctional SR. Additionally, it has been demonstrated that the level of another SR Ca buffer (HRC) that binds to the RyR complex is significantly lower in failing myocytes from human and animal models [138]. Finally, the levels of triadin and junctin were reported as being dramatically reduced in human end stage HF [139]. Future studies are needed to assess whether restoration of the levels of proteins that confer luminal Ca sensitivity to RyR in failing heart will lead to normalization of SR Ca release channel function.

Calmodulin. It has been reported that failing hearts have a reduced amount of CaM associated with the RyR complex [79, 140]. A molecular mechanism of CaM dissociation may be a defective interaction between N-terminal and central domains within the RyR [140]. However, it is not clear yet how CaM dissociation from the RyR affects SR Ca release. Several studies showed that CaM decreases single RyR channel activity and Ca spark frequency [92, 141], whereas other shows that CaM can increase the channel and spark activity [142]. Such discrepancies can be explained by the dual role of CaM: (1) it can serve as a stabilizer of RyR via direct binding to the channel and (2) it can be involved in regulation of phosphorylation–dephosphorylation balance of the RyR as an essential component for activation of CaMKII and phosphatase PP2B (also known as calcineurin or PP3).

Regardless of the molecular mechanisms, it is clear that during HF many important regulatory proteins detach from the RyR complex, and the channel simultaneously becomes more phosphorylated and oxidized (Fig. 5). These modifications of the RyR can cause SR Ca mishandling, contractile dysfunction, and arrhythmias.

Functional Alteration of the RyR in HF

SR Ca release activation. Global SR Ca release is a summation of thousands of Ca sparks activated during an AP. Thus, a greater Ca transient can be achieved by more synchronized recruitment of individual SR Ca release units [39]. The degree of synchronization of SR Ca release depends on the strength of the trigger and the efficiency of coupling between the LTCC and RyR cluster. It has been suggested that decreased SR Ca release in HF is a result of decreased fidelity of LTCC–RyR coupling, leading to less organized and less synchronized SR Ca release [87]. HF myocytes from a myocardial infarction model showed

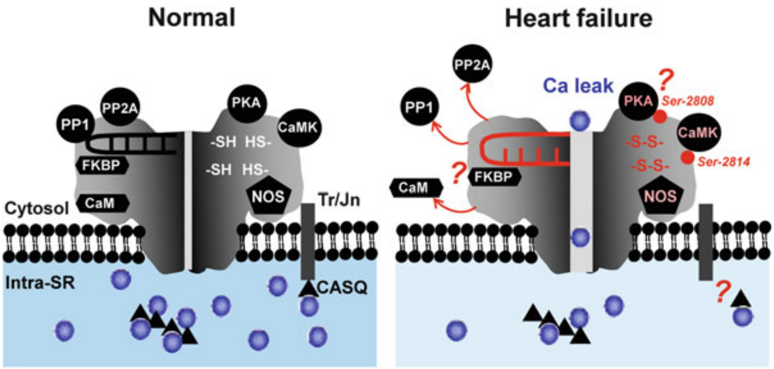

Fig. 5 Alteration of the RyR complex structure in failing heart. On the cytosolic side, RyR interacts with at least calmodulin (CaM), FKBP, nitric oxide synthase (NOS), two major protein kinases (PKA and CaMKII), and two phosphatases (PP1 and PP2A). Moreover, triadin and junction (Tr/Jn) form the luminal Ca sensor via interactions with the SR Ca-chelating protein calsequestrin (CASQ). RyR also contains ~100 reactive cysteine residues (SH–). During heart failure, CaM and phosphatases (PP1 and PP2A) dissociate from the RyR complex. The channel becomes more phosphorylated at the CaMKII and the PKA sites. Cysteine residues become oxidized forming the disulfide bonds. Dissociation of FKBP12.6 from the channel causes N-terminal and central domain unzipping. CaMKII and PKA become more active and NOS uncoupled

diminished Ca transients, which are associated with slow rising and decay phases of the transient. It appears that on the subcellular level some RyR clusters have a delayed response to the stimulus. This desynchronization of SR Ca release in HF is due to downregulation of LTCC current [82, 143]. A similar effect can be achieved in healthy myocytes by partially inhibiting LTCC current [144]. However, in failing myocytes from spontaneously hypertensive rats, desynchronization of SR Ca release was a result of remodeling of the T-tubule system rather than a reduction in LTCC current [84, 87]. It appears that the reorganization of the T-tubular system leads to a substantial mismatch of the T-tubules with the junctional SR. Such spatial dissociation of triggering LTCCs and RyRs has been attributed to downregulation of junctophilins [145], proteins that tether T-tubules to the junctional SR [5]. It seems that this leaves some RyR clusters without local control by LTCC. At these "orphaned" clusters, Ca release is delayed because their activation depends on Ca diffusion from intact SR Ca release units instead of LTCC. Reports that the T-tubule system is significantly disorganized in cardiomyocytes from human [83, 85, 86] and animal HF models provide compelling evidence for this theory [82, 85, 104, 145]. However, other studies found that the T-tubule system remains relatively intact in HF [146].

Although the vast majority of SR Ca release during ECC is mediated by LTCC, it has also been proposed that Ca influx via NCX can trigger CICR [147]. It has been suggested that Ca entry via LTCC and NCX interacts synergistically to trigger greater SR Ca release during β-AR stimulation and PKA activation [148].

In HF induced either by chronic administration of β-adrenergic agonist isoproterenol or by volume overload, this mechanism has been found to be significantly compromised. This alteration in Ca regulation could account for the reduced SR Ca release and low response to β-AR stimulation in HF [148].

ECC gain. ECC gain quantifies the amplification strength of CICR. It is often calculated as SR Ca release divided by the amplitude of LTCC current [149]. Due to sensitivity of the RyR to luminal [Ca], ECC gain steeply depends on SR Ca load [55]. Thus, a small decrease in $[Ca]_{SR}$ would lead to significant decrease in Ca transient amplitude. Indeed, several studies of different HF models showed that the decrease in Ca transient amplitude was mainly due to depletion of the SR Ca load, but not to the LTCC current density [73–77]. Thus, it has been concluded that diminished SR Ca uptake, but not ECC gain, plays a crucial role in suppression of systolic SR Ca release in HF. However, experiments on HF myocytes from hypertensive rats showed that Ca transient amplitude was significantly reduced as a result of a decrease of ECC gain, not of SR Ca load [87]. The decreased ECC gain in this HF model was explained by inefficient coupling between LTCCs and RyRs, presumably due to loss of T-tubules. Additionally, a study that analyzed the changes of Ca handling during progression of HF found a significant increase in ECC gain at the early stage of HF, but diminished at advanced stage [70].

Fractional SR Ca release and Ca release termination. Recent studies suggest that depleted SR Ca load in HF would reduce Ca transient even more dramatically if it were not compensated for by an increase in RyR activity. Local depletion of $[Ca]_{SR}$ causes a decrease in the RyR open probability, and this mechanism is believed to be responsible for termination of Ca sparks and for refractoriness of SR Ca release [27,28]. As a result of the remodeling processes that occur in HF, the channel becomes more sensitive to $[Ca]_{SR}$ [11, 81]. It has been shown that under conditions of matched SR Ca load, the fraction of total $[Ca]_{SR}$ released to the cytosol (or fractional SR Ca release) was significantly larger in HF when compared to normal hearts [24, 41, 150]. This compensatory mechanism partially prevents reduction in Ca transient amplitude at a lower SR Ca load. Figure 6 shows examples of cytosolic Ca transients (*red*) and corresponding $[Ca]_{SR}$ depletions (*green*) recorded under different experimental conditions. Partial inhibition of SERCA in non-failing myocytes led to significant decrease of the Ca transient amplitude and fractional SR Ca release as a result of depletion of SR Ca load (Fig. 6, second images). Although SR Ca load was similarly decreased in myocytes from HF, the fractional SR Ca release and, consequently, Ca transient amplitude was significantly larger (Fig. 6, far right images). Similar to RyR activation with caffeine (Fig. 6, third images), the modifications of the RyR that occur in HF increase SR Ca release by decreasing the $[Ca]_{SR}$ level at which release terminates. It appears that during the early stages of HF, sensitization of the RyR allows myocytes to maintain Ca transients of nearly normal amplitude, despite the diminished SR Ca load [41, 70]. However as a drawback, these RyR modifications increase diastolic SR Ca leak (largely due to lowered SR [Ca] threshold for Ca spark

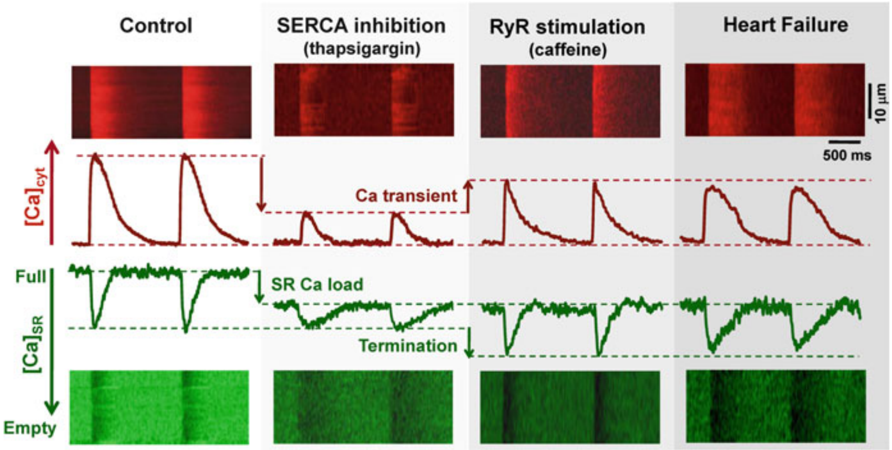

Fig. 6 Cytosolic Ca transients, SR Ca load, and SR Ca release termination. From *left* to *right*: Cytosolic Ca transients induced by action potentials (*top*) and corresponding global $[Ca]_{SR}$ depletions (*bottom*) recorded in a non-failing myocyte at control conditions; in a non-failing myocyte after partial SERCA inhibition with thapsigargin (0.5 µM); in a non-failing myocyte after activation of the RyR with caffeine (200 µM); and in a myocyte from a failing heart. Cytosolic Ca transients were measured using the high-affinity Ca indicator Rhod-2 loaded into the cytosol. Global $[Ca]_{SR}$ depletions were measured with the low-affinity Ca indicator Fluo-5N loaded into the SR

activation and termination) and increase the propensity of cardiac arrhythmias [53, 107, 150–152]. At more advanced stages of HF, severe dysfunctions of the RyR cause a significant reduction of the Ca transient as a result of a massive loss of SR Ca content [70].

SR Ca release refractoriness and RyR-mediated SR Ca leak. Abnormally high activity of RyRs due to increased sensitivity to luminal Ca has also been linked to shortened refractoriness of SR Ca release during diastole. Premature reopening of RyRs slows relaxation, exacerbates reduction of SR Ca content, and promotes generation of spontaneous Ca sparks which can ignite arrhythmogenic Ca waves [51, 70] contributing to diastolic and systolic dysfunction of HF. A growing body of evidence indicates that RyR-mediated Ca leak is significantly increased in the failing heart [53, 70, 78, 79, 81, 99, 107, 150, 152]. Augmentation of SR Ca leak is the one of the earliest alterations of Ca handling during the progression of HF that precedes changes in the amplitude of the Ca transient [70]. Increased RyR-mediated Ca leak can contribute to a reduction of SR Ca content and trigger Ca-dependent arrhythmias. This also implies that during diastole SERCA cannot achieve its maximal thermodynamic efficiency and more ATP must be consumed to balance the increased SR Ca leak. Thus, SR Ca leak is energetically costly, particularly in the metabolically compromised failing heart.

During diastole, Ca sparks serve as the main pathway for SR Ca leak [8, 53, 59]. Studies of Ca sparks in HF revealed varied results, ranging from decreased [41, 153] to increased spark frequency [11, 70, 85, 113]. Such diversity can arise from species- and model-specific differences in Ca regulation, and also from differences in experimental conditions. For example, a study of myocytes from human HF showed significantly lower Ca spark frequency compared to non-failing cells [153]. When SR Ca load was elevated in HF cells, spark frequency was increased to a level higher than control myocytes, suggesting that RyR activity is increased in human HF. Similar results were obtained in a study of rabbit HF induced by combined aortic insufficiency and stenosis [41]. In a rabbit pacing-induced HF model Ca spark frequency was similar to non-failing hearts, but the amplitude was significantly decreased [101]. Due to the observed decrease in RyR and SERCA expression levels, the authors concluded that alteration of spark properties was a result of combined defects in SR Ca release and uptake. In a study of a canine rapid pacing-induced HF model, Ca spark frequency was significantly higher in failing myocytes, despite a decreased SR Ca load [11]. These seemingly contradicting results were explained by increased sensitivity of the RyR to luminal [Ca] which was directly confirmed by single RyR measurements. It appears that increased RyR sensitivity to luminal [Ca] during HF contributes not only to the augmentation of spark frequency but also to the augmentation of non-spark-mediated Ca leak.

It has been shown recently that in ventricular myocytes a significant portion of SR Ca leak can occur as undetectable openings of the RyR, referred to as the spark-independent SR Ca leak [53]. This component of Ca leak becomes particularly predominant at low SR Ca loads. Because failing myocytes are characterized by depleted SR Ca content, this would imply that a significant portion of SR Ca leak in HF is mediated by spark-independent pathways. The specific role of increased non-spark-mediated Ca leak in HF is yet to be elucidated.

Other Pathways for Ca Release from the SR

Although RyR is the major Ca release channel on the SR of adult ventricular myocytes, contributions from other Ca pathways need to be considered. The existence of RyR-independent Ca leak in ventricular myocytes has been suggested before [53, 154], however the molecular mechanisms responsible for this Ca leak have not yet been identified.

IP_3 receptors. A potential candidate in this context is the inositol-1,4,5-trisphosphate receptor (IP_3R) SR Ca release channel. There are three IP_3R isoforms referred to as type 1, 2, and 3, encoded by three distinct genes. Type 2 is the primary isoform of the IP_3R expressed in ventricular myocytes [155]. Cardiac IP_3R is more sensitive to IP_3 and almost insensitive to $[Ca]_i$ over the physiological range of $[Ca]_i$ [156] suggesting that the channel acts purely as a IP_3 sensor. Although expressed at lower densities compared to RyRs, IP_3R-dependent Ca release can facilitate CICR and even trigger arrhythmogenic Ca waves in cardiomyocytes [157, 158]. It has been shown that IP_3Rs are upregulated during HF [79, 98]. Recently we have shown

that activation of IP_3Rs in normal myocytes nearly doubled RyR-independent Ca leak [53]. These results suggest that activation of the IP_3R can contribute to the increased SR Ca leak during HF, particularly under conditions associated with elevated neurohumoral tone (e.g., endothelin-1) and stimulation of the phospholipase C-IP_3 signaling pathway. Furthermore, it has been proposed that stimulation of IP_3R-mediated Ca leak can regulate gene transcription during development of hypertrophy and HF [159]. Clearly more studies are needed to determine the pathological role of the IP_3R in HF.

Phospholamban (*PLB*). PLB is a small transmembrane protein that regulates SERCA activity [49]. PLB exists in dynamic equilibrium between three different forms: in complex with SERCA, as a single molecule, and as a pentamer. Phosphorylation of PLB by PKA (Ser-16) shifts this balance toward formation of PLB pentamers [160]. It has been shown that PLB pentamers can function as Ca channels in lipid bilayers [161] and thereby can contribute to SR Ca leak. Thus, changes in PLB phosphorylation level that occur during HF [71, 79, 162, 163] can potentially affect diastolic SR Ca leak. However, Ca leak measured from SR vesicles isolated from wild-type and PLB-knockout mice was not significantly different [164]. Some studies have even reported that the PLB pentamer does not possess Ca channel activity [165]. Thus there is yet to be any conclusive evidence on this theory.

Other pathways. Cardiac SR, besides being the major organelle to store Ca, is responsible for other primary functions of endoplasmic reticulum as for any cell type (such as protein folding). Therefore the same may be said for other Ca leak pathways which exist in non-muscle cells. These pathways may include ATPase back-flux, Ca leak mediated by a small apoptosis-related protein BCL2, and the protein complex associated with the translocation of nascent polypeptides across membranes called translocon [166]. In addition, the transient receptor canonical type 1 (TRPC1) involved in capacitative Ca entry via sarcolemma was recently found to reside on the SR membrane of skeletal muscle and overexpression of this Ca channel led to accelerated loss of Ca from the SR [167]. Despite the potential importance of the aforementioned pathways in health and disease their role in cardiac Ca homeostasis has not been studied yet.

Structure and Function of SERCA in HF

Relaxation of cardiac muscle relies on removal of Ca from the cytosol. One mechanism by which this is achieved is through re-sequestration of Ca into the SR by SERCA. By hydrolyzing one ATP molecule, SERCA can transport two Ca ions from the cytosol into the SR lumen. SERCA activity also plays a critical role in setting of diastolic $[Ca]_i$ and SR Ca load. A specific SERCA isoform, SERCA2a, is one of the most abundant proteins of the cardiac SR. This Ca pump contains ten transmembrane domains (M1–M10) and several cytoplasmic domains, including a nucleotide binding (N) domain, an actuator (A) domain, and a phosphorylation (P) domain [168]. SERCA is freely mobile in the SR membrane [169], suggesting that the pump can be equally distributed throughout the SR network. Although SERCA interacts with a wide array of proteins (including HRC, PP1, calreticulin, S100A, and sarcolipin), PLB is the most important regulator of the pump activity [49]. Under the basal (unphosphorylated) state, PLB inhibits SERCA activity by lowering the apparent affinity of the pump for Ca. Phosphorylation of PLB at Ser-16 by PKA [170] largely relieves this inhibition, increasing the pumping rate by several fold. Stimulation of SR Ca uptake by PLB phosphorylation appears to be the major mechanism of acceleration of relaxation (lusitropic effect) during β-AR stimulation. SERCA activity can also be increased via phosphorylation of PLB by CaMKII at Thr-17 [170]. This mechanism has been suggested to be responsible for a stimulation frequency-dependent acceleration of SR Ca uptake. In addition to protein interactions, SERCA is highly sensitive to changes in the metabolic status of the cytosol, including the ATP/ADP ratio, pH, and the redox potential [1, 171].

Structural Alteration of the SERCA/PLB Complex in HF

SERCA and PLB expression. It is still a matter of debate whether the decreased SR Ca uptake in HF is due to a decrease in SERCA expression level or in SERCA activity. Downregulation of SERCA2a at the mRNA and protein levels has been shown in numerous studies of human and animal HF models [74, 75, 97, 101, 163, 172–176]. All these studies highlight the critical role of SERCA in abnormal Ca homeostasis and contractile dysfunction of the failing heart. For example, it has been shown that the transition from hypertrophy (induced by aortic banding in rats) to HF is associated with significant decrease in the SERCA mRNA level [172]. It has also been shown that increasing the level of SERCA protein via adenoviral infection can improve the function of hypertrophied hearts and can delay its transition to HF [177]. However, there are a significant number of publications that did not find any alteration in the SERCA expression level in human HF [162, 178–180] and animal HF models [11, 181].

It has been shown that HF is also associated with a decrease in the PLB expression level [74, 75, 97, 101], but to a lesser degree than SERCA [175, 176, 182]. It has been suggested that the decreased SERCA–PLB ratio can lead to inhibition of SR Ca uptake in HF, because an increased SERCA fraction would be inhibited by PLB binding. On the contrary, the level of PLB remains unchanged in HF models characterized by an unchanged SERCA level [11, 79, 162, 178, 179].

Posttranslational modifications of SERCA and PLB. In addition, the diminished SR Ca uptake in HF can be a result of posttranslational modifications of the SERCA/PLB complex. It has been shown that the responsiveness of SERCA to activation either by cAMP or by PKA was significantly decreased in human failing heart compared to non-failing myocardium [162]. Furthermore, the PLB phosphorylation level at the PKA-specific site was significantly decreased in HF. Another group showed that SERCA activity and its Ca sensitivity were significantly decreased in HF compared to normal heart [71, 180]. These changes in the pump's activity were associated with decreased PLB phosphorylation at both the PKA and the CaMKII sites; however, the total level of SERCA and PLB remained unchanged [71, 180]. A decrease in PLB phosphorylation level can result from increased PP1 activity, observed in failing hearts [183]. In contrast, another study showed that the total level of PLB phosphorylation was higher in HF preparations [163]. Decreased SR Ca uptake in failing heart was explained by downregulation of the SERCA expression level. Another study showed that PLB phosphorylation level was increased at the CaMKII site but was decreased at the PKA site [79]. The authors suggested that the overall PLB phosphorylation level in HF would have only a minor effect on SERCA activity.

It has been recently established that several naturally occurring mutations of PLB can give rise to human dilated cardiomyopathy. For example, the missense mutation of Arg-9 to Cys (R9C) impairs SERCA regulation by preventing PKA-mediated phosphorylation of PLB [184]. Moreover, this mutation may also increase the sensitivity of PLB to oxidation, mediating disulfide bond formation between adjacent proteins in the PLB pentamer [185]. Such cross-linking prevents dissociation of PLB into the active monomers, and may increase association of protein into nonphosphorylatable aggregates. Thus, the R9C mutation may cause impairment of Ca handling leading to development of HF.

There is only a small amount of information regarding posttranslational modifications of the SERCA protein that affect Ca handling in HF. It has been reported that the contractile dysfunction in Gαq-induced cardiomyopathy was mediated by inhibition of SERCA activity as a result of oxidation of cysteine residue(s) on the pump [186]. Another study has shown that the depressed SR Ca uptake of failing myocytes is associated with dissociation of the small ubiquitin-related modifier 1 (SUMO1) from SERCA [187]. It has been suggested that restoring the sumoylation level of SERCA can stabilize the pump structure and improve SR Ca uptake in mouse and human failing hearts. However, these results are controversial, since a protein of the molecular weight corresponding to the sumoylated SERCA has never been detected in many previous immunoblotting studies of normal and failed human hearts [188].

Alteration of SERCA Activity in HF

In human and different animal HF models, evidence from SR Ca uptake studies indicates there is a reduction of SERCA activity [71, 75, 163, 178, 189, 190]. In many cases (but not all), this effect was linked to the downregulation of SERCA at the protein and mRNA levels. Furthermore, numerous studies of failing cardiomyocytes showed that the decrease in Ca transient amplitude was associated with a depletion of the SR Ca load [73–77]. Based on these results, it has been suggested that abnormal SR Ca uptake (rather than SR Ca release and Ca current) is one of the primary causes of depressed SR Ca release and contraction in failing heart. Decreased SR Ca uptake can also contribute to an increase in diastolic $[Ca]_i$. Thus, reduction in SERCA activity can cause the impairment of both systolic and diastolic function in the failing myocardium. Studies by another group showed that the decrease in SR Ca load was a result of a significant increase in NCX activity and only a modest decrease in SERCA function [181, 191]. It has been proposed that upregulation of NCX together with downregulation of SERCA plays a key role in alteration of Ca regulation that leads to depletion of SR Ca load in the failing heart [182, 192]. Notably, several recent publications showed no significant alteration in SERCA-mediated Ca uptake during ECC in intact failing myocytes as well as during rest in permeabilized cells [70, 152]. To explain a similar HF phenotype, the authors reported significant augmentation of RyR-mediated Ca leak which causes depletion in SR Ca load.

A normal heart responds to increased stimulation frequency by generating larger force (positive FFR or the Bowditch effect). This mechanism allows increased cardiac output, thereby, providing a sufficient energy supply to the body during stresses. It has been shown that at low stimulation frequencies the failing myocardium has similar contractile characteristics as the non-failing heart. However, increasing the pacing rate causes contractility to decrease in HF [63]. The blunted or negative FFR is one of the prominent characteristics of failing hearts [193]. It has been suggested that the negative force–frequency response of the failing heart stems from an inability of the SR to increase Ca load at increased stimulation frequencies. This effect was explained by a decrease in SERCA activity and an enhancement of NCX extrusion of Ca [189, 190, 194]. Furthermore, the same mechanism was attributed to a decreased response to inotropic agents of human failing heart at the end stage [195]. Alternatively, it has been proposed that defective regulation of the RyR by CaMKII plays a critical role in the blunted FFR of the failing heart [44].

SERCA is regulated thermodynamically (i.e., by ΔG_{ATP}) as well as kinetically (i.e., via changes in ATP hydrolysis products) (for review see [1, 171]). The dependence of cardiac SR Ca uptake on energy supply has been demonstrated in different experimental conditions [196–198]. Furthermore, SERCA activity also depends on the removal of ATP hydrolysis products, ADP and P_i [199]. Since SR Ca uptake is highly dependent on the effective energy supply, SERCA activity can be reduced as a result of metabolic imbalance that commonly occurs during HF [200]. It has been demonstrated that a glycolytic metabolite pyruvate is able to improve SERCA activity and the contractile performance of isolated failing human myocardium [201]. It has also been proposed that an increase in phosphorylation potential (i.e., the ratio of [ATP] to [ADP] $\times$ $[P_i]$) would improve the efficiency of ATP-dependent processes, especially those participating in the ECC cycle, such as the acto-myosin ATPase and the SR Ca-ATPase.

Normalization of SERCA and RyR Function as a Treatment Strategy in HF

Abnormalities in SR Ca regulation play a critical role in contractile dysfunction and arrhythmogenesis of the failing heart, making the RyR and SERCA clinically relevant sites for potential therapeutic interventions. Normalization of SR Ca regulation in HF can be achieved in two conceptually different ways—either by improving SR Ca uptake or by preventing loss of SR Ca during diastole.

An increase in SR Ca uptake can be achieved by overexpression of the SERCA pump, knock-out of PLB, or overexpression of phospho-mimetic PLB. These approaches have been demonstrated to improve contractile performance and delay progression of HF in several animal HF models (for review see [202]). It seems, however, that these approaches would be less beneficial for conditions of HF that are associated with increased RyR sensitivity to luminal [Ca] [11, 70, 150]. An attempt to increase SR Ca load in these HF models would also enhance SR Ca leak, thus increasing the likelihood of life-threatening arrhythmias. Moreover, increasing SERCA activity without reducing SR Ca leak would further increase energy consumption in already energy compromised tissue. In support of this reasoning, it has been shown that the loss-of-function PLB mutation has been associated with the development of cardiomyopathy in humans [203]. Interestingly, however, restoration of SERCA expression level in HF can stabilize SR Ca load and prevent arrhythmias by decreasing SR Ca leak and normalizing RyR phosphorylation [204]. It is possible that in this experimental setting the beneficial effect of SERCA overexpression primarily stems from increased $[Ca]_i$ buffering, as in the case of parvalbumin overexpression [205], and not due to increased SR Ca uptake.

The therapeutic approaches that target RyR-mediated Ca leak are considered to be another promising strategy to improve Ca homeostasis in the failing heart. Initially, it seems counterintuitive since systolic Ca release is already reduced in HF and inhibition of RyR may worsen systolic dysfunction. In practice, however, moderate inhibition of RyR activity in HF was proven to restore Ca cycling to a substantial degree. Partial RyR inhibition efficiently prevents diastolic loss of SR Ca, thus increasing SR Ca content. Subsequently, increased $[Ca]_{SR}$ helps to override RyR block during systole resulting in nearly normal Ca transients. Several groups showed potential benefit of using dantrolene, an anti-malignant hyperthermia drug, for normalization of cardiac RyR function in failing hearts. It has no effect on cardiac RyR from normal hearts. However, in HF it has been demonstrated to prevent arrhythmogenic Ca release and improve FFR and β-AR responsiveness [206, 207]. The proposed mechanism of these beneficial effects is that dantrolene stabilizes aberrant inter-domain interactions within the RyR from failing hearts, similarly to another pharmacological agent, a 1,4-benzothiazepine derivative JTV519 (K201) [208, 209]. It has been suggested that one of the beneficial effects of beta-blockers is to reduce phosphorylation of the RyR by PKA, restore FKBP12.6 binding, and normalize SR Ca leak [78, 99]. More recently, inhibition of CaMKII and restoration of the RyR redox status have emerged as promising strategies to normalize RyR function in diseased hearts [79–81, 113, 127]. The apparent disadvantage of these approaches is that available pharmacological agents cannot reduce phosphorylation or oxidation of the RyR specifically, without affecting other cellular systems and producing adverse effects.

Conclusion. Heart failure remains an incurable disease with the highest mortality rate. Over the last 2 decades, significant progress has been made in the understanding of mechanisms of SR Ca mishandling that leads to contractile dysfunction and arrhythmogenesis of the failing heart. It has become increasingly clear that functional impairment of two major Ca regulatory systems, the RyR and the SERCA/PLB complexes, contributes in great degree to aberrant SR Ca homeostasis. However, the previous studies indicate that the role of RyR or SERCA malfunction in SR Ca mishandling varies significantly, possibly depending on the stage and etiology of HF. Therefore, the development of individualized therapeutic strategies that specifically target the malfunctioning component of SR Ca machinery remains the highest priority.

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Ca-Homeostasis and Heart Failure: Focus on the Biophysics of Surface Membrane Ca-Fluxes

Kathrin Banach

Introduction

In the cardiac excitation cycle Ca plays a significant role in cell excitation, contraction, and relaxation. Upon stimulation, the upstroke of the cardiac action potential (AP) is initiated by the rapid voltage-dependent activation of sodium channels. The enhanced depolarization of the membrane potential (E_m) activates L-type Ca channels (LTCCs), which allow Ca entry into the cell. Ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR) are activated by this Ca entry and release Ca into the cytoplasm (Ca-induced Ca release: CICR) further increasing $[Ca]_i$ for contraction. The depolarization of the AP is attenuated by the closing of Na channels and activation of the transient outward potassium channels (I_{to}). This initial repolarization enhances Ca influx through LTCCs. LTCC-dependent Ca entry determines the plateau of the AP and is limited by Ca- and voltage-dependent inactivation of the channel. The relaxation of the muscle can occur when Ca is removed from the cytoplasm. The major Ca removal mechanisms are the SR Ca ATPase (SERCA) and the sodium-calcium exchanger (NCX). While SERCA sequesters Ca into the SR, NCX transports one Ca ion out of the cell in exchange for the entry of three sodium ions across the plasma membrane. This electrogenic transport depends on the membrane potential (E_m) and contributes a depolarizing current during the late phase of the AP. Besides the relevance of Ca entry as a trigger and regulator of cardiac contractility Ca movement across the plasma membrane can initiate arrhythmic activity or as a signal transduction molecule, promote cellular remodeling by activation of transcription factors.

Heart failure (HF) is accompanied by attenuated contractile strength and systolic dysfunction, which in part is based on a decrease of the Ca transient amplitude.

K. Banach (✉)
Department of Medicine, Section of Cardiology, Center for Cardiovascular Research, University of Illinois, Chicago, IL, USA
e-mail: kbanach@uic.edu

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During the remodeling process that results in HF, the electrophysiological and Ca handling properties of the cardiomyocyte undergo significant changes. The pathways of Ca entry into the cell are modified with the reexpression of T-type Ca channels (TTCCs) and transient receptor potential (TRP) channels which are down-regulated after embryonic development under physiological conditions. Modifications in the AP waveform and ion distribution across the membrane modulate the time course and extend of the contribution of LTCCs and NCX to the Ca entry. As a consequence these alterations further modify the AP waveform and cellular excitability. However, due to the role of Ca as a signaling molecule the different mechanisms of Ca entry can further enhance the cardiac remodeling process by activating "hypertrophic" transcription factors.

In the following chapter we will focus on the voltage-dependent Ca entry mechanisms (LTCC, TTCC, and NCX) that contribute current to the cardiac AP. Their molecular structure and voltage-dependent properties will be reviewed and the contribution of these proteins to the electrophysiological phenotype observed in HF will be discussed. Consideration will be given to the role that subcellular remodeling has for the plasma membrane Ca flux and which Ca-fluxes further the activation of transcription factors that enhance the remodeling process. The understanding of these mechanisms is critical for the development of pharmacological strategies that attenuate HF induced cardiac remodeling and arrhythmia.

Ca Entry During Excitation-Contraction Coupling

The L-type (LTCC) and TTCC are the major two voltage-dependent Ca channels expressed in the cardiac muscle. The two channels types have distinct characteristics in their spatial expression, voltage dependence of activation and inactivation, their open time and permeability. Their individual properties give them distinct roles during the cardiac excitation cycle as well as intracellular signaling.

L-Type Ca Channel

Molecular structure: The LTCC family consists of four members ($Ca_v1.1$–$Ca_v1.4$). Expression of $Ca_v1.1$ was described in neonatal myocytes and $Ca_v1.3$ contributes to $I_{Ca,L}$ in cardiac pacemaker cells [88, 92]. The predominant isoform in ventricular muscle cells however is $Ca_v1.2$ (α1C, CACNA1C). The protein makes up the pore forming alpha subunit of the channel, which consists of four homologous domains with six transmembrane helices each [93]. While $Ca_v1.2$ can form a fully functional channel in heterologous expression systems, in the heart it associates with the auxiliary subunits $\alpha 2$, β, and δ. The expression of a γ subunit has been described in cardiomyocytes but the physiological relevance remains yet to be determined [32]. In the cardiac muscle four cytoplasmic β-subunits ($Ca_v\beta$ 1, 2, 3, and 4) are expressed with β2 being the predominant isoform [25, 46]. Expression of α1C in combination with the β2 subunit significantly increases $I_{Ca,L}$ density and shifts the voltage-dependent activation to more negative potential [110]. Down-regulation of β2 in neonatal or adult myocytes on the other hand reduces the total current amplitude and shifts $I_{Ca,L}$ activation to more depolarizing potentials [33, 79]. It was proposed that the β-subunit masks the endoplasmic reticulum (ER) retention signal by binding to the first intracellular loop (between membrane domains I and II) of $Ca_v1.2$ [16]. The α2 and δ subunits (125 kDa) derive as post-translational cleavage products of the same gene [66]. They interact through disulfide links that tether the extracellular α2 subunit to the δ transmembrane segment. Their down-regulation in the heart leads to an overall reduction in $I_{Ca,L}$, and decreased voltage-dependent activation and inactivation of the channel [150]; however, their influence remains small compared to the β-subunit.

Channel gating and role in the AP: The voltage-dependent properties of LTCC make it the major source of Ca entry into the cardiac myocyte [103]. $I_{Ca,L}$ is activated by depolarization to membrane voltages more positive than −40 mV [54]. The half activation potential is recorded around −15 mV with a maximum current around 0 mV. After activation, $I_{Ca,L}$ inactivates in a voltage, time and Ca-dependent manner. The steady state voltage-dependent inactivation curve can be analyzed by replacing Ca through Ba as a charge carrier or by measuring the flux of nonspecific cations through the channel [50]. Inactivation is U shaped and the potential of half-maximal inactivation is −35 mV, increasing again at voltages >0 mV. Voltage-dependent inactivation of $Ca_v1.2$ is comparatively slow ($t_{1/2} = 161$ ms) when recorded during Ca buffering or with Ba as the charge carrier. In the presence of Ca however, inactivation significantly accelerates ($t_{1/2} = 17$ ms). Due to its kinetic, Ca-dependent inactivation therefore plays the prominent role in the termination of $I_{Ca,L}$ during the AP. The Ca-dependent inactivation is a function of the α1 subunit [150] and is regulated by $[Ca]_i$ in the direct vicinity of the channels pore. A central role for calmodulin (CaM) has been postulated in the inactivation process [111]. Under physiological Ca concentrations CaM can bind to the pore region of the channel. This close association to the site of Ca entry allows it to be regulated by Ca at the level of single channel Ca entry. Ca-dependent activation of CaM leads to its interaction with an IQ motif in the C-terminus of the channel and the resulting structural changes lead to the inactivation of the channel [40].

Due to the dominant presence of the Na current in ventricular myocytes $I_{Ca,L}$ despite its fast activation kinetic (~5 ms), does not contribute significantly to the upstroke of the cardiac AP. However, it promotes the extended plateau phase of the action potential and thereby regulates its duration (APD). The significance of $I_{Ca,L}$ for cardiac function is supported by the early death (d14.5) of $Ca_v1.2$ deficient mice [126] and the 86 % decrease in cardiac contractility in cardiac-specific inducible KO mice [121]. The extent of Ca entry through LTCCs depends on the driving force for Ca during the AP waveform and the rise of $[Ca]_i$ in the vicinity of the channel. In the voltage clamp configuration during a square voltage pulse $I_{Ca,L}$ rapidly reaches a peak and then continuously declines due to Ca- and voltage-dependent inactivation. However, voltage protocols that mimic the shape of the AP with depolarizing voltage ramps or use APs as the voltage stimulus can visualize Ca entry through LTCC more realistically. While the rapid upstroke of the AP activates the channel, an increased or prolonged depolarization of the AP peak limits Ca entry due to the decreased electrochemical driving force [122]. Only with the repolarization of the AP $I_{Ca,L}$ increases and reaches a peak during the plateau of the AP. The decline of the current then mainly depends on the Ca-dependent inactivation of LTCC activated by the increase of $[Ca]_i$ in the microdomain of the channel. In the ventricular myocyte invaginations of the plasma membrane so-called t-tubules extend the membrane into the depth of the cell. LTCCs are localized to these structures at a high density. The SR interacts with the plasma membrane allowing for the close association of LTCC and RyR. These functional domains are called dyadic clefts and the group of functionally interacting LTCCs and RyR form a Ca release unit or couplon [53]. In the dyadic cleft, the close apposition of LTCCs and RyR leads to a rapid CICR and rise in $[Ca]_i$. Ca-dependent inactivation was shown to critically depend on the release component that is dominated by the load of the SR [128, 132]. In this diffusion-limited space $[Ca]_i$ is believed to reach higher concentrations than bulk cytoplasmic Ca. The direct measurement of $[Ca]_i$ in this domain is technically challenging. Therefore recently Ca-dependent inactivation and recovery of LTCCs from inactivation have been used as reporter mechanisms to derive the changes in local $[Ca]_i$. The results further support the close interdependence of Ca influx and Ca release [1, 84]. Another regulator of Ca in this microdomain is the NCX that removes Ca from the release site. The dependence of $I_{Ca,L}$ on NCX activity was demonstrated using the AP clamp technique [10] and NCX deficient myocytes [116]. Under both conditions, loss of NCX activity significantly attenuated Ca entry into the cell and shortened the cardiac AP. The proposed mechanism is an increased Ca-dependent inactivation of $I_{Ca,L}$ due to the enhanced accumulation of Ca at the site of the channel. Interestingly AP clamp studies that aimed to maintain physiological buffer conditions for $[Ca]_i$ visualized a second peak of $I_{Ca,L}$ at the end of the AP (second dome) and a "residual current" after AP repolarization. This recovery of $I_{Ca,L}$, likely depends on a combination of a recovery of LTCCs from Ca-dependent and voltage-dependent inactivation. Both current components occur during a vulnerable phase of the AP where depolarizing currents can trigger EADs.

Regulation of LTCC: LTCCs are regulated through beta adrenergic stimulation. Activation of β1, β2 receptors (β3 presence in human heart under debate) leads to G-protein-mediated activation of adenylate cyclase, cAMP production, and protein kinase A (PKA) activation [70]. PKA phosphorylates the pore forming portion of LTCC (α1C) at Ser1928 but further PKA phosphorylation sites were identified at the β2-subunit (Ser-459, Ser478, Ser479) [21]. PKA activity enhances the probability and duration of channel openings and therefore overall enhances the Ca influx. Isoform-specific stimulation of the β-adrenergic receptors yields distinct results of $I_{Ca,L}$ activation. Whereas the stimulation of β1 adrenergic receptor results in a persistent cell wide PKA-dependent phosphorylation of Ca handling proteins such as LTCC and phospholamban, the stimulation of β2 has only a transient and spatially limited effect on a subpopulation of LTCCs that are localized in caveolae [8]. The β2-mediated increase in $I_{Ca,L}$ was eliminated by disruption of caveolae or the deletion of caveolin 3 (CAV3) [8, 23]. The localized increase in cAMP is mediated by the recruitment of phosphodiesterases (PDE4D) to these specific membrane domains [38]. Recently the association of AKAP5 and CAV3 was demonstrated to be relevant for the regulation of β-adrenergic signaling. Deletion of AKAP5 prevented the Iso induced increase in Ca transient amplitude after β-adrenergic stimulation [101]. Interestingly with the loss of AKAP5 stimulation of LTCCs was preserved but under these conditions only non-CAV3-associated channels seemed to be affected. The relevance of this signaling domain for the activation of the hypertrophic remodeling process will be discussed later.

T-Type Ca Current

In contrast to LTCC, the low voltage-activated TTCCs expresses in the heart in a spatially and temporally defined pattern. Prominent expression of the channel occurs during embryonic development and declines after birth [36, 45, 104, 117]. Expression levels in the adult heart are confined to the cells of the cardiac pacemaker and atrium as well as to the conduction system in rabbit, cat, and rat [129, 149]. Under pathophysiological conditions like hypertrophy, pressure overload, or post-infarction the reexpression of TTCCs has been reported in adult ventricular myocytes [13, 14, 60, 64, 105]. The role TTCCs have in the cardiac muscle is not yet fully resolved.

Molecular basis: TTCCs are structurally closely related to L-type channels with four functional domains each consisting of six transmembrane segments, although the channels exhibit low sequence homology. The two major isoforms identified in the heart are $Ca_v3.1$ and $Ca_v3.2$ whereas $Ca_v3.3$ was described only at the mRNA level in Purkinje cells [108, 136]. $Ca_v3.1$ KO mice exhibit bradycardia and conduction slowing in the Purkinje system supporting the role of TTCC in the pacemaker and the conduction system [89]. A switch in the isoform expression has been described from $Ca_v3.2$ to $Ca_v3.1$ after birth [45, 104] that is repeated in vitro during the differentiation of mouse embryonic stem cell-derived cardiomyocytes [96]. In contrast to LTCC where auxiliary subunits play a central role in the trafficking and gating of the channel, so far no substantial evidence supports the relevance of auxiliary subunits for the function of TTCCs; however, an increased surface expression of $Ca_v3.1$ has been reported when it was coexpressed with α2, δ1 and β subunits [4, 42]. A more significant variation in channel function is attributed to the differential splicing of the channel protein. Splice variants of $Ca_v3.1$ and $Ca_v3.2$ channels confer differences in the activation and inactivation kinetic as well as in their voltage dependence [97, 109]. For cardiomyocytes a high variability in the presence or absence of exon 25 was identified that is localized in the cytoplasmic III–IV linker domain; however, the functional relevance is yet to be determined.

Voltage dependence and relevance for cardiac AP: TTCCs belong to the category of low voltage-activated Ca channels. Their voltage-dependent gating properties differ significantly from that of LTCC. From a holding potential of −80 mV, T-type channels are activated positive to −60 mV with a peak current around −30 mV (with half-maximal activation at ~−40 mV) [27]. TTCCs exhibit a voltage-dependent inactivation and in contrast to LTCCs, their activity does not depend on $[Ca]_i$. This allows for a functional distinction of the channels when Ba is used as a charge carrier. TTCCs completely inactivate at voltages more positive than −40 mV. The significant overlap of their inactivation and activation curve allows for a window current between −60 and −30 mV [27, 103]. The voltage-dependent properties of TTCCs in cardiac pacemaker cells results in the activation of $I_{Ca,T}$ during the diastolic depolarization. Ca influx through TTCCs can trigger spatially defined Ca release events (sparks), which further enhances the diastolic depolarization of E_m through the activation of NCX [62, 136]. Due to their low expression under physiological conditions, the role of TTCCs in ventricular myocytes is difficult to determine. TTCC activation is expected during the upstroke of the AP and its ability to trigger CICR has been demonstrated [74]. However it has to be pointed out, that the contribution of $I_{Ca,T}$ to CICR was only significant at specific voltages and required an increased loading of the SR. Since TTCC exhibit a high channel open probability around the diastolic potential it was further speculated that it can provide a persistent low level entry pathway for Ca into the cell that contributes to the load of the SR. Suppressed contractile function in the presence of the T-type blocker mibefradil supported this hypothesis. While increased TTCC activity could enhance contractile Ca, its persistent activity during the repolarization phase of the AP actually could increase a cells propensity for EADs and DADs. The regulation and expression of TTCCs during the pathophysiological remodeling of the cardiac muscle could play a significant role in the increased arrhythmicity of the cardiac tissue.

Sodium-Calcium Exchanger

In the cardiac muscle the NCX is the major extrusion mechanism that removes Ca from the cytoplasmic space. However depending on the thermodynamic conditions, it can also transport Ca into the cell. NCX thereby plays an important role in the regulation of the intracellular Ca-homeostasis. While its major function is the regulation of $[Ca]_i$, due to its electrogenic nature NCX current (I_{NCX}) can contribute to the induction of spontaneous activity under physiological and pathophysiological conditions.

Molecular basis: NCX activity in cardiac cells was first described as a Na-dependent Ca efflux [120] but it took until 1990 that the cardiac exchanger was cloned (NCX1) [102]. Two additional isoforms (2 and 3) were later identified in brain and skeletal muscle. The working model of NCX predicts nine transmembrane domains (108 kDa) with a glycosylated extracellular amino terminus, a cytoplasmic loop between transmembrane segments 5 and 6 and a cytoplasmic C-terminal domain. The cytoplasmic loop or central hydrophilic domain contains a XIP (eXchange Inhibitory Peptide)-binding domain that resembles a calmodulin-binding domain. Target peptides to the XIP domain can inhibit NCX activity from the intracellular side [31]. Further located in the loop are a catenin-like domain and two calcium-binding domains which have a beta strand arrangement [55]. The Ca-binding domains have distinct affinities for Ca (Kd1: 120, 240 nM; Kd2: 820 nM and 8.6 μM) and are both required for allosteric Ca-dependent activation of NCX. The dynamic changes in $[Ca]_i$ vary between 100 and 1,000 nM indicating that NCX is activated by Ca under physiological conditions. It has further been shown that Ca dissociates only slowly from the protein-binding sites which implies that NCX remains activated during the entire cardiac excitation cycle [12]. In contrast to Ca, the allosteric binding of Na to the exchanger inactivates NCX. Under physiological conditions NCX activity further depends on Phosphatidylinositol-4,5-bisphosphate (PIP2). PIP2 is described to interact with the XIP region of the exchanger and to enhance its activity by preventing its auto-inhibitory interaction with another part of the protein. As shown in giant membrane patches prevention of PIP2 depletion, eliminates Na-dependent inactivation. Since intact cardiac myocytes usually maintain a high level of PIP2 the relevance of Na-dependent inactivation under physiological conditions remains to be determined.

Voltage dependence and relevance for cardiac AP: NCX current (I_{NCX}) was early recorded and described in ventricular myocytes [72, 91]; however, it has been more challenging to determine the contribution of NCX to the AP. Difficulties arose from the lack of specific blockers and due to its dynamic regulation by $[Na]_i$ and $[Ca]_i$. During the early phase of the AP, E_m reaches potentials that promote NCX reverse-mode activity. This condition has been reported to be further enhanced by the accumulation of Na in the microdomains where the exchanger is localized [80]. While reverse-mode activity was widely confirmed, it remained a matter of debate how much Ca entry through reverse mode NCX contributes to CICR. Recent experiments using mice deficient for the expression of NCX reported a Na current dependent increase in the Ca transient that is absent in NCX deficient mice indicating a measurable contribution of NCX reverse mode to cardiac contractility [78]. In AP clamp experiments in rat ventricular myocytes the contribution of NCX to the initial Ca transient spike was confirmed but the mechanism proposed was a modulation of $I_{Ca,L}$ rather than NCX-dependent Ca entry [118]. AP clamp experiments that aimed to isolate I_{NCX} and $I_{Ca,L}$ under low intracellular buffering conditions further support an outward current component that NCX contributes at the onset of the AP [116]. Outward I_{NCX} is indicative of reverse-mode activity and Ca entry however it was not yet evaluated in this study how much this Ca entry mechanism contributes to the Ca transient. NCX then contributes an inward current second phase of the AP dome and a residual current at the end of the AP that could contribute to the formation of EADs [10].

Regulation: Besides the allosteric regulation of NCX through $[Ca]_i$ and $[Na]_i$ post-translational modifications of NCX have been described. The phosphorylation-dependent regulation of NCX is controversial. In the cardiac muscle phosphorylation of the NCX protein through PKC [63] and PKA [125, 143] has been reported, with the occurrence of a hyperphosphorylated NCX protein in failing hearts. However, the catalytic forms of the two PKC and PKA were not shown to change NCX current. Studies that carefully controlled $[Ca]_i$ and $[Na]_i$ could not determine an increase in NCX activity upon β-adrenergic stimulation. A recent study reports that the potential PKA phosphorylation site in the mammalian NCX protein may not be accessible under physiological conditions again making a phosphorylation induced regulation of NCX less likely [140]. While a phosphorylation-dependent regulation of NCX is controversial, an increase of NCX activity by reactive oxygen species (ROS) was more consistently reported [48, 119]. Mobility shift essays suggest changes in the conformation of the exchanger through the oxidation of cysteine residues [124]. While a ROS-dependent increase in NCX activity was described to depend on thiol modifications and on a decreased Na-dependent inactivation of NCX [76]. In heart failure increased levels of oxidative stress are reported that lead to changes in cardiac ECC. The SERCA activity which is relevant for the termination of the Ca transient is significantly reduced and the reciprocal regulation of NCX by ROS was suggested to prevent the increase in diastolic Ca [76]. In heart failure the direct ROS-dependent NCX regulation has not yet been evaluated. The regulation of NCX in this setting is multifactorial and if NCX activity is further increased by ROS under conditions where protein levels are already increased remains to be determined. However, a ROS-dependent regulation of NCX has been suggested for rapid NCX reactivation in a model of hypoxia-reoxygenation [43] supporting a potentially dynamic physiological function for this regulatory mechanism.

Changes in Ca Entry During Cardiac Hypertrophy

During heart failure the Ca transient amplitude is decreased and results in a reduction of contractile strength. A reduced SERCA activity and increased leak of Ca through the RyR result in a reduced SR load that forms the basis for the decreased Ca transient amplitude. In the following, we will discuss the mechanism by which the previously described Ca entry mechanisms in HF contribute to changes in ECC and to the increase in cardiac arrhythmia.

L-Type Calcium Channel

While the Ca transient and contractility is significantly reduced, overall $I_{Ca,L}$ density does not seem to undergo significant changes during cardiac remodeling in ventricular myocytes isolated from human [13, 15], dog [69], rabbit [114], mouse [112], guinea pig [2] heart failure tissue. However, in some cases decreased currents in heart failure models have been described [20] as well as a transient up-regulation of LTCC channel protein that maintained $I_{Ca,L}$ density constant with increasing cell size. The variability in the experimental results could in part be explained by the fact that a decreased amount of channel protein in heart failure cells can be compensated by an increased Ca channel activity. A reduced sensitivity of $I_{Ca,L}$ to BayK 8644 a positive inotropic agent that acts directly on the Ca channel protein, supports this hypothesis [29]. Hullin et al. described an increased $I_{Ca,L}$ based on an increased open probability and prolonged channel open states in myocytes from failing human hearts (mode 2 gating) [61]. In this preparations increased $I_{Ca,L}$ coincided with increased expression of the accessory β2 subunit [61]. In an animal model the over-expression of β2 promoted Ca-overload and EADs. This phenotype depended on the activation of two CaMKII phosphorylation sites on β2-subunit and could be attenuated by CaMKII inhibition. Lack of β2 expression prevents LTCC to enter into mode 2 gating and thereby limits enhanced Ca entry [75].

In contrast to the maintained Ca current density, changes in Ca current kinetic are commonly reported in isolated myocytes of HF models. Inactivation of $I_{Ca,L}$ was shown to be slowed in cells isolated from a guinea pig heart failure model [2, 17]. The difference in Ca-dependent inactivation was eliminated when Ba was used as a charge carrier or $[Ca]_i$ was buffered. The results indicate that changes in $I_{Ca,L}$ kinetic reflect changes in Ca-dependent inactivation and depend on $[Ca]_i$. As previously discussed $I_{Ca,L}$ sensibly reports [Ca] in the microdomain of the channel and therefore CICR from the SR modulates specifically its first time constant of inactivation. Since SR Ca load is decreased in HF animals, the change in $I_{Ca,L}$ inactivation likely reflects the attenuated and delayed rise of the Ca transient. This change in Ca-dependent inactivation significantly prolongs Ca influx and as a consequence the AP duration. In addition to Ca-dependent inactivation, a shift in the steady state inactivation of LTCC to more positive voltages has been described in human, dog, and sheep heart failure tissue [3, 20, 137]. This shift increases the so-called "window current" that occurs in voltage range (LTCC: −30 to 0 mV) where the activation and inactivation relationships overlap. In this "window" channel openings can occur and generate a depolarizing inward current. Since the voltage range of the window current coincides with the repolarization of the AP, an increased window current enhances the propensity for EADs [20, 137]. Another change in the current kinetic of $I_{Ca,L}$ has to be considered. Besides changes in the Ca handling properties, changes in the expression and current density of potassium current are described. This way the transient outward potassium current (I_{to}) is significantly decreased in rabbit HF myocytes [115]. The loss of I_{to} results in an attenuated early repolarization of the cardiac AP and a prolongation of APD. The extended duration of the AP peak at depolarized Ems attenuates Ca entry due to a decreased driving force in the early phase of the AP [34, 83, 123]. As a consequence, the trigger for CICR is reduced and Ca release from RyRs becomes more heterogeneous. Overall a delay in the Ca transient rise and a decrease in the Ca transient amplitude are the consequence.

T-Type Calcium Channel

Cardiac TTCCs are reexpressed in the heart during pathophysiological conditions such as hypertrophy, cardiomyopathy, and cardiac infarction [13, 14, 105]. Current recordings in isolated cardiomyocytes from diseased hearts show a high nickel sensitivity of the Ca current indicative of the expression of the $Ca_v3.2$ channel isoform. However, the up-regulation of $Ca_v3.1$ has also been demonstrated on mRNA and protein level [133]. Recent experimental data further demonstrate the preferential up-regulation of the $Ca_v3.2(+25)$ splice variant during hypertrophy. The change in splicing enhances a channel population with an activation curve that is shifted to more negative voltages [37]. Different factors have been shown to induce the expression of TTCCs. This way long-term treatment of angiotensin II induced up-regulation of $Ca_v3.2$ in cardiomyocytes in vitro and in vivo [44]. The insulin-like growth factor promotes TTCC expression in vitro and increased levels of $Ca_v3.1$ and $Ca_v3.2$ were demonstrated to coincide with increased levels of IGF-1 in atrial tissue [35]. Both factors are well-established regulators of cardiac hypertrophic remodeling. In the $Ca_v3.1$ gene corticosteroid response elements have been indentified consistent with the observation that mineralocorticoid, glucocorticoid hormones and the synthetic glucocorticoid, dexamethasone up-regulate TTCCs in cardiomyocytes [11]. It has been suggested that the increased propensity for cardiac arrhythmia during corticosteroid treatment is related to a TTCC reexpression [90].

The reexpression of $I_{Ca,T}$ can result in enhanced spontaneous activity of the cardiomyocytes however it could provide a positive inotropic effect by enhancing Ca influx. In a study of coronary artery ligation a decrease in contractile activity was reported when TTCCs were blocked by mibefradil [98] and also during right ventricular hypertrophy TTCCs were proposed to contribute a positive inotropic effect [133]. In contrast, mibefradil was shown to improve the β-adrenergic response of the myocardium and improve cardiac function [94, 95]. While mibefradil is one of the more specific TTCC blockers, drug-related effects of these studies have to be considered and recent transgenic studies might give more insight in the functional contribution of TTCCs to cardiac ECC. This way over-expression of $Ca_v3.1$ in the mouse heart, significantly increased $I_{Ca,T}$ and prolonged the APD but exhibited only a mild increase in contractility [65]. Interestingly the Ca transient and SR Ca load remained unchanged in these myocytes. The authors therefore suggested a differential function of LTTCs and TTCCs in the cells [65]. The distinct subcellular distribution of the channel could be one mechanism by which this functional difference is achieved. While LTCCs predominate in the t-tubular system of the myocytes, TTCCs localize to the sarcolemmal membrane. The different localization of LTCCs and TTCCs could be significant since TTCC over-expression does not result in pathophysiological changes while a similar increase in $I_{Ca,L}$ promotes hypertrophic remodeling [28].

While the impact of TTCC on cardiac contractility seems to be limited, the occurrence of arrhythmia and sudden cardiac death has been more readily related to increased expression of TTCCs. Mibefradil treatment prevented sudden cardiac death in a mouse and rat model of heart failure [73, 98]. If the mechanism of increased arrhythmic is not specified in these disease models but it would be anticipated that increased TTCC enhances the propensity for EADs given the lack of over-expression induced Ca overload that could favor DADs.

The role of TTCCs in pathophysiological remodeling is further complicated due to the fact that it seems to have regulatory functions that go beyond cardiac ECC. Horiba et al. [58] provide evidence that TTCC activity actually contributes to the remodeling process itself. In an in vitro hypertrophy model they showed that activation of the transcription factor NFAT3 (nuclear factor for activated T-cells) was attenuated in the presence of the t-type channel blocker mibefradil. This result is confounded by the fact that $Ca_v3.2$ KO mice are protected from pressure overload induced cardiac hypertrophy [30]. The TTCC-dependent activation of cardiac transcription factors seems to be isoform specific since $Ca_v3.1$ deficient mice displayed enhanced hypertrophic remodeling in response to pressure overload [99]. Over-expression of $Ca_v3.1$ on the other hand conferred protection from hypertrophic stimuli. The authors propose a functional interaction between $Ca_v3.1$ and eNOS that leads to a protective activation of the NO/PKG signaling pathway. The mechanism by which these functional differences between $Ca_v3.1$ and $Ca_v3.2$ are induced or how the comparably small Ca entry through TTCCs can regulate cardiac hypertrophy remains to be determined. However, the functional interaction between $Ca_v3.2$ and the phosphatase calcineurin (CaN) the up-stream activator of NFAT might be favored by co-localization in specific membrane domains, or the selective interaction of CaN with $Ca_v3.2$ [30].

Overall reexpression of TTCCs during cardiac pathophysiological remodeling seems to have a low impact on overall cardiac contractility. However, voltage-dependent properties of TTCC favor a depolarizing $I_{Ca,T}$ in cells with a depolarized resting membrane potential as well as during the late phase of the cardiac AP that can increase the propensity for arrhythmic activity. In addition the functional impact of TTCCs seems to be in the isoform-specific regulation of signal transduction pathways, where the $Ca_v3.2$-dependent activation of the transcription factor NFAT can actually serve as a positive feedback mechanism to enhance cardiac remodeling.

Sodium-Calcium Exchanger

During cardiac hypertrophy the Ca transient amplitude is decreased due to attenuated SERCA activity and enhanced RyR leak. NCX represents the major Ca extrusion mechanism that allows for the termination of the Ca transient. In animal models (rabbit, dog) as well as in humans an increase in NCX activity and protein level has been described [56, 113]. NCX compensates in part for the decrease in SERCA function and promotes the decrease of the Ca transient preventing diastolic dysfunction. The extrusion of Ca from the cytoplasm on the other hand further decreases SR load and enhances contractile dysfunction. The AP duration in heart failure myocytes is already prolonged due to attenuation of repolarizing potassium current [115] and decreased Ca channel inactivation. An enhanced depolarizing inward current at the end of the AP therefore can increase the propensity for arrhythmia induced by EADs. This would be prevalent especially during decreased beating frequencies where APD is prolonged.

NCX activity depends on the $[Ca]_i$ and $[Na]_i$ and functions in the reverse mode during the peak of the AP particularly when $[Na]_i$ increases in sub-sarcolemmal membrane domains as it has been proposed [78]. In HF myocytes increased intracellular Na concentration were reported. The increase in $[Na]_i$ is potentially based on a decreased Na extrusion through the Na/K ATPase, an increased activity of the Na-H exchanger or increased Na entry through the late Na current [6, 7]. The increased [Na]i favors reverse-mode NCX activity which is even further enhanced by the reduced $[Ca]_i$ during the Ca transient [142]. As a consequence, a potential contribution of NCX-mediated Ca entry is discussed for the plateau phase of the AP [5]. In human heart failure myocytes NCX-dependent Ca entry was shown to significantly contribute to myocyte contractility [41, 144].

Due to their antiarrhythmic and positive inotropic effect inhibitors of NCX are discussed in the treatment of different cardiac diseases. In heart failure rabbits, treatment with the NCX inhibitor SEA0400 shortened the action potential duration, decreased the dispersion of repolarization and suppressed early after depolarizations. Changes in contractility would also be expected due to reduced extrusion of Ca from the cytoplasm and consequently an increased SR load.

Regulation of Ca Influx Through Spatial Remodeling

Skeletal as well as ventricular myocytes develop membrane invaginations called t-tubules that substantially increase the membrane surface area. T-tubules develop postnatally and are confined to the edge and periphery of the muscle fiber in neonatal myocytes [130]. During the maturation process the interaction of membrane invaginations with the SR coincides with the appearance of junctophilin2, junction, and triadin in the SR membrane [47]. The membrane scaffolding protein Bin1 is critically important in the generation of the t-tubules and later in the transport of LTCCs to the maturing dyads where they closely interact with the ryanodine receptor [57]. At the end of this developmental process, in rat ventricular myocytes 25 % of dyads are localized at the surface membrane whereas 75 % are localized along the t-tubular membrane. The extension of the surface membrane allows for AP propagation and voltage-dependent Ca entry in the depth of the cell where the close apposition of LTCCs and RyRs then guarantees the rapid and spatially homogenous rise of Ca throughout the myocyte [53]. During pathophysiological remodeling changes in the t-tubular structure occur. A decrease in t-tubular density was described in a dog and rabbit model of HF [9, 49, 51], during chronic ischemia [52], as well as during atrial fibrillation [81]. In a mouse model of heart failure the t-tubular density was maintained but $I_{Ca,L}$ at the site of t-tubules was significantly reduced [59]. In healthy human cardiac tissue an overall more coarse arrangement of the t-tubular system is described [68] where specifically a decrease in the longitudinal extensions occurs during HF [24, 85, 86].

T-tubules contain a high density of membrane proteins. To better understand the impact that t-tubular remodeling has on the cellular electrophysiology and ECC VMs have been de-tubulated by short exposer to an osmotic shock. De-tubulation of VMs results in a 30 % reduction of membrane surface area, however since the density of LTCCs is sixfold higher in t-tubules, de-tubulation results in the loss of about ~60 % of total $I_{Ca,L}$ [19, 71]. As a consequence a significant shift in the ratio of $I_{Ca,L}$ from the t-tubules vs. the sarcolemmal membrane occurs [59]. Functionally a loss of t-tubules or a loss of $I_{Ca,L}$ in the t-tubular system was shown to reduce the synchrony of Ca release from RyR. Dys-synchrony can be increased by the decrease in the trigger current ($I_{Ca,L}$) at a given sight, a decrease in the number of dyads that allow for rapid release, an increase in the number of RyR that are located outside such dyads (orphaned RyR) as well as by changes in SR load and RyR open probability. In all cases a reduced cardiac gain is expected which is defined as the ratio between the amount of SR Ca release in relation to the Ca entry at a given voltage [53]. While the total current density in heart failure models might be unaltered, the loss of dyadic structures leads to delayed activation of release sites and consequently a slower Ca transient rise time and therefore amplitude. This reduction in Ca transient amplitude is again a reflection of the decreased contractile strength of the muscle.

De-tubulation of ventricular myocytes further revealed differences in the function and regulation between $I_{Ca,L}$ at the sarcolemmal and the t-tubular membrane. It was shown that $I_{Ca,L}$ exhibits a faster inactivation kinetic at the sarcolemmal membrane [18]. This change in kinetic was dependent on Ca as the charge carrier which indicates spatial differences in the Ca-dependent inactivation of LTCCs [19]. Decreased inactivation at the sarcolemmal membrane leads to a prolonged Ca entry at this location and could explain why de-tubulation does not coincide with a decrease in the load of the SR [18, 71]. A differential role for t-tubular Ca entry as the trigger of Ca release and sarcolemmal Ca entry for the refilling of the SR has been proposed. The mechanism of the differential regulation of $I_{Ca,L}$ inactivation remains to be determined.

Also NCX was described to preferentially localize to the t-tubular membrane. De-tubulation reduced I_{NCX} to 40 % indicating a threefold higher density of NCX in the t-tubular membrane [39, 148]. The exact localization of NCX within the t-tubules is still debated. CAV3-binding motives in the NCX protein have been identified and several studies have proposed the localization of NCX in a multi-protein complex. Ankyrin-dependent interaction of NCX with the sodium-potassium ATPase as well as the IP_3 receptor has been proposed. However, other studies could not identify a close interaction between CAV3 and NCX [26, 67, 82]. Overall NCX localization seems to be distinct from that of $Ca_v1.2$ and therefore the dyadic junctions but close nonrandom proximity of NCX to the dyadic cleft and RyRs seem to exist [67]. The close proximity of NCX to the site of Ca influx and Ca release can have substantial influence on ECC. A close spatial association of NCX and RyR could facilitate NCX reverse mode induced CICR in particular when NCX activity is enhanced by $[Ca]_i$ at the site of Ca influx [131, 138].

Experimental evidence that NCX is localized close to the release site is provided by simultaneous recordings of $[Ca]_i$ and I_{NCX}. When Ca is released from the SR by caffeine, the activated I_{NCX} is higher at the upstroke of the Ca transient than at the corresponding $[Ca]_i$ during the decay phase of the transient [1, 135]. This discrepancy is believed to result from the difference in $[Ca]_i$ between the dyadic cleft and the cytoplasm at the moment of Ca release from the SR. As previously described for $I_{Ca,L}$ this way also I_{NCX} can be used as an indicator for $[Ca]_i$ near the release sites [1, 84]. The dependence of NCX on this local $[Ca]_i$ however also means, that it can dynamically regulate $[Ca]_i$ at the site of release. As a consequence, changes in NCX activity can modulate the local $[Ca]_i$, and as a consequence the Ca-dependent inactivation of $I_{Ca,L}$, Ca entry and AP waveform. A recent model explores the dynamic interaction between [Ca], $I_{NCX,}$ and I_{Ca} in a functional microdomain where they interact with RyR through changes in local [Ca] [84].

During hypertrophic remodeling a decrease in the t-tubular density, an increase in orphaned RyRs and an increase in NCX are described. Future experiments will have to determine what portion of NCX under these conditions is still situated or controlled within these microdomains and which consequences this structural remodeling has on the regulation of the current.

Contribution of Ca Entry Mechanisms to the Induction of Hypertrophic Remodeling

On top of its important role in cardiac excitation-contraction coupling Ca also plays a central role in the induction of the cardiac hypertrophic remodeling process. Ca binding to calmodulin leads to the activation of the protein phosphatase 2B calcineurin, which in turn dephosphorylates the Nuclear Factor of Activated T-cells (NFAT) and enhances its translocation into the nucleus. NFATc3 was demonstrated to be required for the induction of the pathophysiological hypertrophic remodeling process [145, 146]. In a cardiac myocyte $[Ca]_i$ changes by a factor of 10 on a beat-to-beat basis making it unlikely that CaN is activated through ECC induced changes in Ca. The Ca release or Ca entry mechanism responsible for CaN/NFAT activation therefore still remains a matter of debate. Potential trigger could allow changes in $[Ca]_i$ in a spatially defined microdomain through entry mechanisms that do not directly contribute to cardiac ECC. Potential sources of such Ca triggers will be discussed.

Role of $I_{Ca,L}$ in hypertrophic remodeling: LTCC is activated and the major source of Ca entry during each cardiac excitation cycle. However these beat-to-beat changes fail to activate the signaling molecules that promote the remodeling process. Enhancing $I_{Ca,L}$ by over-expression of α1C or the β2 subunit, leads to a significant increase in Ca transient amplitude and to enhanced contractility [134, 139]. Both animal models were demonstrated to develop a hypertrophic phenotype which can be suppressed by inhibition of $I_{Ca,L}$, CaMKII, or CaN [28]. CaN was shown to bind to the C terminus of LTCC and was also demonstrated to associates with $Ca_v1.2$, AKAP5, and CAV3 in a multimolecular signaling domain. The contribution of the CAV3-associated LTCCs was recently evaluated by their spatially restricted inhibition [87]. These studies indicate that LTCCs localized in caveolae contribute only a small current to cardiac ECC while their inhibition can prevent the pacing induced CaN-dependent NFAT translocation into the nucleus. The results would argue that induction of hypertrophic cardiac remodeling is mediated by Ca entry in a spatially defined microdomain.

Role of TTCCs in hypertrophic remodeling: Also Ca entry through TTCCs has been discussed as a potential trigger of CaN/NFAT activation. TTCCs are expressed during cardiac development and expression is again up-regulated during the cardiac remodeling process. In neonatal mouse myocytes mibefradil was shown to prevent in vitro the serum induced NFAT activation. In $Ca_v3.2$ and $Ca_v3.1$ KO mice Chiang et al. [30] demonstrate an isoform-specific role of TTCCs in the cardiac remodeling process. $Ca_v3.2$ deficient mice are protected from pressure overload. As a mechanism for a $Ca_v3.2$-specific NFAT activation, the authors suggest a potential association of the channel with CaN in WT myocytes. Mice deficient for the expression of $Ca_v3.1$ exhibit exacerbated cardiac remodeling upon isoproterenol infusion while the cardiac-specific over-expression of $Ca_v3.1$ protects the animals from pressure overload induced hypertrophy [99]. In the latter case, $Ca_v3.1$ over-expression results in a significant increase in the Ca transient amplitude and fractional shortening further indicating that global change in $[Ca]_i$ is not the predominating trigger for the CaN-dependent remodeling process. The authors suggest a $Ca_v3.1$-dependent activation of eNOS as the potential cardioprotective signaling mechanism. Further experiments will have to determine if the TTCC/eNOS-dependent signaling depends on the localized interaction of the proteins and how CaN can differentiate between the Ca influx through the different TTCC isoforms.

Role of transient receptor potential (*TRP*) *channels in hypertrophic remodeling*: In recent years Ca entry through TRP channels has gained increasing attention. The TRP channels comprise six related protein families: A for ankyrin, C for canonical, M for melastatin, ML for mucolipins, P for polycystin, and V for vanilloid. Overall 28 genes have been cloned and linked to these families. The channels of the different families share a common membrane topology consisting of four protein subunits with six transmembrane domains each. N- and C-termini are cytoplasmic and contain different regulatory or protein interaction domains. In human cardiomyocytes C5, C6, P1, P2, and M4 have been identified [22] in mouse in addition to that the expression of C1, C2, C3, C6, C7, V2, V4, M4, and M7 was reported [141]. The up-regulation of the TRP channels C1 [106, 127], C3 [22], C4 [147], C6 [77, 107], and C7 [107] was demonstrated after aortic banding or stimulation with the hypertrophy inducing factors angiotensin II, phenylephrine, or endothelin-1. All TRPC channels are nonselective cation currents that are also permeable for Ca. As indicated above the channels can be activated by different stimuli but for TRPCs the activation through diacylglycerol (DAG) during increased phospholipase C activity was demonstrated. This receptor-mediated activation (ROC) can be paralleled by Ca entry that is induced by IP_3-mediated depletion of the intracellular stores (store operated Ca entry). TRP C1, C3, and C6 have been shown to be activated by SOCE but also the DAG-mediated activation of TRPC1 and C3 was demonstrated [127].

Besides the up-regulation of TRPC channels during hypertrophy, their activity has been linked to the activation of the remodeling process itself. For TRPC3 and TRPC6 it was demonstrated that down-regulation reduced agonist induced hypertrophy while their over-expression exaggerated the remodeling process [100, 107]. The relevance of the TRPC channels was further established by the over-expression of either one of the dominant negative TRPC isoforms C3, C6, and C4 in the cardiac muscle. Either of these strategies attenuated the hypertrophic response which could indicate that the TRP channels exist as hetero-multimers or in protein complexes [147]. In all cases TRP channel activity is closely linked to the activation of the CaN/NFAT pathway again supporting the hypothesis that TRP channels allow for Ca entry in defined microdomains that allow a readout of changes in $[Ca]_i$ that is distinct from cardiac ECC. Interestingly TRPC1, C3, and C6 have consensus NFAT-binding sites in their promoter region, which indicates that their expression is increased by a positive feedback mechanism once the remodeling process is initiated. A recent study addressed the question if Ca entry through the TRP channels themselves activates CaN/NFAT signaling. In neonatal and adult feline cardiomyocytes pacing induced NFAT activation could be inhibited by the LTCC blocker nifedipine but not by inhibitors of TRP channels. The over-expression of TRPC3 induced hypertrophic remodeling; however the remodeling process was only attenuated by TRPC inhibitors but blocked by nifedipine. This study suggests that while TRPCs promote hypertrophic signaling, they do so by modulation of the Ca entry through LTCC. This again raises the question if the subcellular interaction of the proteins or their assembly in a functional signaling domain is required for the regulation of hypertrophic signaling and how subcellular remodeling can influence this process.

Conclusion

During the development of heart failure the cardiomyocyte undergoes a substantial electrophysiological remodeling process that results in decreased Ca transients with prolonged rise and decay phases. While a decreased SR load is assumed to form the basis for the reduced transient amplitude, changes in the Ca entry mechanisms can further contribute to this phenotype. The Ca entry during HF is modified through changes in the expression level of channel proteins, changes in the AP waveform as well as the subcellular remodeling of the proteins in the t-tubular structure. In addition to the modulation of the contractile strength this affects the cellular propensity for arrhythmic activity, which is increased in HF myocytes. While the expression levels and the electrophysiological properties of different Ca entry mechanisms in the myocytes are well described, many questions remain regarding the relevance of the subcellular arrangement of the proteins. It is clear that functional signaling domains exist that allow for the defined neurohumoral regulation of channel proteins and allow Ca itself to induce a hypertrophic signal transduction cascades in defined membrane domains. At this point it needs to be determined how subcellular remodeling itself can contribute to the hypertrophic response through modification of the functional interaction of the proteins. A clear understanding of the mechanism by which changes in Ca entry modulate cardiac contractile function, cellular excitability, and the remodeling process itself is a prerequisite for the development of effective therapies that prevent or potentially reverse the progression into heart failure.

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Biophysics of Membrane Currents in Heart Failure

Man Liu, Vikram Maddikunta Brahmanandam, and Samuel C. Dudley Jr.

Introduction

The cardiac action potential (AP) is made possible by active and passive processes that maintain highly regulated electrochemical gradients for sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) ions through cell membrane ion channels, transporters, and energy-dependent pumps. The delicate balance between depolarizing and repolarizing ionic currents is subject to disruption in heart failure (HF), leading to arrhythmias and mechanical pump dysfunction. Altered membrane currents are implicated in sudden cardiac death (SCD), which claims up to 450,000 lives annually in the United States [1, 2]. While ischemic heart disease is responsible for three quarters of these deaths, the rest are largely attributable to HF, and a 2.6–6.2-fold increased risk of SCD exists in the HF population compared to those without left ventricular (LV) dysfunction [3]. Moreover, worsening LV dysfunction and higher New York Heart Association HF classes correlate with an increased risk of SCD [4]. Cell surface membrane proteins are altered in numerous ways in HF, including regulation of gene expression, posttranslational modifications, protein assembly and trafficking, membrane insertion, functional control and degradation.

M. Liu
Section of Cardiology, University of Illinois at Chicago, Chicago, IL, USA

The Jesse Brown VA Medical Center, Chicago, IL, USA
e-mail: liuman99@uic.edu

V.M. Brahmanandam
Section of Cardiology, University of Illinois at Chicago, Chicago, IL, USA
e-mail: vikramb@uic.edu

S.C. Dudley Jr. (✉)
Section of Cardiology, University of Illinois at Chicago/Jesse Brown VA Medical Center,
840 S. Wood Street, MC715, Chicago, IL 60612, USA
e-mail: scdudley@uic.edu

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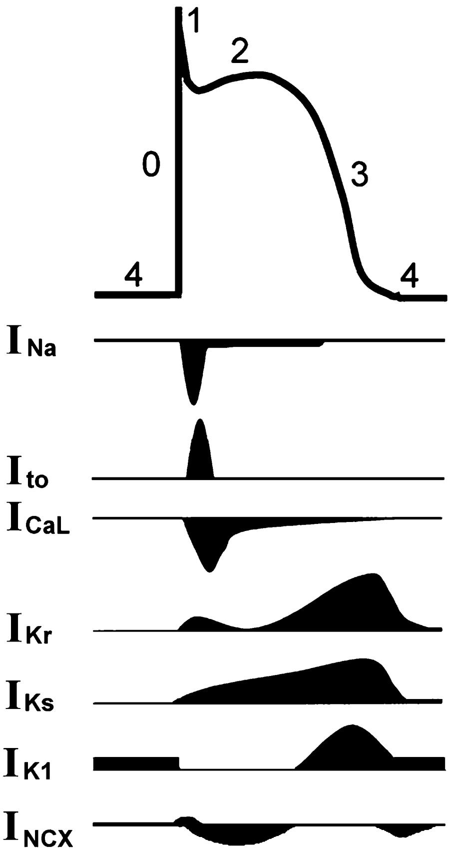

Fig. 1 A schematic representation of the cardiac action potential (AP) with the major contributing cardiac ion channel currents after [5, 6]

New research continues to offer potential therapeutic targets by elucidating the pathways for these observed changes.

While not every contribution to membrane potential during the cardiac AP in myocytes is fully understood, consensus does exist with respect to the main contributing ion fluxes resulting in the five phases of the cardiac AP cycle. As shown in Fig. 1, in a representative cardiac AP of ventricular myocytes [5, 6], Phase 0 is characterized by a rapid depolarizing inward Na^+ current (I_{Na}) that depends upon the voltage-gated Na^+ channel ($Na_v1.5$) encoded by SCN5A. These channels are inactivated within milliseconds of opening. This upstroke is followed by Phase 1, which is characterized by a brief repolarization phase as a result of activation of voltage-gated K^+ channels ($K_v4.x$) that allow for a transient outward K^+ current (I_{to}). Phase 2 is a plateau phase in which an inward Ca^{2+} current (I_{CaL}) through voltage-gated L-type Ca^{2+} channels ($Ca_v1.2$) is balanced by at least two outward K^+ currents: the rapid and slow outward delayed rectifier K^+ currents (I_{Kr} and I_{Ks}), which also contribute to Phase 3, a rapid repolarization phase. I_{Kr} is conducted by HERG and MiRP-1, while I_{Ks} by K_vLQT1 and minK. Additionally, an inward rectifier K^+ current (I_{K1}) conducted by the Kir2.x family contributes to Phase 3 and maintains the resting membrane potential at baseline or Phase 4 by shifting the membrane potential towards the equilibrium potential of K^+. Other contributing membrane proteins include the Na^+-Ca^{2+} exchanger (NCX), the voltage-dependent T-type Ca^{2+} channel, and the Na^+-K^+ ATPase. Nerbonne and Kass provide an excellent review of the cardiac AP [7]. In HF, changes to cardiac $Na_v1.5$ channel I_{Na} contribute to clinical disease including arrhythmias. While the focus of this chapter will be on $Na_v1.5$, we will briefly review known changes in the other ion channels occurring during HF.

HF affects K^+ currents, and while these changes may be adaptive initially, they have the potential to lead to arrhythmias. For example, downregulation of K^+ currents allows for increased depolarization and time for excitation-contraction (EC) coupling that improves mechanical function in HF. Nevertheless, these changes also lead to afterdepolarizations, heterogeneous repolarization patterns, and ventricular arrhythmias [8–10]. Prolongation of the AP duration (APD) is observed in human HF, and animal models reveal that reductions in I_{to}, I_{K1}, and I_{Ks} are responsible [11–13]. The reduction of these currents has been linked to reduced transcription, translation, and expression of the corresponding channel subunits, such as $K_v4.3$, K_vLQT1, Kir2.1, and accessory proteins including minK and K^+ channel interacting protein 2 [14–17]. Reduced K^+ currents increase membrane resistance, leading to greater depolarization and delayed afterdepolarizations (DADs) [18, 19]. In HF, most studies find that I_{to} is downregulated, although there are conflicting data between human and other large animal studies [14, 18, 20, 21]. In humans, downregulation of I_{to} has been observed at the transcriptional and translational level as $K_v4.3$ mRNA levels are decreased and channel protein processing is altered [16, 22]. One mechanism is that the trafficking of $K_v4.3$ channel is downregulated by forming complexes with angiotensin receptors and increasing $K_v4.3$ internalization [23], which may explain the decreased channel density in HF [20, 21]. I_{K1} is found to be downregulated in HF at the mRNA level leading to increased susceptibility to spontaneous membrane depolarization and increased automaticity [13, 16, 20, 24]. Changes of I_{Kr} and I_{Ks} in HF are not yet well described in humans. The ATP-sensitive K^+ current and the pacemaker current (I_f), are activated at negative membrane potentials. Both are associated with increased automaticity in HF [25–27]. Of note, a repolarization reserve (redundant existence of several K^+ channels with different properties [28]), shields the heart from some pathologic changes seen in HF. Extreme reductions in one current or more may impair this reserve, leading to arrhythmogenic consequences [29]. Abnormalities of repolarization reserve also lead to exaggerated repolarization disturbances under circumstances that further exacerbate repolarization, such as with channel mutations causing decreased K^+ current [30]. Delayed ventricular repolarization can prompt early afterdepolarizations (EADs) generation and lead to ventricular tachycardia [11].

Ca^{2+} currents are dysregulated in HF as well. I_{CaL} has been shown to increase or decrease during HF depending on which channel alterations are dominant. Membrane density of the channel decreases because of reduced channel expression, however, this is opposed by an increase in channel phosphorylation and a slowing of inactivation, which both serve to prolong the AP thereby increasing EC coupling time but also increasing arrhythmic risk [31–34]. Cardiomyocytes from patients with end-stage HF show decreased amplitude of the peak Ca^{2+} current at higher stimulation frequencies [35], which is because of incomplete recovery and accumulated inactivation of the $Ca_v1.2$ channel accompanied by slow Ca^{2+} removal [36]. This may cause repolarization failure, EADs and DADs. T-tubule loss observed in HF [37, 38] can also result in a loss of $Ca_v1.2$ channel availability and a decrease in I_{CaL}. HF has also been found to alter $Ca_v1.2$ channel modulators, such as the sarcoplasmic reticulum (SR) Ca^{2+} release channels—ryanodine receptors (RyR) and a membrane scaffolding protein, bridging integrator 1. Bridging integrator 1 is found to be reduced in HF, causing impaired $Ca_v1.2$ trafficking to T tubules [39]. A defective coupling of Ca^{2+} influx via the $Ca_v1.2$ channel has been noticed in reduced SR Ca^{2+} release in HF [40]. The NCX is upregulated in HF, which also causes AP prolongation and repolarization instability [41–44]. These examples highlight that there are multiple changes that take place during HF, and Nass et al. provide a detailed review of calcium handling in HF [45].

Changes in the Na^+ Channel with Heart Failure

The voltage-gated cardiac $Na_v1.5$ channel is responsible for the generation of the upstroke of the myocyte AP (Phase 0), which is the main current for excitation propagation [46–48]. The AP upstroke velocity determines AP impulse propagation and conduction velocity in heart tissue, along with the extent of intercellular communication via gap junctions. The cardiac $Na_v1.5$ is involved in determining the APD, since some channels may reopen during the plateau phase, generating a persistent or “late” inward current. The importance of cardiac $Na_v1.5$ channels for functional cardiac electrical activity is highlighted by the emergence of potentially lethal arrhythmias in inherited and acquired sodium channel diseases. Changes of the cardiac $Na_v1.5$ have been implicated in the increased risk of sudden death in HF [49–51]. The macroscopic $\boldsymbol{I}_{Na}$ is reduced in HF [52, 53] and $Na_v1.5$ inactivation is also impaired, causing repolarization failure and EADs and DADs [54–56]. Under pathological conditions such as myocardial ischemia and HF, altered Na^+ channel function causes conduction disturbances and ventricular arrhythmias.

The cardiac $Na_v1.5$ is a transmembrane protein containing a principal pore-forming α-subunit composed of four homologous domains, each containing six transmembrane segments (S1–S6). The four domains are attached to one another by cytoplasmic linker sequences. The positively charged S4 segment of each domain forms the voltage sensor, responsible for increased channel permeability during membrane depolarization [57]. In physiological situations, activation and inactivation properties of $Na_v1.5$ channels are tightly regulated, while during channel dysfunction the gating properties and current kinetics may be altered. The α-subunit interacts with smaller accessory proteins known as the β-subunits that modulate the channel expression levels and function. The cardiac K^+ and Ca^{2+} channels share similar structures although with different components that result in different regulation and functions.

Since SCN5A, encoding the α-subunit of the cardiac $Na_v1.5$, was cloned and mapped to the chromosomal region 3p21 [58, 59], more than 100 mutations have been found in the gene [60], which cause inherited sudden death syndromes such as Brugada syndrome (BrS) [61], the third variant of long QT syndrome (LQT3) [62], and sudden infant death syndrome (SIDS) [63]. Mutations in the Na^+ channel have been associated with inherited diseases of the conducting system and more commonplace arrhythmias such as atrial fibrillation [64–71]. Loss of function mutations that show decreased or no I_{Na} are thought to confer arrhythmic risk by virtue of slowing conduction or altering the AP that both favor reentry. This can induce BrS, progressive cardiac conduction disease, sick sinus syndrome, or combinations thereof. In cardiomyopathic conditions, treatment with Na^+ channel-directed antiarrhythmic drugs increases sudden death risk, consistent with a downregulation of the channel [72–76]. On the other hand, gain of function mutations that mainly exhibit an increased late Na^+ current lead to AP prolongation, cellular Ca^{2+} overload, and afterdepolarizations. Alterations of I_{Na}, either up- or downregulation, lead to arrhythmias.

Na^+ Channel Current During HF

Various abnormalities of cardiac $Na_v1.5$ have been shown in HF, mainly emerged as reduced macroscopic I_{Na}, which contributes to slower conduction in HF facilitating reentry and ineffective cardiac contraction [18, 77, 78]. The underlying mechanisms for decreased I_{Na} density have been studied in animal models, including posttranscriptional [79] and posttranslational [52, 80, 81] deficiency of $Na_v1.5$. In a canine model of HF, a ~34 % decrease of I_{Na} and a ~30 % reduction of $Na_v1.5$ protein expression are observed without changes in channel mRNA level and gating properties in ventricular cardiomyocytes, indicating a downregulation of $Na_v1.5$ at posttranscriptional level [79]. I_{Na} reduction because of posttranslational alterations in protein kinases dysregulation, channel deglycosylation, channel trafficking disruption, metabolic and oxidative stress-induced channel dysfunction has been reported [52, 80, 81]. In a mouse HF model, a decreased I_{Na} is observed with altered gating properties as a result of deficient $Na_v1.5$ glycosylation, contributing to longer AP and a higher probability of EADs [52]. The cardiac $Na_v1.5$ channel changes in HF on gating, trafficking, and regulation by protein kinases and oxidative stress will be discussed separately. Here, we will focus on the metabolic regulation of cardiac $Na_v1.5$ in HF.

Cardiac injury from many causes is associated with altered metabolism and downregulation of $Na_v1.5$ [52, 53, 82, 83]. HF has been associated with less β-nicotinamide adenine dinucleotide (NAD^+) and increased reduced nicotinamide

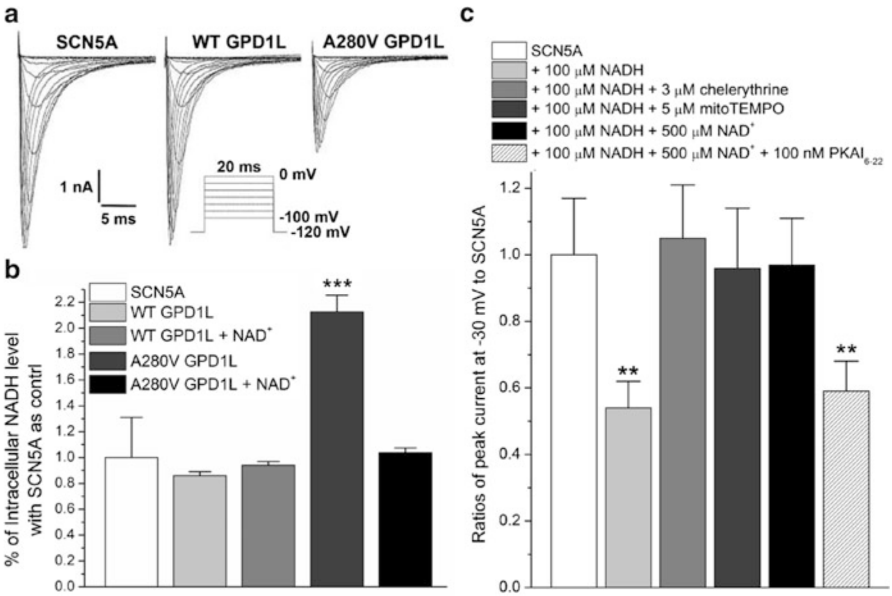

Fig. 2 The A280V GPD1L mutation decreases I_{Na} and increases intracellular NADH level. (**a**) Representative Na^+ current traces from HEK293 cells transfected with SCN5A (*left*), or cotransfected of SCN5A and WT (*middle*) or A280V GPD1L (*right*). (**b**) The intracellular NADH level is increased by A280V GPD1L and is reversed by incubation of NAD^+. ***$P < 0.001$ vs. all other groups. (**c**) The peak current of cardiac $Na_v1.5$ channel is downregulated by NADH and restored by NAD^+, a PKC inhibitor chelerythrine, or mitoTEMPO. The NAD^+ effect can be blocked by a PKA inhibitor, $PKAI_{6-22}$. **$P < 0.01$ vs. all other groups (modified from refs. [81, 89, 91] with permission)

adenine dinucleotide (NADH) [84–86], which can cause a reduction of I_{Na} [53, 87, 88]. For example, mutations of glycerol-3-phosphate dehydrogenase 1 like (GPD1L) protein have been found to induce BrS and SIDS with a decrease of I_{Na} (Fig. 2a) [89, 90]. An elevated NADH level has been associated with the reduction of I_{Na} observed in these cases (Fig. 2b) and the signaling events involve activating protein kinase C (PKC) and inducing oxidative stress, as shown in Fig. 2c [81]. Reduced I_{Na} may contribute to conduction block and arrhythmic risk known to exist with reduced cardiac contractility. Application of NADH results in reduction of I_{Na} and the maximum upstroke velocity of the AP on rat neonatal cardiomyocytes [81, 91]. The NADH-induced decrease of cardiac I_{Na} has also been observed in isolated cardiomyocytes from a mouse model of nonischemic systolic dysfunction HF [92]. This model shows a significantly increased NADH level, decreased I_{Na}, and the conduction velocity. The NADH-induced decrease of I_{Na} in HF can further exacerbate the changes in conduction velocity and contraction that lead to arrhythmic risk. These studies may help explain the link between metabolism and arrhythmic risk [87, 93].

Being in a redox couple with NADH, NAD^+ has been found to antagonize the $Na_v1.5$ channel downregulation by NADH (Fig. 2c) and may represent a new type of antiarrhythmic therapy. In our study of a mouse HF model, we have found that treating either isolated cardiomyocytes or HF animals with NAD^+ is able to increase the cardiomyocyte I_{Na} back to control levels [92]. Nevertheless, the NAD^+ effect does not seem to occur by the same signaling mechanism as does NADH and can be recapitulated by a protein kinase A (PKA) activator or prevented by a PKA inhibitor (Fig. 2c).

While it appears that HF can cause reduced I_{Na}, it is also possible that reduced I_{Na} can cause HF. HF has been reported in patients with SCN5A missense and truncation mutants [70, 94, 95]. For example, the mutation D1275N is associated with a variably expressed phenotype of conduction delay, dilated cardiomyopathy, atrial fibrillation, and impaired automaticity [70]. Two SCN5A mutants R814W and D1595H are reported to be associated with atrial and ventricular arrhythmia [96]. The mechanism whereby loss of Na^+ channel current contributes to HF is unknown.

Abnormal Na^+ Channel Gating in HF

In physiological situations, the biophysical gating properties of the cardiac $Na_v1.5$ are tightly regulated to maintain normal cardiac excitability. Altered gating properties or pathologic gating defects comprises delayed activation, earlier inactivation, faster inactivation, and enhanced slow inactivation [95]. In a mouse model of HF, altered $Na_v1.5$ channel gating properties with decreased I_{Na} has been found to contribute to prolonged APD and a higher probability of EADs [52]. For WT cardiac $Na_v1.5$ channels, there is a well-described component, called late current (I_{NaL}). I_{NaL} was first described in isolated Purkinje fibers in animal models and was found to prolong the AP [55, 97–99]. In disease states including HF and ischemia, more $Na_v1.5$ channels remain in the active state or reopen during Phase 1 and Phase 2 of the AP, causing increased I_{NaL} and further depolarizing the cell membrane. Silencing the $Na_v1.5$ gene expression leads to a 75 % decrease of I_{NaL} and results in reduction of APD and beat to beat AP variability in a HF model, implicating SCN5A as the underlying cause of I_{NaL} [100]. Augmented I_{NaL} and the concomitant increased intracellular Na^+ load in HF lead to dispersion of repolarization and EADs that predispose to ventricular arrhythmias [101–103].

There are likely many contributing factors to allow I_{NaL} to occur and persist in HF and ischemia. $Na_v1.5$ is complex, consisting of a core α subunit and β subunits, and the channel is affected directly by kinases, phosphatases, and trafficking proteins. While $Na_v1.5$ is downregulated in HF, the β subunits remain unchanged and the relatively higher membrane content of these subunits could be involved in generating I_{NaL} [79]. Ca^{2+}-mediated changes may also be responsible for I_{NaL}. There is evidence that Ca^{2+} can directly augment I_{NaL} by binding to the Na^+ channels [104]. Also, the Ca^{2+} binding protein calmodulin may also directly augment I_{NaL} [105]. Calmodulin may regulate $Na_v1.5$ channel gating via Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). A study that used adenovirus-mediated overexpression of CaMKII found a Ca^{2+}-dependent increase in I_{NaL} [106]. While there are many questions remaining about the underlying mechanisms responsible for I_{NaL}, these different possibilities also offer potential targets for future therapies to block I_{NaL}.

Na^+ Channel Trafficking in HF

Some cases of decreased I_{Na} in HF are because of altered trafficking of cardiac $Na_v1.5$, which results in decreased membrane expression of the channel. Channel trafficking is complex and includes the translocation from the endoplasmic reticulum (ER) to the Golgi apparatus, subsequent trafficking to the sarcolemma, association with accessory subunits, and anchoring to the cytoskeleton, and regulation of endocytosis. Trafficking plays a pivotal role in posttranslational regulation and functional expression of the cardiac $Na_v1.5$ channels under both physiological and pathophysiological conditions. Intracellular trafficking may prove to be a useful tool in manipulating the expression of the cardiac Na^+ channel, and has been shown to be a possible therapeutic target in arrhythmia, epilepsy, anesthesiology, and cancer applications [107–110].

Trafficking changes in human HF are not well studied to date. Downregulation of $Na_v1.5$ channel trafficking has been hypothesized in a chronic canine HF model, which revealed a 30 % decrease of I_{Na} without changes of channel gating properties and channel RNA levels in the failing hearts compared to normal hearts [111]. In spite of lack of knowledge of $Na_v1.5$ trafficking changes in HF, mutations in cardiac $Na_v1.5$ channel and Na^+ channel interacting proteins that cause significant decreases of channel trafficking have been investigated intensively and shown to underlie cardiac arrhythmias and SCD [69, 112–118]. For instance, A G1743R mutation of $Na_v1.5$ was identified in a patient with typical ECG pattern for BrS and ventricular fibrillation [117]. Immunostaining experiments confirmed the retention of G1743R in the ER, which lead to the barely expressed mutant channels on the cell membrane surface and hardly detectable I_{Na}. Baroudi et al. [119] found in BrS patients that a R1432G mutant of human cardiac $Na_v1.5$ colocalized with calnexin in the ER instead of the cell surface. The trafficking deficiency results in significant reduction of $Na_v1.5$ membrane expression and I_{Na}, which can cause slower conduction and increased risks of arrhythmias.

Causes of Changes in Sodium Channel Biophysical Properties in Heart Failure

Studies have revealed that the expression level, channel localization, and biophysical properties of the cardiac Na^+ channel are finely regulated by mediators and modulators in many processes such as gene transcription, RNA processing, protein synthesis and assembly, and posttranslational processes. Some of the mediators and modulators have been mentioned above, including the cellular metabolic state and $Na_v1.5$ interacting proteins. These mediators are modulated in HF, which in turn affects cardiac $Na_v1.5$ and the susceptibility to arrhythmias. In this section, we will focus on the posttranslational regulation of the cardiac $Na_v1.5$ by protein kinases and the reactive oxygen species (ROS), which have been investigated the most. We will also discuss the genetic control of the $Na_v1.5$ by transcription factors and the roles of neurohumoral systems, G proteins, and coupled receptors on regulation of the $Na_v1.5$ in HF.

Protein Kinases

HF usually develops progressively, during which time the reduced output leads to an increase in neuroendocrine factors like catecholamine, which activate PKC. Activated PKC evokes many signaling molecules that further affect the heart function [118]. PKCs are a family of serine/threonine kinases, comprising three subgroups with at least ten isoforms: the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, θ, and η), and the atypical PKCs (ζ, ι/λ). They have been found to play various roles in HF and cardiac ion channels functions. For example, human HF is associated with elevated activation of some conventional PKC isoforms [120, 121]. In HF, the translocation and activation of PKCα are increased [122, 123]. An upregulation of PKCα is observed in spontaneously hypertensive rat model of HF [124] and in a rat model of cardiac hypertrophy [123]. Further activation of PKCα leads to a lethal cardiomyopathy in a hypertrophy heart model, whereas inhibition of PKCα activity improves both systolic and diastolic function of the sick heart [125]. Activation of PKCα has been reported to downregulate the cardiac $Na_v1.5$ channel and decreases the macroscopic I_{Na} through channel phosphorylation, decreasing channel distribution on the plasma membrane, and increasing ROS levels [80, 81, 126]. In our recent study, PKCα seems to play a role in downregulating the $Na_v1.5$ channel in response to NADH [127].

PKCα also regulates other cardiac ion channels. Its activation plays a role in a T-type Ca^{2+} current increase, which seems to contribute to triggering arrhythmias [128]. Activation of PKCα leads to a decrease of the $K_v4.3$ channel current I_{to}, causing shortening of the APD that can cause diastolic dysfunction and arrhythmias [129]. PKCα also shows inhibition on acetylcholine-regulated K^+ current in canine atrial cardiomyocytes of atrial tachycardia-induced remodeling [129].

A significant elevation of PKCβ level and activation has been observed in human HF and in adult cardiac myocytes under hypertrophic stimuli [120, 121, 130–132], although the regulation of PKCβ on cardiac ion channels in HF is not known yet. Nevertheless, a specific study on PKCβII shows that overexpression causes cardiomyopathy exhibiting LV hypertrophy, cardiomyocytes necrosis, multifocal fibrosis, and decreased LV performance in mice [131]. Ablation of PKCβ is detrimental to pressure-overload-induced HF [133]. Similarly, mice with overexpressed PKCβ show better recovery following ischemia [134], indicating a protective role of PKCβ against HF. Further studies are necessary to understand the roles of PKCβ subtypes on cardiac ion channels in HF.

Studies on PKCε and PKCδ have shown their levels and translocation towards the myocyte membrane are increased, unchanged, or decreased in different types of HF models [135, 136]. The absolute mRNA and protein expression level of PKCδ are enhanced in a rat model of cardiac hypertrophy, while those of PKCε are unaltered [123]. Similar results were obtained in rat aorto-caval fistulas [137]. An increase of PKCε content and activation in myocyte membrane is observed in aortic banding with rats, guinea pigs, and humans [120, 122, 138] and a rat model of hypertrophy [130], but a decrease of PKCε levels have been reported in human and rabbit HF [121, 139]. In the case of cardiac ion channels, activation of PKCδ shows downregulation of the cardiac $Na_v1.5$ channel by inducing an overproduction of mitochondrial ROS level [127], resulting in a significant reduction of I_{Na} that can contribute to slower conduction speed in HF and facilitate reentry and ineffective cardiac contraction. PKCε increases the acetylcholine-regulated K^+ current in canine atrial cardiomyocytes of atrial tachycardia-induced remodeling [129].

PKA is a tetrameric serine/threonine kinase activated by cAMP binding. General effects of PKA on its target present in two ways: protein phosphorylation and protein synthesis. In protein phosphorylation, PKA directly changes the target protein activity, which is a fast process in seconds; while in protein synthesis, PKA first activates CREB, which binds the cAMP response element, altering the transcript and protein synthesis, which may take hours to days. Some of these functions have been found to be modified in HF. For example, β-adrenergic overstimulation-induced PKA activation in HF decreases in $I_{Ca,L}$ through modifications of $Ca_v1.2$ channel together with CaMKII and phosphatases [140], which is different from observations with normal cardiac myocytes where activation of β-adrenergic receptor (β-AR) and consequent PKA increase $I_{Ca,L}$ [141, 142]. A decreased response of the NCX to PKA phosphorylation is observed in a pig model of HF, indicating an increased level of baseline phosphorylation [143]. PKA hyperphosphorylation of the cardiac type RyR2 has been observed in human HF [144] and various animal models of HF [145, 146], causing RyR2 dysfunction with an increased sensitivity to Ca^{2+}-induced activation that can alter $I_{Ca,L}$ and cause prolonged APD and increase arrhythmic risks.

PKA activation upregulates cardiac Na^+ channels and increases I_{Na} through at least three different ways: channel phosphorylation, increasing channel trafficking, and decreasing cellular ROS level that downregulate I_{Na} [81, 92, 147, 148]. In our recent study of a mouse model of nonischemic systolic dysfunction cardiomyopathy, activation of PKA shows salutary effects by decreasing oxidative stress and increasing cardiac I_{Na} [92, 149]. Therefore, PKA may be a therapy to mitigate decreased I_{Na} in HF. PKA also modulates cardiac Ca^{2+} and K^+ currents [150, 151], although the implications in HF are unknown.

The specificity of PKA action is achieved by subcellular targeting of the holoenzyme A-kinase anchoring proteins (AKAPs) [152], which guarantee the correct spatial and temporal action of PKA through generation of multimolecular complexes with PKA and other signaling molecules that participate in the signaling cascades. AKAP binding to PKA is regulated by regulatory subunit II (RII) autophosphorylation. A decrease of PKA RII is observed in HF that may affects PKA relocation and its phosphorylation of other proteins involved in cardiac function [153].

In summary, PKC and PKA are altered in HF. These alterations may explain some of the ion channel changes in HF, such as decreased I_{Na} and $I_{Ca,L}$, and increased I_K. These changes can alter the APD and increase arrhythmic risks in HF.

Reactive Oxygen Species

Oxidative stress is common in HF and cardiovascular disease [84, 86, 154]. Large clinical trials have shown that ROS scavenging by antioxidant vitamins is ineffective or even harmful. On the other hand, prevention of ROS overproduction by targeting various sources of ROS may achieve intriguing benefits. The major sources of ROS overproduction in HF include mitochondrial electron transport chain, uncoupled nitric oxide synthase (NOS), the NADPH oxidase (Nox), and the xanthine oxidase (XO). Elevated ROS levels from these sources are accompanied by an elevation of corresponding enzyme expression and/or activity, or a decrease of antioxidants. For example, the mitochondrial ROS level increases and myocardial antioxidant reserve decreases in HF [155, 156]. Mitochondrial ROS overproduction is observed in mutated GPD1L-induced BrS [91]. Elevated mitochondria ROS decrease cardiac I_{Na} in a mouse model of systolic dysfunction [92]. Elevated mitochondrial ROS in rat neonatal cardiomyocytes [91] and mouse adult cardiomyocytes [127, 149] downregulate cardiac I_{Na} in vitro. The reduction of I_{Na} induced by mitochondrial ROS in HF can increase arrhythmic risk and aggravate cardiomyopathy [53, 87, 88].

Uncoupling of endothelial NOS (eNOS) has been found in diastolic HF, atherosclerosis, diabetes mellitus, ischemia reperfusion injury, and cardiac hypertrophy [157]. Patients and experimental animals with congestive HF have been found to express increased levels of the inducible isoform of NOS in cardiomyocytes [158]. In animal hypertensive HF model studies, uncoupled eNOS is shown to be the major source of superoxide production in the aorta [159]. In the Nox family, the subtypes Nox2 and Nox4 are major sources of superoxide in vascular cells and myocytes and play important roles in atherosclerosis and hypertension [160]. In human HF, higher expression, activity and translocation are observed with Nox4, the regulatory subunit of Nox, $p47^{phox}$ and $p67^{phox}$ [161–163]. The Nox is determined to be a major source of superoxide in a pressure-overload-induced guinea pig model of cardiac hypertrophy [164], with observations of an increased expression and activity of p22^{phox}, gp91^{phox}, p67^{phox}, and p47^{phox} in cardiomyocytes. An increased XO activity is reported to contribute to abnormal energy metabolism in human dilated HF [165] and in patients with chronic HF [166]. In vitro studies of isolated rat hearts, progressive development of HF is associated with oxidative stress induced by increased myocardial XO levels [167].

Cardiac ion channels have been found to be affected by oxidative stress stemmed from all the sources discussed above. The cardiac Na$_v$1.5 is downregulated by direct application of H_2O_2 and by mitochondrial ROS overproduction [91, 168]. ROS-dependent CaMKII activation enhances I_{NaL}, leading to cellular Na^+ and Ca^{2+} overload and afterdepolarizations, which can contribute to arrhythmias [169] that can be suppressed by the antioxidant N-acetylcarnosine and CaMKII inhibitors [170, 171]. The oxidative stress has been shown to reduce repolarizing K^+ currents by affecting K^+ channel mRNA and protein levels, potentially causing abnormal QT prolongation and arrhythmias in HF [172, 173]. Direct treatment with H_2O_2 triggers an initial K^+ channel HERG activation and subsequent acceleration of channel deactivation, increasing the risks of ectopy [174]. In a chronic rat HF model, the gene expression reduction is observed with the K$_v$4.3 [173]. NO increases the amplitude of the inward rectifying I_{K1} current [175] by increasing the Kir2.1 channel opening probability. Since this channel adjusts the resting membrane potential and influences the APD, redox-dependent changes would be expected to influence arrhythmic risk.

Cardiac Ca^{2+} channels and calcium-handling proteins contain sulfhydryl groups or disulfide linkages that are susceptible to the changes of redox states. A ROS-induced reduction of $I_{Ca,L}$ is observed in the ventricular myocytes of guinea pigs, rats, and rabbits, which contributes to shortening of the APD and an increased potential of reentrant arrhythmias [176–180]. In human cardiomyocytes, ROS can induce a decrease expression of Ca$_v$1.2 [181], but the total I_{CaL} remains unchanged [182, 183]. Single channel recordings of this Ca^{2+} channel from failing human hearts have revealed an increased activity, probably resulting from an increased phosphorylation state [34], which may compensate in part for the decrease in the number of channels and explain the constant Ca^{2+} current. In ferret ventricular myocytes, redox-sensitive thiols in Ca$_v$1.2 diminish $I_{Ca,L}$ under oxidizing conditions, and this mechanism is regulated by NO and *S*-nitrosothiols [184]. The effect of NO on Ca$_v$1.2 has also been studied extensively in different experimental models with variable results, depending on the animal species and the concentration of NO (for review see [84]). Oxidative stress in HF also affects the sarco/endoplastic reticulum Ca^{2+}-ATPase (SERCA), the SR RyR, and the NCX.

In summary, oxidative stress is not only elevated in HF, it also affects cardiac ion homeostasis and structure/function of cardiac ion channels and ion handling proteins, contributing to contractile dysfunction, myocyte apoptosis and necrosis, and aggravating the development of HF. Targeting the specific oxidative stress sources may represent a novel strategy to prevent arrhythmias, which could be safer than the conventional ion channel blockers.

Others Ion Channel Modulators Changed in Heart Failure

Besides protein kinases and ROS, there are many other enzymes and molecules functioning as modulators of cardiac ion channel gene expression, protein folding, trafficking, distribution, and gating properties in HF. In this section, we will discuss some representative modulators and how their dysfunction is linked to HF.

HF may result in changes in ion channel gene transcription. Nuclear factor (NF)-κB, a ubiquitous transcription factor that activates genes expression, has shown effects on many cardiovascular diseases, such as myocardial ischemia/ reperfusion injury, cardiac hypertrophy, and HF [185]. NF-κB activation is enhanced in human HF and is required for the development of cardiac hypertrophy in mice and rats and for the transition from hypertrophy to HF in humans [186–190]. Increased NF-κB binding to the cardiac Na^+ channel gene SCN5A promoter can lead to reduction in $Na_v1.5$ transcriptional activity and eventually in I_{Na}. Interestingly, Grabellus et al. show that the elevated activation of NF-κB in human end-stage HF is reversed after installing left ventricular assist devices, indicating the involvement of NF-κB in the process of reverse remodeling [191].

SCN5A gene expression is also regulated by alternative mRNA splicing. In human HF, three C-terminal truncated splice variants have been discovered [87, 168]. Not only are these variants unable to form functional channels, but also they affect the expression of wild-type channel, both of which result in a reduction in I_{Na}. Stimuli for this alternative splicing include hypoxia and angiotensin II, factors associated with ROS and arrhythmic risk. Hypoxia inducing factor-1α (HIFα), a key transcriptional regulator in hypoxia and inflammation, is highly associated to NF-κB regulation of SCN5A [192]. This mechanism is mediated by SCN5A splicing factors RBM 25 and hLuc7A [193, 194]. Targeting these splicing factors may be a possible therapy to increase the membrane expression of wild-type cardiac $Na_v1.5$ and I_{Na} in failing hearts.

Potential Therapies

Despite the fact that a quarter of cardiovascular deaths are to the result of arrhythmias, the therapies available for clinical use are largely limited to ion channel blockers that have proarrhythmic properties and device therapy that does not specifically target the underlying mechanism of each unique arrhythmia [195]. The largest trials of antiarrhythmic medications include the CAST and SWORD studies that both found channel-blocking agents having a negative effect on mortality [196, 197]. Later trials focused on device therapy vs. medical therapy with the consistent finding that devices improve mortality despite treating arrhythmias on a level without focus on specific mechanisms [198–200]. New agents and approaches are needed to manage arrhythmias at the clinical level and the above biophysical considerations suggest possibilities (Table 1).

Table 1 A summary of new therapeutic approaches mentioned in this chapter

Therapy	Targets	Potential roles	Limitations
Gene therapy			
Upregulation of expression	Kir2.1	Stabilize fibrillatory rotors in mice [201] and abbreviate APD without suppressing contractility in HF [203]	Regulation of gene delivery and expression
Adenoviral gene transfer	Drosophila Shaker B K^+channel	Improve AP prolongation and cell mechanical function of failing cardiac myocytes [202]	
New drugs			
Ranolazine	$Na_v1.5$	Suppress I_{NaL} to improve arrhythmia and contractile dysfunction in HF [204]	Unknown efficacy, potential proarrhythmia, and possible off target effects
PD-118057	HERG K^+ channel	Increase I_{Kr} and shorten APD and suppress EADs development [212]	
SEA-0400	NCX	Suppress EADs and DADs, and abolish triggered arrhythmias [213]	
Endothelin antagonists	L-type Ca^{2+} channel and several K^+ channels	Prevent AP prolongation in HF [214, 215]	
Verapamil	L-type Ca^{2+} channel	Convert VF into stable VT [216]	
NAD^+	$Na_v1.5$	Upregulate $Na_v1.5$ [79]	

AP action potential, *APD* action potential duration, *DADs* delayed afterdepolarizations, *EADs* early afterdepolarizations, *HF* heart failure, I_{Kr} rapid delayed rectifier K^+ currents, I_{NaL} late Na^+ current, NAD^+ β-nicotinamide adenine dinucleotide, $Na_v1.5$ cardiac Na^+ channel, *NCX* Na^+-Ca^{2+} exchanger, *VF* ventricular fibrillation, *VT* ventricular tachycardia

Few new antiarrhythmic drugs have been introduced clinically in the last decade, and research has focused on gene therapy to improve perturbations to membrane currents seen in HF. I_{K1} upregulation has been found to accelerate yet also stabilize fibrillatory rotors in mice [201]. Adenoviral gene transfer of an inactivation-defective Drosophila Shaker B K^+ channel in failing cardiac myocytes has been found to improve AP prolongation and cell mechanical function [202]. Similarly, gene therapy with Kir2.1 was found to abbreviate excitation without suppressing contractility in a HF model [203]. Genetic approaches to normalizing membrane currents in disease states will likely prove to be an important area of future clinical research. Nevertheless, there are quite a few difficulties that need to be addressed. Gene delivery systems are not perfected for in vivo models, and furthermore, transgene expression needs to be effectively regulated before this approach can become relevant clinically.

New drug-based approaches to normalizing membrane currents in addition to traditional ion channel-blocking attempts have the potential to yield useful clinical

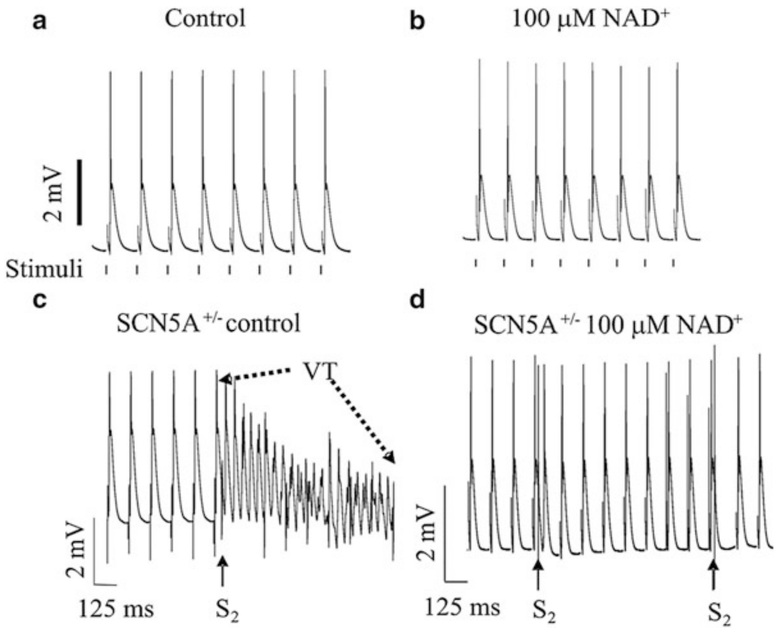

Fig. 3 Representative traces of monophasic action potentials (MAPs) from left ventricular epicardium of Langendorff-perfused SCN5A$^{+/-}$ heart during standard pacing at basic circle length of 125 ms in (**a**) the control condition and (**b**) after 20 min of perfusion with 100 mM NAD$^+$. *Vertical lines* below the MAPs represent the times when electrical stimulations were delivered. (**c**) Representative MAPs recorded during programmed electrical stimulation (PES) showing PES-induced ventricular tachycardia (VT) in SCN5A$^{+/-}$ hearts under control condition. The final six paced beats at 125 BCL (S1) were followed by an extra stimulus (S2) delivered at a S1–S2 interval of 42 ms. PES induced a ventricular tachycardia with cycle length of 20–40 Hz that was sustained for ~19 s. (**d**) Representative trace of PES-induced MAPs recording in same SCN5A$^{+/-}$ heart after 20 min perfusion with 100 mM NAD$^+$. S2 stimuli delivered at a 35 ms S1–S2 interval produced a single MAP but failed to induce VT (modified from ref. [81] with permission)

therapies. For example, ranolazine, a new drug with a 38-fold higher potency for I_{NaL} than for peak I_{Na}, has reported antiarrhythmic effects for both supraventricular and ventricular arrhythmias by suppressing I_{NaL} [204], but there are no large randomized controlled trials to support these findings to date [205–208]. The exact mechanism by which ranolazine alters I_{NaL} is a matter of debate. Two earlier studies identified two binding sites on the $Na_v1.5$ [209, 210]. A recent study suggests that ranolazine blocks I_{NaL} by inhibiting the mechanosensitivity of $Na_v1.5$ independent of previous established binding sites [211]. Evidence suggests that I_{NaL} blockade could be useful to normalize Na^+ and Ca^{2+} handling to treat both arrhythmias and contractile dysfunction in HF. Nevertheless, interpreting the body of literature related to *r* is difficult since off target effects cannot be excluded and a positive control for the effects of the drug is not available.

New compounds have been identified that enhance HERG K^+ channels and result in increased I_{Kr} that can suppress AP prolongation and the development of

EADs [212]. New selective NCX inhibitors have also been found to suppress EADs and DADs in Purkinje fibers [213]. Endothelin antagonists have been developed to prevent AP prolongation in HF, and more importantly, they have been found to counteract the downregulation observed in I_{K1} and I_{to} [214, 215]. Current work on older agents is also promising. A study of verapamil found that it can convert ventricular fibrillation into stable VT by reducing the frequency of rotors and by decreasing wavefront fragmentation, which in turn decreases fibrillatory propagation from the rotor [216].

NAD^+ has emerged as a putative metabolic regulator of transcription, longevity, and several age-associated diseases. Our recent studies show that NAD^+ may represent a new type of antiarrhythmic therapy [81, 91]. NAD^+ modulates the cardiac $Na_v1.5$ channel and mitochondrial ROS formation through PKA (Fig. 2c). This implies that NAD^+ can potentially be given to humans to raise I_{Na} in HF and reduce sudden death risk. NAD^+ has shown antiarrhythmic properties in heterozygous SCN5A knockout mouse hearts that show VT and BrS (Fig. 3) [81]. This opens the possibility of an entirely new paradigm for treatment of arrhythmias by raising rather than blocking $Na_v1.5$.

Further understanding of the changes in currents during HF is likely to lead to new, safer, more effective therapies for arrhythmia. In many senses, we have just begun to reap the benefits of a half century of ion channel biophysics.

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Biophysical Mechanisms for the Metabolic Component of Impaired Heart Function

E. Douglas Lewandowski

This chapter explores metabolic basis of cardiac decompensation through maladaptative changes in metabolic enzyme expression that leads to dysregulation of lipid and carbohydrate metabolism. The consequences of this metabolic dysregulation are examined in detail with respect to inefficiencies in energy production in the myocardium that contribute to the energy starved heart and dysregulation of lipid metabolism that contributes to the potential for lipotoxicity. The initial section considers the link between early metabolic changes and early manifestations of impaired contractility in the decompensating, myopathic heart. Changes in the metabolic fate of the primary fuel for ATP in the heart, long chain fatty acids, occur in the decompensated and failing heart at the level of gene expression. As discussed below, long chain fatty acid oxidation and storage are both impaired in failing hearts, leading to a general dysregulation of lipid dynamics in the cardiomyocyte that contributes to the energy starved condition of the heart. The consequential metabolic adaptations in the cardiomyocyte invoke changes in the cytosolic and mitochondrial metabolism of not only lipids but also carbohydrates, as glucose is inefficiently metabolized for the production ATP. With changes in glycolytic activity and the reduction/oxidation state in the cytosolic space, transport of metabolic intermediates across the mitochondrial membrane serves to transduce the pathophysiological state of the cytosol to the mitochondrial matrix, while reciprocal exchange of intermediates from oxidative pathways links mitochondrial activity to the reduction/oxidation state of the cytosol. The latter sections of this chapter examine the details of altered mitochondrial transporter activity in response to the bioenergetic and biophysical state of the cell.

E.D. Lewandowski, Ph.D. (✉)
Departments of Physiology and Biophysics, Medicine, and Bioengineering,
University of Illinois at Chicago College of Medicine, MC-901,
835 South Wolcott Avenue, Chicago, IL 60612, USA

UIC Center for Cardiovascular Research, 909 S. Wolcott Ave., M/C 801,
Chicago, IL 60612, USA
e-mail: dougl@uic.edu

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Early Signatures of Cardiomyopathy

Examinations of left ventricular (LV) wall contractility link early changes in metabolism to functional deficits in the heart that can predate both overt cardiomyopathy and the onset of complex disease [1, 2]. Indeed, proton magnetic resonance spectroscopy (MRS) detection of triglyceride content in the human myocardium demonstrates a close association of cardiac steatosis to glucose intolerance in patients, development of type II diabetes, and the ultimate development of diabetic cardiomyopathy [1]. As discussed in this section, studies in animal models lead to the recognition that indices of LV function derived from magnetic resonance imaging (MRI) tagging experiments provide sensitive indicators of early functional impairment of disease as well as early indicators of therapeutic value. Practical application of this realization has led to an increasing volume of clinical and preclinical studies to evaluate the efficacy of treatments to improve LV function in diabetic cardiomyopathy [3, 4]. Such changes in the pathophysiology of the heart are also distinguished by a signature reduction in the bioenergetic potential of the cell. Ample evidence for the notion that the failing heart is energy starved exists in the current literature, and examinations of phosphorylation potential and alterations in phosphoryl group transfer also serve as prognostic indicators of patient survival rates and help define the early signatures of cardiomyopathy. This section presents the early changes in LV wall mechanics, metabolic indices, and energetic changes that characterize the early development of heart failure.

Two-Dimensional Strains and Early Decompensation

Biophysical parameters for assessing ventricular wall mechanics with MRI of the heart have evolved to provide noninvasive indices of transmural and regional contractility through the examination of two-dimensional Lagrangian strains, strain rates, circumferential and radial strains, and ventricular torsion, via cardiac tagging methods. The process of MRI detection involves superimposing a grid pattern across the image of the heart, through selective saturation of proton nuclear spins, and tracking the deformation of the grid at systole and/or diastole (Fig. 1). This approach of cardiac tagging with MRI has contributed greatly to characterizing and understanding the functional impact and manifestation of early, subcellular alterations in the contractile apparatus from early contractile defects to decompensated cardiomyopathy to over heart failure.

Interestingly, the two-dimensional values termed E1 and E2, associated with the positive component and negative component, respectively, of the Lagrangian strains reflect very early changes in regional ventricular function [5]. For example, in a study by Hankeiwicz et al., utilizing very high resolution tagging grids of the order of 0.3 mm, the positive strain component, E1, during systole was compromised in a murine mouse model of dilated cardiomyopathy 2 months prior

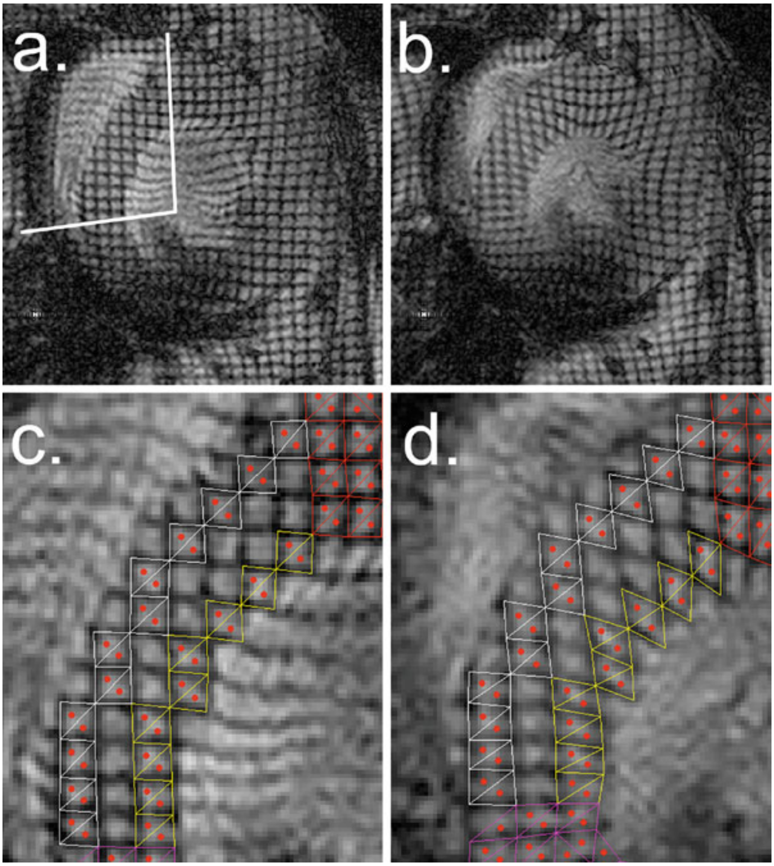

Fig. 1 Cardiac MRI tagging of in vivo mouse heart. Tagged images shown are short-axis tagged, with a 0.33 × 0.33 mm grid and a 0.1 mm grid line. (**a**) Tagged mouse heart image at end diastole with septal segment demarcated by white lines; (**b**) image at end systole; (**c**) zoomed image of septum at end diastole; (**d**) zoomed image of septum at end systole. (**c**, **d**) Display triangulated tagging elements for homogeneous strain measurement with endocardium shown in *yellow* tagging elements and epicardium tagging elements shown in *white*. Centroids of the *triangulated* tagging sections are marked *red*. From Hankiewicz et al., Circulation: Cardiovascular Imaging 2010;3:710–717

to any significant wall thinning in the LV [6]. The initially surprising findings suggest that such biophysical parameters are more closely associated with fundamental changes at the level of regulation of sarcomere activity and responsiveness than are other traditional indices of ventricular performance. Thus, such approaches in examining strain in the ventricular wall appear to reflect fundamental alterations in contractility of the finite elements within the myocardium, such as the sarcomere [6, 7].

In a later study, Li et al. further demonstrated that strain measurements from cardiac-tagged MRI studies showed impaired regional function in the mdx mouse model of muscular dystrophy in the absence of any changes in temporally matched indices of global ventricular function [8]. The notion of strain measurements detecting fundamental changes in the sarcomere is further confirmed by subsequent findings of early reduction in torsion and strain in the LV of a mouse model of decreased expression of cardiac myosin binding protein C that corresponded to altered stretch-activated, cross-bridge kinetics in skinned fibers. Altered torsion and strains also occur in the left ventricles of hypothyroid rats that only express the slow β-myosin heavy chain compared to rats predominantly expressing the α-MHC isoform, as a likely consequence of slowed cross-bridge performance [9].

Contractility and Metabolic Dysregulation

The close association of MRI-based measures of strain in the LV to fundamental changes in sarcomere function, that is not as evident in gross measures of LV function, may also account for the sensitivity of MRI-detected strains to metabolic disruptions that lead to the development of cardiomyopathy. As discussed in greater detail below, the metabolic signaling processes that are invoked by impaired fat and carbohydrate oxidation and storage in the cardiomyocyte may serve as mediators of myocyte contractility and thereby changes in the generation of LV strains.

While the overstorage of cardiac triglyceride is associated with the eventual development of type II diabetes and the occurrence of diabetic cardiomyopathy [1], whether a more direct relationship between myocardial triglyceride and contractile dysfunction exists is addressed by a study of a cardiac-specific mouse model of low, overexpression of the nuclear hormone receptor, peroxisome proliferator-activated receptor-α (PPARα, strain 404-4) [2]. The intramyocellular content of triglyceride has been determined in vivo by proton MRS in hearts of the low overexpressing PPARα mice and non-transgenic littermates over the course of a short-term high fat diet, composed of 60 % calories from fat (Fig. 2).

Using a very high resolution tagging method that enabled resolution of the tagging grid in endocardial and epicardial layers of the LV, increased myocardial lipid after only 2 weeks of a high fat diet was associated with concurrent reductions in E1 and E2 strains in the PPARα overexpressing mice, with the most pronounced reduction occurring in the endocardium (Fig. 3). Myocardial triglyceride was consistently lower in the non-transgenic littermates than in the MHC-PPARα mice and the strains were only attenuated at the highest levels of lipid accumulation, suggesting a threshold response. The importance of elucidating these relationships is that 2D strains are impaired early and without left ventricular diastolic dysfunction, owing to cardiac steatosis, and that only in hearts predisposed to cardiac steatosis did a short-term high fat diet affect contractility. This study was the first evidence that cardiac steatosis is not only prognostic but also directly involved in the development of cardiac dysfunction. The evidence for the role of lipid metabolism in the pathogenesis of cardiomyopathy presented above is supported by a wealth of recent data in the literature to indicate the direct role

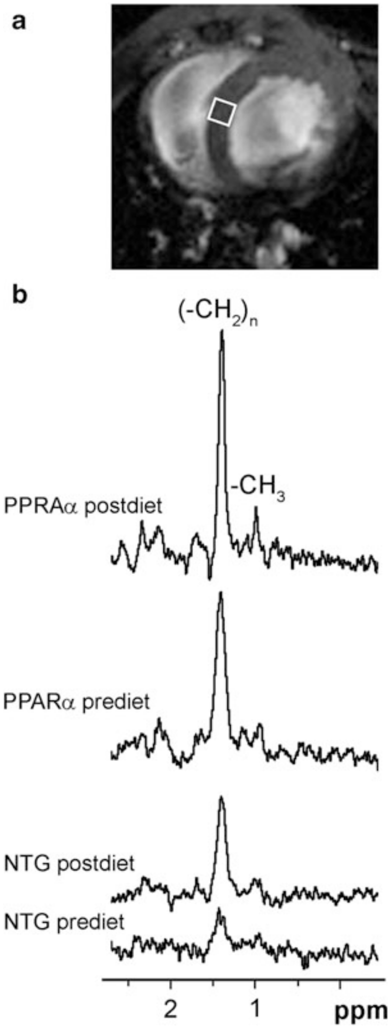

Fig. 2 Localized ^{1}H MRS (septum) of endogenous cardiac lipid content ($1 \times 1 \times 1$ mm volume of interest). (**a**) Axial scout image of in vivo mouse heart with indicated localized volume (*white square*). (**b**) Water suppressed ^{1}H spectrum showing triglyceride signals in pre- and post-high fat diet hearts of non-transgenic littermate mice and MHC-PPARα transgenic mice. From Hankiewicz et al., Circulation: Cardiovascular Imaging 2010;3:710–717

that disruption of metabolic balance, including energy yielding pathways that influence bioenergetic state, plays in the pathogenesis of the failing heart.

Bioenergetic Markers of Cardiomyopathy

The pathogenesis of cardiomyopathy has long been associated with reduced cellular energy potential of the cardiomyocytes. The myocardium that progresses to decompensation and overt failure is characterized by impaired availability and production of chemical energy in the form of ATP to support cell maintenance and contractile function [10–13]. In studies in animal models, ^{31}P NMR measurements have clearly

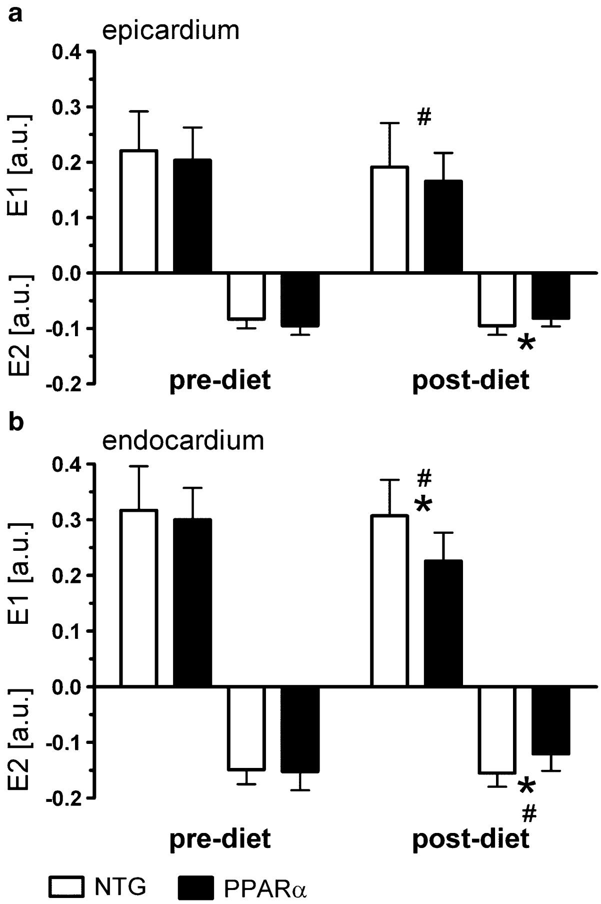

Fig. 3 Effect of high fat diet on values for myocardial 2D principal strains, E1 and E2. (**a**) E1 and E2 in epicardium; (**b**) E1 and E2 in endocardium. Note reduced endocardial strains in MHC-PPARα hearts after 2-week high fat diet. $^*P < 0.05$, post-diet MHC-PPARα vs. post-diet NTG; $^\#P < 0.05$, pre-diet MHC-PPARα vs. post-diet MHC-PPARα. From Hankiewicz et al., Circulation: Cardiovascular Imaging 2010;3:710–717

demonstrated a general reduction in free energy charge in the hypertrophied or dilated myocardium, as evidenced by reduced ratios of phosphocreatine (PCr) to ATP that trend below 1.8 PCr:ATP [14–20]. Similar observations have been made in human subjects across a range of cardiomyopathies, including both hypertrophic and dilated hearts [21–26]. This PCr/ATP ratio serves as an index of the phosphorylation potential within the cell:

$$\Delta G_{\mathrm{P}} = -30.5\ \mathrm{kJ/mol} + RT\ln[P_{\mathrm{i}}]/100$$

where ΔG_{p} is the phosphorylation potential and P_{i} represents inorganic phosphate. The equation is determined from the free energy of ATP hydrolysis in the cell which is given by:

$$\Delta G_{\mathrm{ATP}} = \Delta G_{\mathrm{o}} - RT\ln[\mathrm{ATP}]/[\mathrm{ADP}][P_{\mathrm{i}}]$$

where ΔG_{ATP} is ATP hydrolysis under standard conditions at -30.5 kJ/mol, R is the gas constant (9.314 J/mol K), and T is temperature in Kelvin, and which can be assessed by the relationship of the high energy phosphates to the other reactants and products across the creatine kinase (CK) equilibrium reaction:

$$K_{eq} = [\text{ATP}][\text{free creatine}]/[\text{ADP}][\text{PCr}][^{+}\text{H}]$$

where K_{eq} is a known constant of 1.66×10^9 at pH 7.1 and 37 °C.

In accordance with the CK equilibrium kinase reaction, ^{31}P NMR measurements of whole tissues, as in the heart, have made major contributions to the ability to assess phosphorylation potential because NMR spectroscopy provides measures of ATP, PCr, and intracellular pH (from the pH sensitive chemical shift of inorganic phosphate (P_i)). Importantly, destructive tissue assays for ADP content include both the bound and unbound fractions, when the unbound ADP participates in the CK reaction. Thus, when a known assay result or literature value for creatine content is applied, NMR measurements of ATP, PCr, and pH enable the determination of the actual free ADP content of the cell from the CK equilibrium reaction shown above [27]. Consequently, determined changes in ADP/ATP of an intact tissue reflect a change in the free energy of ATP hydrolysis at steady state conditions.

This PCr/ATP ratio, as an index of the phosphorylation potential, drops in the decompensated, cardiomyopathic heart and has been suggested as a prognostic indicator of mortality in patients with dilated cardiomyopathy [28]. Limitations occur in bioenergetic state and phosphoryl group transfer among high energy phosphates, and both are implicated in the energetic inefficiency of the hypertrophied myocardium [12, 29] (Fig. 4).

One limiting factor in the energetic state of the failing heart is the consequence of reduced cellular content of both ATP and PCr along with reductions in myocardial creatine content due to reduced creatine transporter proteins on the sarcolemma [30]. However, restoration of creatine levels by increasing expression of the creatine transporter in mouse hearts proved ineffective in supporting normal PCr levels [31]. Instead, the induced increases in intracellular creatine produce an abnormally low fraction of phosphorylated creatine that drives down the chemical driving force for the ATPase reactions, with a deleterious effect on contractile function.

More recently the unidirectional rate of ATP synthesis across the creatine kinase reaction has been found to decline in left ventricular hypertrophy (LVH) due to pressure overload, with further, distinguishing reductions in this rate in congestive heart failure (CHF) [12, 29]. These bioenergetic distinctions of LVH and CHF are not only early signatures of the presence of disease but also contribute to the underlying metabolic basis of cardiomyopathy via the consequence of the failing heart being an energy starved heart. Indeed, a mouse model of myofibrillar CK overexpression showed improved ATP flux through CK and contractile function during pressure overload induced by transverse aortic constriction (TAC) [32].

Based on the observed deficiencies in the energetic state of the decompensated myocardium, clinical efforts have focused on restoring both PCr and ATP contents in failing human hearts. For example, blocking the progressive catabolism of the purine

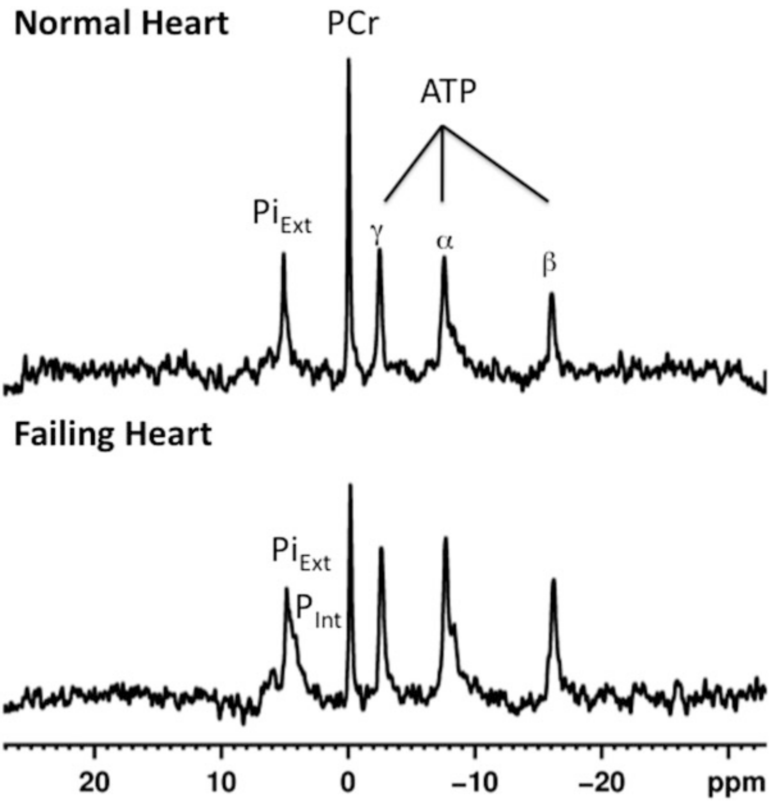

Fig. 4 Phosphocreatine (PCr) and ATP contents are reduced in failing hearts as shown by ^{31}P NMR spectroscopy of intact, beating hearts. ^{31}P spectra enable detection of extra- and intracellular contents of inorganic phosphate (Pi), PCr, and the three phosphate groups of ATP, γ, α, and β. Due to chemical shift effects induced by local electromagnetic environments, the β phosphate group is the only phosphate group that is purely from ATP, while the other two have signal contributions from other phosphates. *Top panel*, ^{31}P spectrum from normal rat heart. *Bottom panel*, ^{31}P spectrum from failing rat heart. Note reduced PCr and elevated intracellular Pi content relative to ATP, represented by the β phosphate signal that indicates low energy potential in the failing heart

moiety from ATP breakdown, through chronic allopurinol administration to inhibit the breakdown of hypoxanthine, has met with some success in maintaining cardiac PCr/ATP ratios, PCr concentration, and CK flux in the failing human heart [33].

Metabolic Dichotomy of Heart Failure

Early changes in metabolic activity, as consequence of altered metabolic enzyme expression, are now well recognized to contribute to the pathogenesis of heart failure [20, 34–40]. As discussed above, the myopathic heart is often characterized as “energy starved”; by virtue of a reduced bioenergetic potential and altered free energy of ATP hydrolysis. An increasing awareness and focus on the defects in metabolic pathways that generate the reducing equivalents, that serve as the currency for oxidative ATP synthesis, has yielded important new findings in the metabolic basis of heart failure. However, the metabolic distinctions associated with the pathogenesis of different cardiomyopathies are not always clearly understood, and often confused. For example, the metabolic changes that contribute to the development of cardiomyopathy in diabetes or metabolic syndrome are different, and at times converse to changes associated with chronic pressure overload. This section examines the underlying changes and restrictions in intermediary metabolism that are associated with the spectrum of cardiomyopathies leading to heart failure.

Distinctions in Fuel Supplies

As discussed in greater detail in later section, the blood-borne, carbon-based fuels that support energy synthesis in the cardiomyocyte include primarily long chain fatty acids, to a somewhat lesser extent carbohydrates, and under variable circumstances ketones. Of these, long chain fatty acids are the most energy rich, yielding more ATP per mole than can be produced from the complete metabolism of either glucose or ketones. Indeed, the normal heart has been characterized as an “omnivore,” able to utilize a variety of substrates and adjust to transient changes in either the availability of substrates for energy production or demand, as during workload jumps. Nonetheless, the preferential and predominant fuel for supporting ATP synthesis in the myocardium is the energy rich long chain fatty acid supply.

A well-recognized metabolic response to chronic pressure overload in the heart is the reduced oxidation of long chain fatty acids. In the pressure overloaded heart, glycolytic flux increases but does not lead to a commensurate increase in flux through pyruvate dehydrogenase (PDH) on the mitochondrial membrane for concerted oxidative production of mitochondrial NADH from glycolytic end products. Thus, a clear reduction in the potential energy provided by the carbon-based substrates that fuel the oxidative intermediary metabolism of the mitochondria occurs. The principles behind the shift away from fat oxidation and the inefficiencies in carbohydrate metabolism that occur in cardiac hypertrophy are discussed in detail below, but from the discussion presented above, the state of low energy charge in the failing heart is consistent with this model of low fat oxidation. However, in addressing the dichotomous nature of metabolism of the failing heart, this section contrasts the low fat metabolism in the pressure overloaded heart to the high fat oxidation state of the diabetic heart, which also develops cardiomyopathy progressing to overt failure.

Consequently, the notion of an energy starved heart may seem paradoxical to the diabetic heart, in which long chain fatty acid metabolism is disproportionately elevated in the presence of limited glucose uptake. Yet, the myocardial PCr/ATP ratio in diabetic cardiomyopathy is also reduced, as in other forms of cardiomyopathy, while improving glucose uptake into the heart with rosiglitazone does elevate PCr/ATP in hearts of patients with type 2 diabetes [41]. Thus, the diabetic heart develops cardiomyopathy and energy deficits amidst an abundance of high energy fuel availability.

Restricted Metabolic Flexibility

Clearly, different pathophysiological states, all leading to heart failure, can present opposing metabolic deficits that challenge a simplistic understanding of fueling cardiac energetics and infer distinctions in metabolic signaling. Caution is indicated in generalizing the metabolic shifts of the failing heart, and investigators must recognize that the pathophysiological condition introduces different metabolic variables. This dichotomy leads to the notion of restricted metabolic plasticity as a deleterious consequence in the myopathic heart. The common metabolic feature in cardiomyopathies is the lack of flexibility in the ability of the cardiomyocyte to adjust substrate utilization for energy production to either changes in substrate supply in the blood or energy demand in response to chronic changes in workload [42, 43].

An example of an inability to adjust to transient changes in work and metabolic demand is the simple model of workload jumps by the heart. During increased work, as can be induced by adrenergic challenge to the beating heart, the additional energy requirement has been found to be supplied by the recruitment of additional carbohydrate metabolism, on top of a high baseline level of long chain fatty acid oxidation [44, 45]. The inability to accommodate this recruitment due to metabolic restrictions, through either impaired glucose uptake in diabetic hearts or reduced oxidation through PDH in hypertrophied hearts, eventually leads to a chronic state of energetic deficiencies in the cardiomyocyte, which are now believed to eventually contribute to the development of impaired energy balance and cardiomyopathy [40, 42, 43]. More recently the role of leptin in regulating myocardial metabolism has been recognized and impaired leptin levels or deficient leptin signaling results in restricted glucose metabolism leading to impaired functional responses to stress [46].

Inefficiencies in Fuel Oxidation and Energy Production in the Hypertrophied and Failing Heart

The hypertrophied myocardium that progresses to decompensation and overt failure is characterized by impaired availability and production of chemical energy in the form of ATP to support cell maintenance and contractile function [10–13]. Changes in the energy yielding intermediary pathways are implicated in the hypertrophic gene expression program, and can be related to specific inefficiencies in ATP synthesis [14, 20, 38, 40, 47–50]. But a generalized description of this metabolic remodeling as a simple reduction in fatty acid oxidation with increased carbohydrate use neglects the balance of carbon flux in and out of the tricarboxylic acid (TCA) cycle and changes in the overall dynamics of lipid utilization/storage that produce physiological effects [20, 40, 51, 52].

With direct relevance to the efficiency of ATP production by the heart, the reduced contribution of fatty acid oxidation represents a net loss of ATP production per mole of substrate in comparison to that of carbohydrate. Although the complete oxidation of glucose requires less oxygen consumption than does that of long chain fatty acids, such as palmitate or oleate, these long chain fats yield more ATP per mole of substrate oxidized than glucose. Thus, in the absence of impaired tissue oxygen tension (PO_2), such as in the flow-limited myocardium, oxygen use is not a critical factor in the absence of limited tissue oxygenation, and the most efficient fuels for ATP production by the mitochondria are the long chain fatty acids. Indeed, while energy balance is impaired in CHF, oxygen availability and tissue PO_2 have been demonstrated to be adequate in remodeled myocardium [53]. The generic extrapolation of the reduced use of oxygen by glucose oxidation as a benefit to all forms of heart disease is not always well considered. Therefore, the efficiency of metabolic pathways that support ATP synthesis and adaptive shifts in the metabolic balance of the hypertrophied myocardium can be argued as more critical concerns than oxygen sparing.

When considering the metabolic efficiency of the heart, in the absence of limited oxygen availability, the more concerning parameter becomes the efficiency of ATP production per unit of carbon-based fuel oxidized. In the of the decompensated, pressure-overloaded myocardium that appears "energy starved," the shift away from fuels, such as long chain fatty acids, that can yield the greatest amount of ATP per mole, may be maladaptive. Several key inefficiencies in the metabolism of carbon-based fuels for ATP synthesis during the previous funding period are: (1) β-oxidation: reduced fatty acid oxidation rate and ATP synthesis from energy rich fuel; (2) lipid storage dynamics: elimination of the endogenous triacylglyceride (TAG) pool as an oxidative fuel source in hypertrophied hearts and reduced incorporation of palmitate into the TAG pool with potential implications for lipotoxicity; (3) carbohydrate metabolism: increased glucose uptake and glycolysis, but a shift in carbohydrate oxidation away from entry into the TCA cycle via PDH and toward anaplerosis via malic enzyme (ME), bypassing NADH producing steps for ATP synthesis. These three inefficiencies are examined in the subsequent sections.

Reduced β-Oxidation in Hypertrophied and Failing Hearts

As introduced above, a classic metabolic distinction of the hypertrophied myocardium is a reduction in long chain fatty acid oxidation in the support of mitochondrial oxidative ATP production. Changes in the expression of enzymes associated with long chain fatty acid oxidation mediate this shift. The mitochondrial membrane is

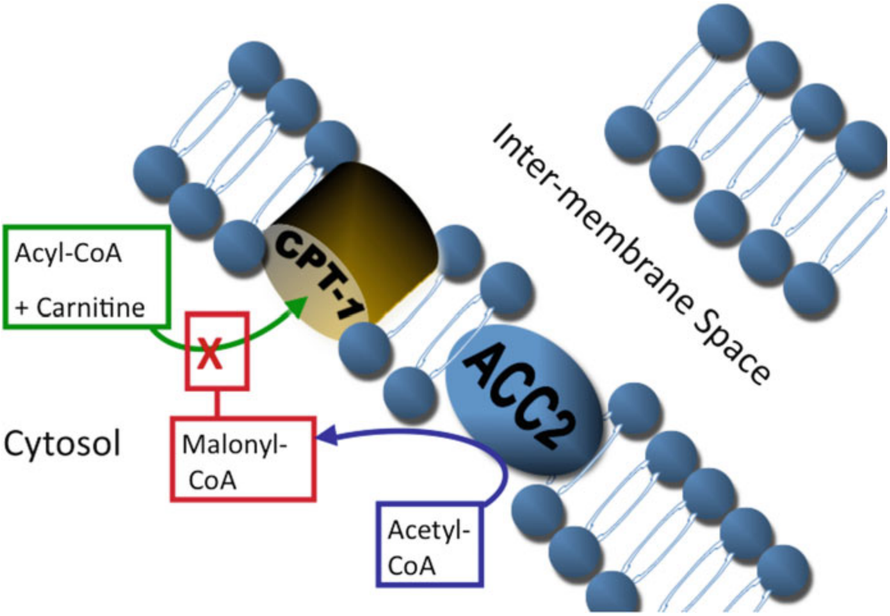

Fig. 5 Regulation of the rate-limiting activity of CPT1 on long chain fatty acid entry into mitochondria for β-oxidation. CPT1 and ACC-2 are integrated into the outer mitochondrial membrane. The muscle isoform of CPT1 (M or β) is inhibited by malonyl CoA levels produced by the carboxylation of acetyl CoA by ACC-2. Questions remain as to whether other factors regulate the activity of the liver CPT1 isoform (L or α) that is expressed in greater abundance in fetal and hypertrophied cardiomyocytes

impermeable to long chain free fatty acids (LCFA) which are oxidized via β-oxidation and the TCA cycle in the inner matrix (Fig. 5) and long chain fatty acids enter the mitochondrial matrix via a shuttle system. Carnitine palmitoyl-transferase 1 (CPT1) serves as a translocase for fatty acyl esters, by spanning the outer mitochondrial membrane and catalyzing acyl group transfer from Coenzyme A to carnitine in the intermembrane space. CPT2 converts fatty acyl-carnitine ester back into fatty acyl CoA in the matrix. Generally considered the rate-determining process in fatty acid oxidation by heart, CPT1 is regulated by malonyl CoA levels. Malonyl CoA is produced from acetyl CoA, via the action of acetyl CoA carboxylase 1 (ACC-1) at the mitochondrial membrane, which in turn is formed via β-oxidation and other sources of oxidative fuels within the matrix [54]. An ACC-2 isoform also exists in the cytosolic fraction, though in much lower abundance in cardiomyocytes. ACC-2 is associated with supplying malonyl CoA for fatty acid synthesis and chain elongation. The level of malonyl is also determined by its catabolism by malonyl CoA decarboxylase (MCD) [55]. Thus, the extent of long chain fatty acid oxidation by mitochondria is regulated by the activity of CPT1, which in the normal adult heart is primarily the muscle isoform.

In general, the fetal heart, and thus neonatal cardiomyocytes, does not utilize fatty acids as a major fuel for ATP production, as the fetus is provided a constant supply of glucose. Thus, enzymes of fatty acid oxidation, including CPT1, are expressed at very low levels until the immediate postnatal period, when they are rapidly induced [56, 57]. There are two structural genes that encode CPT1, the α or L (liver) gene and the β or M (muscle) gene [57–60]. These genes are differentially expressed among tissues that utilize fatty acids as fuel, and they are co-expressed in heart. The major cognate CPT1 enzymes encoded by these genes have different kinetic properties, such that differing relative expression levels among tissues are reflected in different kinetics [56, 60, 61]. Specifically, L-CPT1 is less sensitive to inhibition by malonyl CoA, and has a lower Km for carnitine. In neonates, the L form has been suggested to be 25 % of the total CPT1 activity in heart, while activity from the M form constitutes ~75 % of the activity [57, 62]. During the progression to maturity, this ratio changes such that only ~2 % of enzyme activity is from the L isoform of CPT1.

A teleological argument has been offered that the relatively high proportion of the L-type enzyme in early life permits activity at the low carnitine levels characteristic of neonatal myocardium and may be an adaptive response to the low fatty acid oxidation rates of the hypertrophied heart [36, 56, 57]. However, such suggestions are based on cell culture studies of unloaded cells, and usually neonatal cardiomyocytes that are predisposed to carbohydrate metabolism and not fat oxidation. However, electrical stimulation of neonatal cardiomyocytes does result in a redistribution of CPT1 isoforms [36]. Later investigation using Northern blot analysis showed no significant differences in transcript level of L-CPT1 between normal control human myocardium and in ten failing human heart samples ranging from idiopathic, peripartum, and ischemic cardiomyopathies [63]. However, with the development of specific antibodies, coupled to flux measurements, the first actual demonstration of elevated L-CPT1 enzyme protein content in hypertrophied myocardium of the pressure overloaded rat heart, with online measures of reduced flux through CPT1 in the mitochondria of the same beating hearts, was reported in 2007 [20].

Recently, a study in the ACC-1 deficient mouse heart indicated that a resulting elevation in fatty acid oxidation coincided with an attenuated hypertrophic response to left ventricular pressure overload [64]. However, baseline levels of malonyl CoA were only reduced by about 50 % despite the absence of ACC-2 and, in that particular study, baseline contributions of long chain fatty acids in the control animals were already a good deal lower than reported previously, and the ACC-2 KO hearts displayed only moderate levels of fatty acid oxidation in comparison to other reports for the contribution of long chain fatty acids to oxidative ATP production [20, 37, 40, 44, 47]. Other studies of the intact and in vivo heart indicate no direct relationship between malonyl CoA content and LCFA oxidation in hearts [65–67]. Studies demonstrating the absence of a link between malonyl CoA content and LCFA oxidation were performed on hearts with a predominantly normal distribution of M-CPT1 and L-CPT1 contents, and these findings suggest that L-CPT1 may be subject to additional levels of regulation that have yet to be fully identified. Indeed, Kim et al. report on a malonyl CoA-resistant level of palmitate oxidation in red versus white skeletal muscle preparations [68]. Therefore, post-translational modifications and as yet undetected levels of regulation beyond malonyl CoA must be considered in the intact functioning myocardium that may limit LCFA oxidation through L-CPT1.

Reductions in fatty acid oxidizing enzymes have been reported in hypertrophied myocardium and are associated with reduced activation of PPARα [35, 38]. However, a shift in CPT1 isoform distribution toward increased content of the liver (L-CPT1) isoform occurs concurrent with reduced flux of free fatty acid into oxidative metabolism [20]. The link between increased L-CPT1 and reduced palmitate oxidation in cardiac hypertrophy is consistent with the reduced fatty acid oxidation rates under conditions of limited carnitine availability in fetal and neonatal hearts and thus the reversion to a general programmatic shift toward fetal isoform expression [20, 36, 38, 50, 57, 60, 63]. However, from recent understanding of inducible gene expression and the realization that the metabolic changes in response to altered workload occur relatively early, preceding anatomical changes in the left ventricle, an early programmatic change in gene expression that induces the L isoform of CPT1 appears to be a more likely scenario.

Interestingly, in a rat model of acute L-CPT1 overexpression, induced by adenoviral-mediated delivery of an exogenous gene, a fourfold increase in L-CPT1 expression over control hearts was associated with a significant drop in fatty acid oxidation [69]. Despite the consideration that L-CPT1 is less sensitive to inhibition by malonyl CoA, the expression of L-CPT1 in the fetal heart, the hypertrophied heart, and hearts overexpressing L-CPT1 is consistently associated with lower oxidation of long chain fatty acids. The findings in the otherwise normal, L-CPT1 expressing rat heart strongly suggest that other factors in the intact myocardium regulate fatty acid oxidation in addition to, or instead of malonyl CoA. In fact, CPT1 activity, in tissues in which the L-CPT1 isoform predominates, is not directly proportional to protein content, and thus both hypertrophied hearts and L-CPT1 overexpressing hearts may also lose this proportionality [20, 68–70].

Reduced Triacylglyceride Content and Metabolism in Cardiac Hypertrophy

The content and turnover of the endogenous form of stored lipid in the cardiomyocyte, TAG, are reduced in hypertrophied hearts, as reported in animal model of pressure overload following TAC [47]. Interestingly, in a rat model of cardiac hypertrophy, TAG turnover is reduced by 40 %, a finding not obvious from TAG content alone [47]. While myocardial TAG turnover is a difficult parameter to monitor in humans, reduction in TAG content in the myocardium of heart failure patients has been shown to match these findings of prior animal studies [71], and has been related to potential deleterious elevations of physiologically active and potential lipotoxic acyl derivatives, such as ceramides and sphingosines [72–74]. However, the presence or absence of altered TAG content in heart failure patients does not indicate the extent of potential changes in TAG dynamics that are evident with ^{13}C NMR of experimental models [47, 75, 76].

Along with this finding of reduced TAG turnover, the contribution of TAG to β-oxidation, by supplying long chain fatty acids produced during TAG lipolysis, is also severely reduced in the hypertrophied heart [47]. Thus, a portion of the reduced fatty acid oxidation rates in hypertrophied myocardium is accountable from a severe reduction in the oxidation of endogenous TAG, which cannot be recruited by adrenergic challenge [47]. As an energy rich source, TAG becomes essentially unavailable to support oxidative ATP production in the mitochondria of the hypertrophied and failing heart. However, potential factors that could affect TAG pool size and synthesis, such as long chain fatty acid uptake into the cell and the different affinities of different fatty acid chain lengths for TAG formation, are not yet completely understood [51]. The potential also exists for any limitations in long chain fatty acid incorporation into the TAG pool to contribute to lipotoxicity, with implications for the pathogenesis of cardiomyopathy [47, 51, 71].

While activation of PPARα has long been associated with target gene expression for the enzymes catalyzing long chain fatty acid oxidation, PPARα is now also known to regulate expression of TAG synthases and lipase that contribute to the rates of TAG turnover in the cardiomyocyte [75]. The reduced activation level of PPAR in the hypertrophied heart is therefore implicated in this downregulation of TAG dynamics and subsequent contributions to β-oxidation.

In contrast to pressure overload hypertrophy, diabetic cardiomyopathy occurs under conditions of high mitochondrial oxidation of LCFA [2, 24–27]. This metabolic phenotype is implicated in the pathogenesis of diabetic cardiomyopathy, but also involves increased TAG storage and accelerated TAG turnover rates [77–81]. Thus, the imbalances in lipid utilization and storage persist in the diabetic heart, promoting formation of lipotoxic intermediates. Such potential contributions of altered lipid dynamics to lipotoxicity in the heart are considered further in a subsequent section.

Considerations of Lipotoxicity as Consequence of Altered Lipid Dynamics in the Heart

Palmitate is a common dietary LCFA that is readily oxidized by the heart, but in minimized cell culture models has been related to cardiotoxic effects and the induction of apoptosis [51, 82–84]. Studies supporting this notion of a direct, palmitate-induced apoptosis are limited to the extreme experimental conditions of unloaded, essentially metabolically inactive neonatal cardiomyocytes that are already predisposed to limited ability to oxidize long chain fatty acids, following incubation with high palmitate concentrations for time periods as extreme as 20–48 h [82, 83]. However, the activation of palmitate to palmitoyl CoA does provide substrate for ceramide production via the activity of the serine palmytoyltransferase. While induction of apoptosis directly by palmitate has never been demonstrated in the intact or in vivo heart, a diet high in saturated fats has been found to induce coincident elevations in ceramides and apoptotic cardiomyocytes in rat hearts, along with changes in PPAR-regulated gene expression [85]. But these static changes in ceramide content and apoptotic cell count occurred independent of function, and the complex relationship between fatty acid metabolism and cardiac performance in vivo remains poorly resolved. Non-ceramide-dependent apoptosis has also been reported in neonatal cardiomyocytes through the formation of reactive oxygen species [86]. On the other hand, the incidence of cardiomyopathy subsequent to the dysregulation of lipid dynamics, and not simple static metrics, in the heart remains as an undeniable link. Therefore, any dysregulation of the balance between LCFA oxidation in the mitochondria and storage into the TAG pool can then result in the accumulation of palmitate and palmitoyl-esters with potential deleterious, or at least physiological effects on the cardiomyocyte, as discussed below.

Consequently, TAG production from activated LCFA has been considered a protective mechanism to reduce the intracellular content of lipotoxic acyl-intermediates, like activated palmitate and their derivatives. However, disruption in the dynamics between lipogenesis and lipolysis of the TAG pool, relative to LCFA oxidation rates, can result in changes in TAG content. Static measures of TAG, particularly changes in TAG content, may then serve as an indicator of intracellular conditions that are conducive to the production of lipotoxic intermediates. Metabolites of these acyl-intermediates that are known to exert lipotoxic affects on the cardiomyocyte include diacylglyceride, which has been associated with cardiomyopathies, ceramide and its metabolite via the enzyme ceramidase, sphingosine [72, 87–90] (Fig. 6). These compounds influence myofilament phosphorylation with resulting increases in myofilament sensitivity to Ca^{2+} [91]. Ceramide is the product of serine palmitoyltransferase activity on palmitoyl CoA. The product of ceramidase, sphingosine, activates p21-activated kinase (PAK1) [92, 93] and recent studies have shown that PAK1 affects myofilament sensitivity to Ca^{2+}, possibly through PAK1 pathway-activated dephosphorylation of cTnI and cTnT [94, 95].

Inefficient Carbohydrate Metabolism in Heart Failure

While glycolytic activity is increased in the hypertrophied heart, oxidation of glycolytic end products through PDH does not keep pace with the increased glycolytic activity [34, 96–98]. However, some of this mismatch between glycolytic flux and pyruvate oxidation via PDH in hypertrophied myocardium is accommodated by the entry of pyruvate into the second span of the TCA cycle, as malate from the carboxylation of pyruvate through the cytosolic, NADP+-dependent malic enzyme-1 (ME-1) (Fig. 7) [20, 40]. Stable isotope studies have

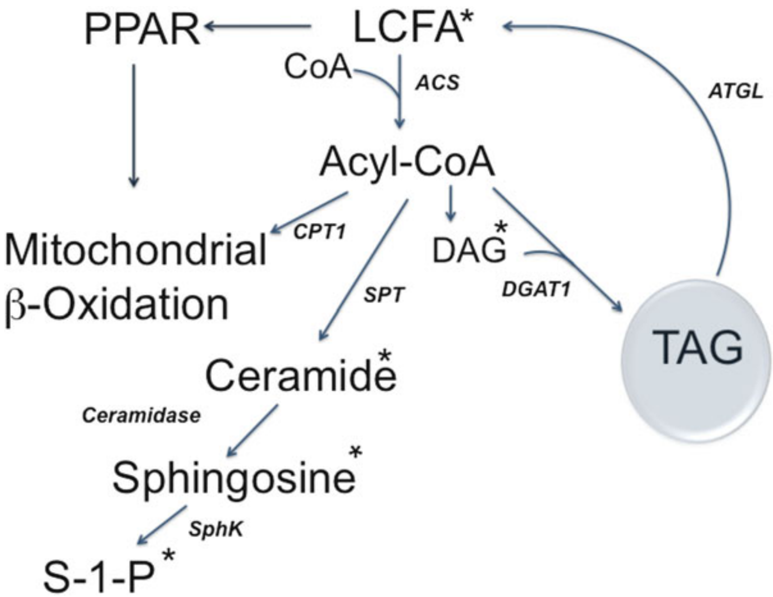

Fig. 6 Metabolic fates of long chain fatty acids in the cardiomyocyte. *Asterisk* indicates active intermediates that induce metabolic signaling pathways with potential downstream effects on nuclear receptor activation, lipotoxicity, apoptosis, Ca^{++} sensitivity at the sarcomere, PKC signaling, and insulin sensitivity. *LCFA* long chain fatty acids, *PPAR* peroxisome proliferator-activated receptor, *DAG* diacylglyceride, *TAG* triacylglyceride, *S-1-P* sphingosine-1-phosphate, *ACS* acyl CoA synthase, *DAGT1* diacylglyceride transferase-1, *SPT* serine palmitoyltransferase, *CPT1* carnitine palmitoyltransferase 1, *ATGL* adipose triglyceride lipase, *SphK* sphingosine kinase

demonstrated entry of isotopically enriched pyruvate from [1,6-$^{13}C_2$] glucose into oxidative metabolism in the absence of direct enrichment of acetyl CoA via PDH with corresponding increases in myocardial malate content [20, 40]. Yet radiotracer approaches that would rely on the trapping of $^{14}CO_2$ gas, released from oxidation of ^{14}C-1 labeled glucose, as an indicator of ^{14}C labeled pyruvate, are insensitive to both $^{14}CO_2$ fixation and any measure of anaplerosis. Indeed, an increase in CO_2 fixation by such carboxylation reactions could be mistakenly attributed to further reductions in pyruvate oxidation. Thus, the fate of glycolytic end products is associated with limited oxidation of pyruvate through PDH, with increased carboxylation of pyruvate due to an increase in ME-1 expression and protein content.

The energetic consequence of pyruvate carboxylation and production of malate, which then enters the second span of the TCA cycle, as opposed to the decarboxylation of pyruvate via PDH is the consumption of pyruvate in the absence of NADH generation. Oxidation of pyruvate through PDH in the normal heart produces the two-carbon acetyl CoA that enters the TCA cycle via citrate synthase. The process of converting pyruvate into acetyl CoA directs this carbon mass from pyruvate metabolism through downstream reactions in the TCA cycle that produce NADH, thereby providing reducing equivalents for electron transport and coupling to

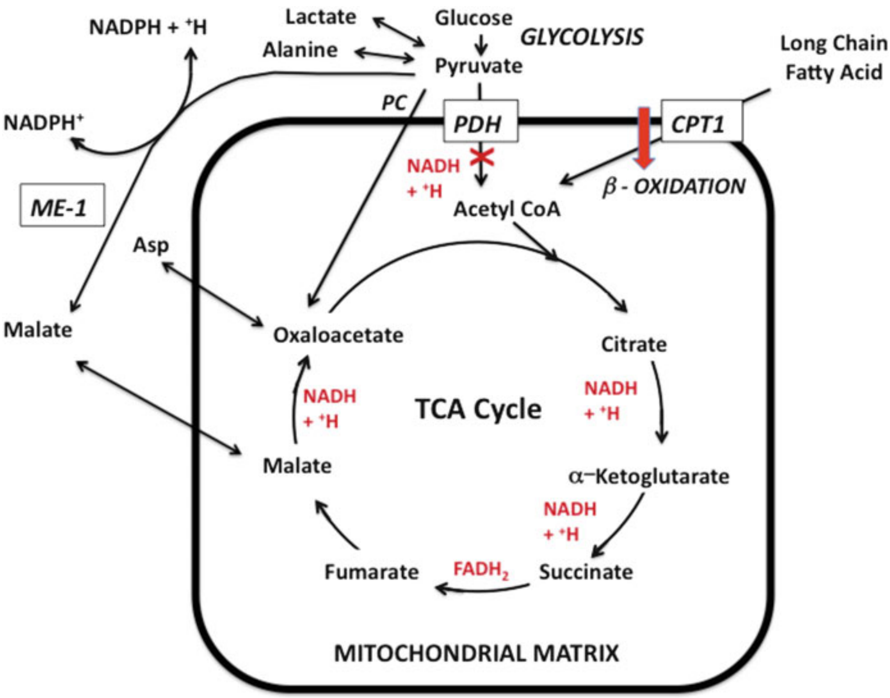

Fig. 7 Carbon unit entry into the TCA cycle from carbohydrates and fatty acids is altered in hypertrophied and failing hearts. Flux through CPT1 is reduced (*red arrow*), resulting in lower fatty acid oxidation rates and less efficient ATP production in hypertrophied hearts. Oxidation and decarboxylation of pyruvate from glycolysis through pyruvate dehydrogenase (PDH) are slowed and do not keep pace with increased glycolytic activity in hypertrophied myocardium. Baseline production of oxaloacetate is generated through the transamination reaction involving aspartate. Carboxylation of pyruvate via malic enzyme-1 (ME-1) is increased, with the production of malate bypassing three of the four reactions of the TCA cycle that produce reducing equivalents to form NADH and the production of $FADH_2$. The carboxylation of pyruvate by ME-1 also consumes NADPH. Conversion of pyruvate to oxaloacetate via pyruvate carboxylase (PC) is relatively inactive in heart muscle and is not increased in hypertrophy. Increased expression and activity of ME-1 in hypertrophied hearts catalyzes inefficient utilization of carbohydrate for ATP production and consumes NADPH required for glutathione reduction and maintenance of redox balance in the cell

oxidative phosphorylation for mitochondrial ATP production. However, in hypertrophied myocardium, the conversion of pyruvate to malate via ME-1 transfers these carbon units to the second span of the TCA cycle, bypassing the NADH and $FADH_2$ production that would otherwise be supported by pyruvate decarboxylation via PDH to produce acetyl CoA and the downstream dehydrogenase reactions of the TCA cycle (Fig. 7). The energetic consequence of this increase in ME-1 catalyzed pyruvate carboxylation is the inefficient utilization and entry of glucose metabolites into the oxidative metabolism of the mitochondria and loss of potential NADH production [40, 48]. Reducing flux through ME-1 and restoring pyruvate decarboxylation through PDH result in improved contractility in the hypertrophied heart, perhaps as a consequence of countering the maladaptive influence of ME-1 expression and activity on ATP synthesis and redox state [40, 48].

The anabolic formation of four-carbon intermediates of the second span of the TCA cycle is referred to as anaplerosis [99]. Normal anaplerosis provides necessary carbon mass within the second span of the TCA cycle [100, 101], and becomes critical for providing substrate for the condensation reaction of citrate synthase in the setting of high acetyl CoA levels that can potentially limit CoA availability for the rate-limiting reaction catalyzed by α-ketoglutarate (αKG) dehydrogenase [102–104]. Thus, under conditions such as ketosis, anaplerosis is critical to maintaining TCA cycle flux [102, 105, 106]. Indeed, elevated pyruvate carboxylation has been reported to be beneficial to the heart during cardio-pulmonary bypass and reperfusion [107].

In hypertrophied hearts displaying reduced in fatty acid oxidation and lack of coupling between glycolytic flux and PDH activity, increased anaplerosis then becomes a compensatory mechanism for both pyruvate oxidation and maintaining carbon mass within the TCA cycle [20, 40]. However, the compensatory recruitment of anaplerosis may come at the cost of other less beneficial adjustments in metabolism. In fact, reducing pyruvate carboxylation via ME for anaplerosis, via increased pyruvate oxidation through PDH, can restore TAG levels and improved contractility [40].

While relatively inactive in the normal heart, in hypertrophied hearts elevated ME-1 activity converts pyruvate to malate, in the process consuming NADPH:

$$\text{Pyruvate} + CO_2 + \text{NADPH} + H^+ \leftrightarrow \text{Malate} + \text{NADP}^+$$

The consumption of increased ME flux may limit other NADPH-dependent reactions [48]. For example, regulation of redox state is affected by the ratio of oxidized to reduced glutathione (GSSG/GSH) [108–110]. NADPH maintains glutathione in the reduced state. Thus, redox balance may be adversely affected by upregulated ME expression in hearts, which can produce contractile dysfunction due to oxidant stress [108]. While sources of mitochondrial and cytosolic NADPH remain somewhat controversial, work by Jain et al. would indicate that anaplerotic activity through the cytosolic NADPH-dependent malic enzyme is maladaptive in limiting cytosolic NADPH for the reduction of glutathione [109, 110] (Fig. 7). Consistent with the notion that ME-1 upregulation in cardiac hypertrophy is a metabolic maladaptation, pharmacologic activation of PDH in the hypertrophied heart, to compete against ME-1-catalyzed pyruvate carboxylation, improved contractility, as determined by the rate of pressure development in the left ventricle, dP/dt [40].

In other tissues, ME-1 is characterized as a lipogenic enzyme, via NADPH production through the reverse reaction converting malate to pyruvate. ME expression in adipocytes is induced by high fat diet [111]. Since NADPH is also required for the reduction of dihydroxyacetone phosphate in the cytosol for TAG synthesis, NADPH consumption by the activity of ME-1 may also contribute to the observed reduction in endogenous triglyceride stores in hypertrophied myocardium [40, 47]. Interestingly, though the exact mechanism has yet to be fully elucidated, the same study of pharmacologic activation of PDH to compete with ME-1 activity for pyruvate also normalized TAG levels in hypertrophied hearts [40].

Role of the Mitochondrial Membrane in Metabolic Communication and Transduction of Pathophysiological State

Shuttling of Reducing Equivalents for Mitochondrial Oxidative Energy Production

The principal outcome of the intermediary metabolic pathways in fueling oxidative ATP production is the generation of reducing equivalents, which are carried by the positively charged, acceptor molecule, nicotinamide adenine dinucleotide (NAD^+). In the mitochondrial matrix, carbon-based fuels are progressively oxidized, being stripped of electrons, and the resulting reduction of the acceptor, NAD^+, forms NADH and a proton (^+H). Other acceptor molecules, such as flavin adenine dinucleotide (FAD) can be linked to oxidative reactions, as well. These reducing equivalents are then carried to the electron transport chain to supply the proton-motive force that then drives the F1-ATPase in the direction of ATP synthesis.

However, nonoxidative, glycolytic metabolism also produces NADH in the cytosolic compartment. Although glycolysis produces a relatively small fraction of ATP in the myocyte compared to the mitochondria, the production of NADH can have profound influence on not only the balance between the metabolic state of the mitochondrial and cytosolic compartments, but the pathophysiological state of the myocyte is also affected. As the rate-limiting glycolytic enzymes require NAD^+ as a cofactor, oxidation of NADH must occur to maintain glycolytic flux. This regeneration of cytosolic NAD^+ can occur either nonoxidatively, through the production of lactate from pyruvate via the lactate dehydrogenase reaction, or oxidatively, by shuttling the reducing equivalents from cytosolic NADH to the mitochondrial matrix via the malate–aspartate shuttle (see Fig. 8).

Of the shuttle mechanisms for cytosolic NADH, the malate–aspartate shuttle predominates in the heart with minimal activity from the glycerophosphate shuttle [112–115]. After birth, the myocardium becomes less reliant on glycolytic metabolism and expression of the proteins for these shuttles is reduced [114–116]. The function of the 2-oxoglutarate (α-ketoglutarate)–malate carrier protein (OMC) on the mitochondrial membrane is important to the maintenance of cytosolic NADH handling and holds particular importance under the conditions of increased glucose metabolism in hypertrophied myocardium [20, 50, 115].

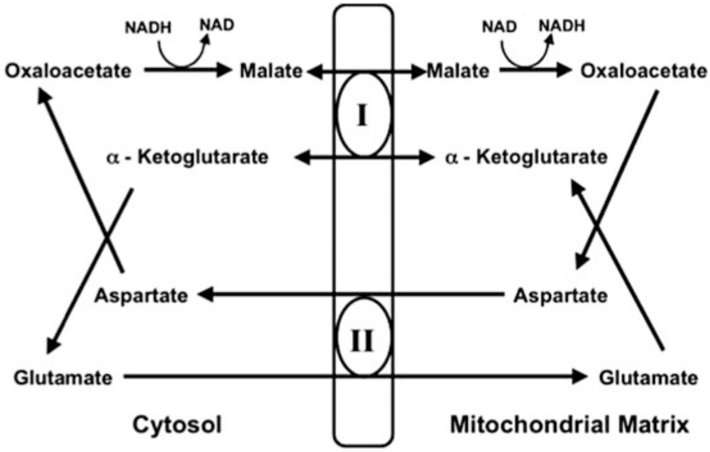

Fig. 8 Transfer of reducing equivalents from the cytosol to mitochondria via intermediate exchange across the malate–aspartate shuttle. High cytosolic NADH drives coordinated flux through the two transporters in the net forward direction. I, denotes the reversible α-ketoglutarate (2-oxoglutarate)–malate carrier (OMC). II, denotes the unidirectional aspartate–glutamate carrier

While it is generally acknowledged that cytosolic NADH content of the myocyte is difficult to accurately measure due to the high NADH content of the mitochondria, Scholz et al. demonstrated that the distinctions in cytosolic redox state can be assessed using glycerol-3-phosphate levels relative to dihydroxyacetone phosphate as an indicator of the cytosolic $NADH/NAD^+$ [117]. In this manner, Scholz et al. [117] were able to confirm the distinctly different cytosolic redox state condition that is to be expected between isolated hearts supplied pyruvate versus hearts supplied lactate. Briefly, the increase in cytosolic lactate concentration establishes a metabolic equilibrium with the reverse flux through lactate dehydrogenase producing a higher baseline level of $NADH/NAD^+$ in the cytosol. This lactate-induced shift in cytosolic redox state can stimulate malate–aspartate shuttle activity, due to elevated $NADH/NAD^+$ in the cytosol [118].

Intermediate Exchange Across the Mitochondrial Membrane and Subcellular Communication

Another important consideration is the competition between the exchange of α-ketoglutarate and malate across the mitochondrial membrane and the α-ketoglutarate dehydrogenase enzyme of the TCA cycle for α-ketoglutarate (also referred to as 2-oxoglutarate) as a substrate [118, 119]. The balance between these two competing processes is central to linking energy metabolism to the metabolic

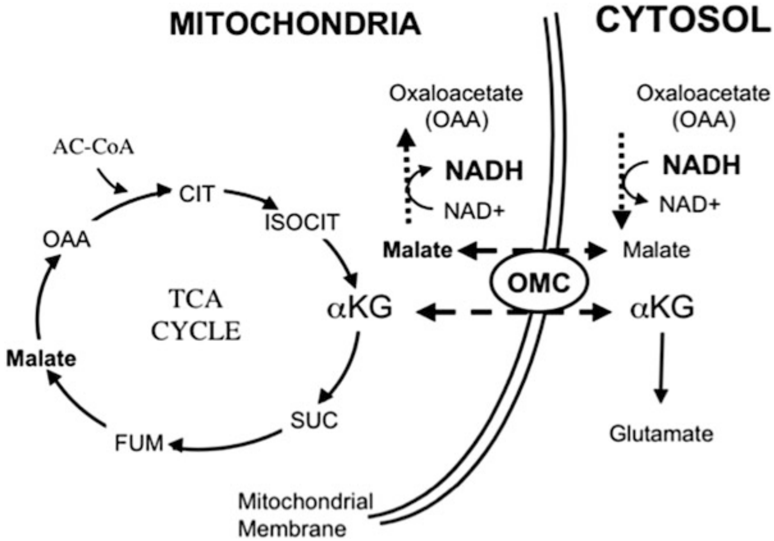

Fig. 9 The malate–aspartate shuttle and the flux through the TCA cycle are coordinated through competition between α-ketoglutarate (αKG) efflux from the mitochondria through αKG OMC and αKG oxidation to succinate (SUC) via the NAD^+-dependent, calcium-activated αKG dehydrogenase

state of the cytosol (Fig. 9). The exchange of α-ketoglutarate and malate is involved in transferring reducing equivalents generated in the cytosol to the mitochondria for oxidative energy production, while the α-ketoglutarate dehydrogenase is a calcium-activated, mitochondrial enzyme of the TCA cycle. This exchange occurs via the OMC on the mitochondrial membrane.

Importantly, α-ketoglutarate dehydrogenase is also very sensitive to the mitochondrial $NADH/NAD^+$, as the enzyme-catalyzed reaction utilizes NAD^+ as a cofactor and produces NADH. The mitochondrial dehydrogenases are also Ca^{++} activated, and thus the activity of α-ketoglutarate dehydrogenase competes with OMC activity for its substrate, α-ketoglutarate. In this manner, the rates of the mitochondrial dehydrogenases, in response to mitochondrial $NADH/NAD^+$ and Ca^{++} levels, and OMC activity, are coordinated to the cytosolic $NADH/NAD^+$ which is also linked to cytosolic Ca^{++} levels, providing a homeostatic mechanism for coordinating the contractile state of the cardiomyocyte with flux through the TCA cycle for NADH production leading to oxidative ATP synthesis [118–121]. Thus, the rate of exchange of metabolic intermediates between the mitochondrial matrix and cytosolic compartment of the cell enables the transduction of pathophysiological state between compartments to coordinate oxidative activity in the mitochondrial matrix with the functional state of the cell.

^{13}C NMR detection of flux through the oxidative intermediary pathways in the intact heart relies on the observation of the large pool of glutamate that becomes labeled through exchange with the TCA cycle. The enzymes for the TCA cycle are inside the mitochondria, while at least 90 % of the glutamate is cytosolic [122–124]. Exchange of ^{13}C enriched TCA cycle intermediates with the NMR-observed glutamate pool in the cytosol is achieved by the coordinated activity of two types of carrier proteins that span the mitochondrial membrane. One carrier is responsible for the reversible exchange of malate and αKG. The other carrier is unidirectional by virtue of being electrogenic, and exchanges cytosolic glutamate for mitochondrial aspartate. This transport system is the malate–aspartate shuttle, and, as discussed above, transports reducing equivalents cytosol to the mitochondrial matrix. At high cytosolic redox state ($NADH/NAD^+$), the two carriers are driven in the forward direction, increasing exchange between αKG and glutamate [50, 118].

NADH Transfer into Mitochondria in Hypertrophied Hearts

The increased reliance on glycolytic metabolism by hypertrophied hearts implies that the heart also becomes increasingly dependent on the oxidation of reducing equivalents produced in the cytosol. The neonatal heart, as it develops to adult, shows reduced expression of both the malate–aspartate shuttle and glycerophosphate shuttle proteins, with minimal contributions from the glycerophosphate shuttle [114]. As discussed above, both shuttles carry reducing equivalents, produced by glycolysis in the cytosol and accepted by NAD^+ across the NADH-impermeable membrane of the mitochondria for oxidative production of chemical energy. This link between the more glycolytic metabolism of the neonatal heart and the expression and activity of these transporters is clear, as the immature myocardium relies heavily on carbohydrates for fueling ATP production. With development, the myocardium becomes less reliant on glycolysis and the expression and activity of the exchange proteins is reduced [114–116].

However, in the hypertrophied myocardium, the activity but not the expression of the OMC, also referred to as the αKG–malate exchanger, is increased in the intact heart [50, 115]. In a study of the intact, hypertrophied rat heart, the activity of OMC was dramatically increased upon pressure overload to the heart, prior to any initial cardiac hypertrophy, and returned to baseline upon evidence of significant LVH [117]. The findings of that study suggest that the immediate stress of pressure overload elevates cytosolic NADH, and upon the initial development of hypertrophy, prior to functional decline, the metabolic stress is transiently alleviated by hypertrophy with OMC activity returned to baseline levels. Subsequently, as the pressure overloaded heart decompensates, the OMC activity rises again. This response of OMC to pressure overload is quite likely induced by an elevated cytosolic $NADH/NAD^+$ ratio in the cytosol due to increased glycolytic activity in cardiac hypertrophy, as discussed in the previous sections. However, the response is purely activity-driven by metabolic regulation, inducing intermediate exchange across the mitochondrial members, because OMC content over the course of the hypertrophic either remains unchanged from the initial induction of the hypertrophic stimulus to the point of compensatory hypertrophy or displays a modest, initial drop [50, 115].

Metabolite exchange across OMC also enables the influx and/or efflux of carbon mass through the first span of the TCA cycle, serving to maintain equilibrium in the carbon mass balance between the first and the second spans of the cycle. The ability to exchange carbon mass, through metabolic intermediate transfer across the mitochondrial membrane, maintains the equilibrium condition required for net forward flux through the TCA, especially in response to the elevated influx of carbon mass in the hypertrophied heart due to increased malate production via ME-1, as discussed in previous sections of this chapter [20, 40].

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Cytoskeletal Nuclear Links in the Cardiomyocyte

Elizabeth McNally

Cytoskeletal Crosslinkers and Mechanical Stress: An Overview

The cytoskeleton maintains the internal architecture of the cytoplasm and also transmits signals to the nucleus while also coordinating information from surrounding matrix and neighboring cells. The myocardium is composed of cardiomyocytes as well as cardiac fibroblasts in addition to the vascular structures that supply oxygen and nutrients to the myocardium. The terminally differentiated mature cardiomyocyte is a binucleate structure surrounded by a laminin–collagen-enriched extracellular matrix. Both cardiac fibroblasts and cardiomyocytes contribute to the formation of this matrix. The cardiomyocyte connects to the extracellular matrix through costameres. Costameres are rib-like structure along the surface of striated muscle cells and are found in both skeletal myofibers and cardiomyocytes [1, 2]. Costameres are in register with the Z bands, the electron dense regions that anchor actin filaments and highly concentrated with action binding proteins (Fig. 1). Therefore, costameres are the sarcolemmal reflection of the underlying intracytoplasmic myofilament structures and positioned to transmit from the Z band to the membrane and extracellular matrix.

Cardiomyocytes, like nearly all animal cells, possess an intrinsic stiffness. In most animal cells, cell stiffness is attributed to the actin-based cytoskeleton, the microtubule network, and intermediate filaments. In addition to these elements, the myofibrillar proteins themselves contribute significantly to the cellular stiffness in cardiomyocytes. Immediately under the plasma membrane in the cytoplasm is the actin cytoskeleton that includes non-sarcomeric γ-actin. The microtubule network densely surrounds the nucleus and is quite dynamic, responding to contraction,

E. McNally, M.D., Ph.D. (✉)
Departments of Medicine and Human Genetics, The University of Chicago,
5841 S. Maryland MC6088, Chicago, IL 60637, USA
e-mail: emcnally@uchicago.edu

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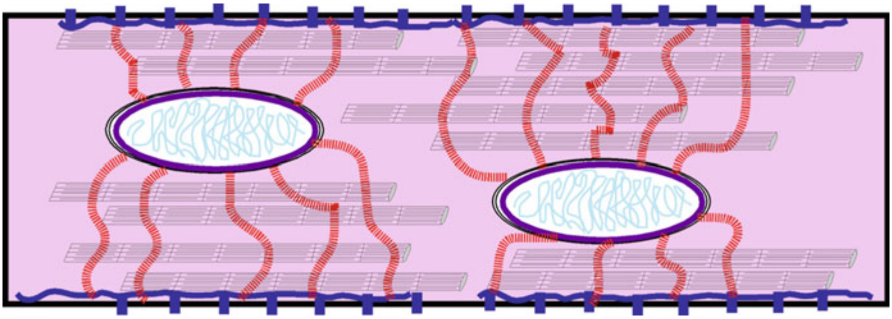

Fig. 1 Cardiomyocyte structural networks. The mature cardiomyocyte is a binucleate terminally differentiated cell. The cytoplasm contains longitudinally arranged myofilaments (*gray striations*). The nuclear membrane is reinforced by the nuclear lamina (*purple line*) and interfaces with DNA within the nucleus (*light blue lines*). The intermediate filament network of desmin proteins (*red dashed lines*) runs through the myofilaments and connects to both the nucleus and the plasma membrane. The cytoskeletal actin network connects to the dystrophin complex through costameric structures (*blue*). All of these components contribute to the cardiomyocyte stiffness

hypertrophy, and heart failure [3]. Intermediate filaments such as desmin provide interweave between myofilaments in addition to providing support to the actin cytoskeleton and reinforcing cytoplasmic and nuclear connections [4, 5].

Desmin is a type III intermediate filament protein and was the first intermediate filament protein linked to inherited cardiomyopathy [6]. Ablation of the desmin gene in mice produces cardiomyopathy and skeletal muscle disease associated with early lethality [7, 8]. Loss of desmin disrupts normal costameric patterns and the subsarcolemmal spectrin and cytokeratin-containing networks [9]. Although desmin is the dominant intermediate filament protein of striated muscle, synemin (also known as desmuslin) and paranemin are also present and can form copolymers with desmin. The intermediate filament network also serves as a scaffold for anchoring signaling molecules, and thus is poised to transmit mechanotransduction [10].

The filamentous networks are also interconnected through crosslinking proteins such as the plakins. The plakin family includes plectin, microtubular actin crosslinking factor (ACF7, MACF), bullous phemphagoid antigen (BPAG, also known as dystonin), and desmoplakin [11]. In the heart, these elements, particularly plectin, are concentrated at the intercalated disk. In humans, mutations in these genes lead to a combination of epidermal, cardiac, and skeletal muscle defects arising from the expression of these genes in skin, heart, and muscle, respectively. Ablation of plectin in the mouse leads to early death from skin blistering but also includes disrupted cytoarchitecture in heart and muscle [12]. As each of these organs or tissues, skin, muscle, and heart, is faced with substantial mechanical deformation to accommodate its function, plectin, as a cellular crosslinker, is an important component of mechanotransduction.

The Nuclear Membrane

Cardiomyocytes, like other cells, have a nuclear membrane composed of two membranes, the inner and outer nuclear membranes. The eukaryotic nuclear membrane is defined by this double membrane structure and between these membranes is the perinuclear space, typically ranging in size from 30 to 50 nm [13]. The inner nuclear membrane faces the nucleoplasm and the outer nuclear membrane faces the cytoplasm. The nuclear membrane is discontinuous and interrupted by nuclear pores that mediate nuclear–cytoplasmic transport [14]. Nuclear pores contain a diverse array of proteins that are thought to mediate transport. The outer nuclear membrane is contiguous with the endoplasmic reticulum and secretory system [15]. In the cardiomyocyte, this secretory system is also contiguous with the sarcoplasmic reticulum. In the cardiomyocyte, sarcomeres abut the outer nuclear membrane and are linked via the intermediate filament protein desmin. The inner nuclear membrane is lined by electron dense material known as the nuclear lamina. The lamina is roughly 20–30 nm in thickness and provides stiffness to the membrane, and its primary protein components are the lamins, A/C and B. The nuclear lamina binds directly to chromatin, especially heterochromatin that is enriched at the nuclear membrane.

Lamins and the Nuclear Lamina

The nuclear lamins are type V intermediate filament proteins [16]. Lamins A and C are produced from a single gene, *LMNA*. Lamins A and C are identical along their first 566 amino acids but differ at their carboxy-terminus [17] (Fig. 2). Prelamin A is 664 amino acids in length. The longer carboxy-terminal extension on lamin A provides a CAAX sequence that is farnesylated and then cleaved, resulting in a 645-amino acid mature lamin A protein. These posttranslational modifications allow lamin A to fix to the nuclear membrane. In contrast, lamin C is 572 amino acids in length and does not undergo this processing. Lamin C adheres to the nuclear membrane primarily through its interactions with other lamins, namely lamin A. The first 33 amino acids of lamin A/C encode a short head-like domain. The central rod domain of lamin A/C is defined by amino acids 33–383, and residues 430–545 of lamin A/C form the globular Ig-like fold [18]. The nuclear localization signal falls between the central rod domain and the Ig fold.

Lamins A/C, like other intermediate filament proteins, dimerize as parallel structures [19]. The central rod of lamin is sufficient to dimerize. Lamin dimers then form head to tail association with other dimers. Antiparallel arrangement of the oligomerized dimers leads to the formation of intermediate filament proteins with approximately 25 nm periodicity. Lamin A/C assembly requires the rod domain and region of the short head region [20, 21]. Lamins A/C are enriched at the nuclear membrane of post-mitotic terminally differentiated cells including cardiomyocytes

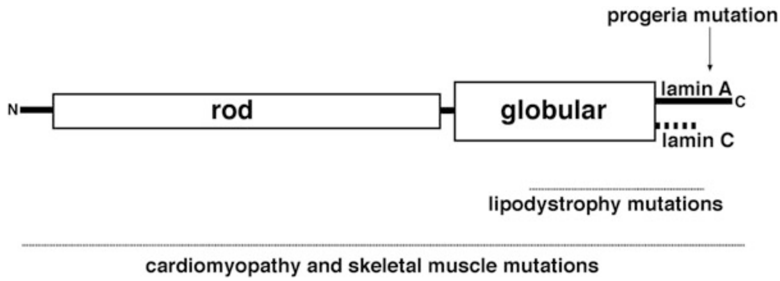

Fig. 2 Lamin A/C structure. Lamins A and C are produced from one gene, *LMNA*. Alternative splicing at the 3′ end produces alternative carboxy-termini forming either lamin A or lamin C. Hutchinson Gilford Progeria is associated with a point mutation at the 3′ end that disrupts the processing of lamin A, leading to an increase in unprocessed prelamin A, sometimes referred to as progerin. More than 300 point mutations along the length of *LMNA* contribute to cardiomyopathy and/or muscular dystrophy. Mutations in specific regions of the globular domain lead to Dunnigan partial lipodystrophy. *LMNA* mutations are associated with many different inherited diseases, but the most common inherited disease associated with *LMNA* mutations is inherited cardiomyopathy that is commonly associated with cardiac conduction system disease

and skeletal myofibers. Lamin B is produced from two genes and is constitutively expressed, and is the major nuclear laminar protein in dividing cells. Lamin A/C binds a series of molecules important for nuclear structure and function [22]. Included as lamin A/C binding partners are lamin B, nesprins, SUN domain-containing proteins, emerin, lamin-associated proteins (also known as LAPs), nuclear pore proteins, and chromatin.

The LINC Complex

The LInks the Nucleus to the Cytoplasm (LINC) complex was described as a transnuclear membrane complex that spans from the nucleoplasm through both the inner and the outer nuclear membranes and to cytoplasmic elements [23] (Fig. 3). The SUN domain-containing proteins SUN1 and SUN2 (so named for their presence in Sad1-UNc84 proteins) embed in both the inner and the outer nuclear membranes and traverse the perinuclear space structure [24]. In *C. elegans*, the UNC-84 gene was first implicated in nuclear positioning [25, 26]. The SUN domain is approximately 200 amino acids in length and, in the broadly expressed mammalian orthologs SUN1 and SUN2, the SUN domain is found at the carboxy-terminus. SUN proteins bind to KASH domains; the KASH domain (named for its presence in Klarsicht, ANC-1, and Syne Homology protein) is found in the nesprin proteins [27–29]. The KASH domain is defined by a single transmembrane pass followed by a region of approximately 30 amino acids that sits within the perinuclear space. LINC complex proteins, the SUNs and nesprins, not only are important for nuclear positioning but likely transmit mechanical signals between

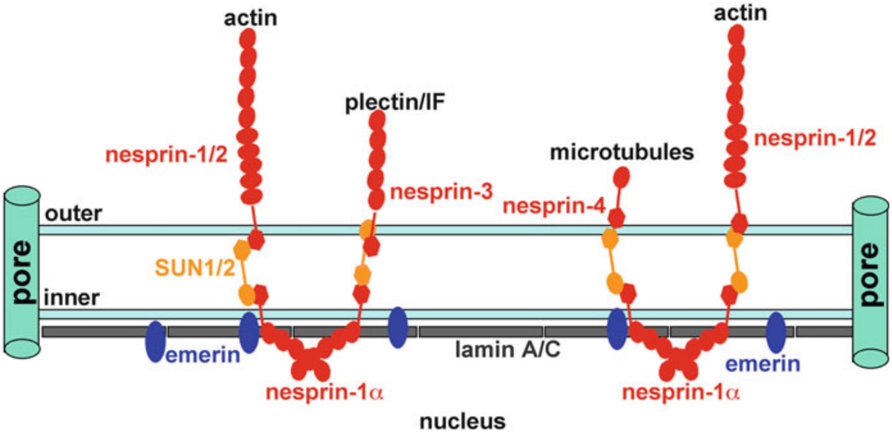

Fig. 3 The LINC complex LInks the Cytoplasm to the Nucleus. The LINC complex is present in many different cell types. The central portion of the LINC complex centers on the SUN–nesprin interaction through the KASH domain. This complex spans both the outer and the inner nuclear membranes. There are at least four nesprins, defined by the nuclear envelope spectrin repeat-containing proteins. Nesprin-1 and nesprin-2 can exist as extremely large forms on the outer nuclear membrane and connect to actin though an amino-terminal calponin homology domain. Nesprin-3 and nesprin-4 form links to plectin and to microtubules, respectively. Nesprin-1 and nesprin-2 mutations have been described in dilated cardiomyopathy

the cytoplasm and the nucleus. Recently, the LINC complex interaction was refined to indicate that trimers form from the SUN domains and it is this trimerized structure that directly interacts with the KASH domain [30, 31]. KASH domain-containing nesprins have also been implicated in nuclear size [32].

Nesprins

Nesprins are nuclear envelope spectrin repeat-containing proteins. Originally referred to as Syne (synaptic nuclear envelope protein) or Myne (myocyte nuclear envelope protein), nesprin-1 and -2 are found in heart and muscle [29, 33]. Both nesprin-1 and -2 undergo extensive splicing to result in an array of different proteins that vary considerably in size and position within the cell [34]. The longest forms of nesprin-1 and -2, called giant nesprin, contain an amino-terminal actin binding domain, a central region with spectrin repeats, and a carboxyl-terminal KASH domain. The giant form of nesprin-1 and -2 is approximately 1 MDa in size. The long forms of nesprin are concentrated on the outer nuclear membrane and their spectrin repeat domains are cytoplasmic (Fig. 3). The carboxy-terminal KASH domains found at the carboxy-terminus anchor into the nuclear envelope. In heart and muscle, specific short forms of nesprin-1 are highly expressed, and the short form, nesprin-1α, does not contain an actin binding domain [33, 35]. Nesprin-1α is

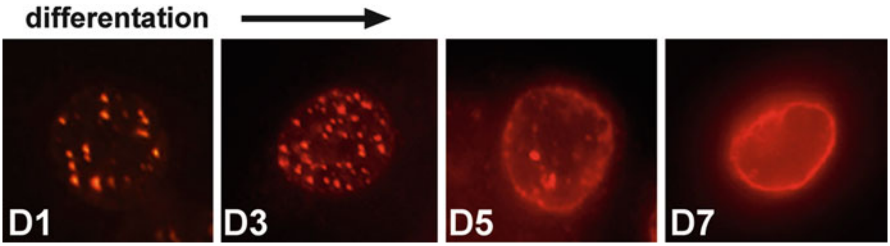

Fig. 4 Nuclear membrane maturation occurs concomitant with cellular differentiation. Each frame shows a single nucleus from the myogenic C2C12 cell induced to undergo differentiation. An antibody that detects nesprin-1α is shown in *red*. Nesprin-1α immunoreactivity is seen on day 1 of differentiation (D1), a time point when cells are just withdrawing from cell cycle and beginning to undergo terminal differentiation. With progressive maturation at day 3 (D3), nesprin-1α expression increases and nesprin-1α foci are seen within nuclei. By day 5 (D5) of differentiation, around the time when myofilament proteins are being expressed, the mature nuclei membrane begins to form and nesprin-1α staining is seen at the nuclear periphery. At day 7 (D7) of differentiation, when sarcomeres are formed, a mature nuclear membrane is seen showing nesprin-1α at the nuclear membrane rim. A similar pattern is seen for lamin A/C immunoreactivity (not shown), and this nuclear rim pattern is what is seen in mature cardiomyocytes and skeletal myofibers

anchored in the inner nuclear membrane and its intracellular localization is dependent on lamin A [36]. The spectrin repeats within nesprins dimerize, and the short forms of nesprin-1, when dimerized, have high affinity for inner nuclear proteins, lamin A/C and emerin [37, 38]. The enrichment of the short form of nesprin-1α in the inner nuclear membrane of cardiomyocytes and skeletal muscle cells demonstrates that the nuclear membrane proteins of the striated muscle cells have unique protein components not shared by other cells.

Nesprin-1 and -2, SUN1 and SUN2, and lamin A/C are all found at the nuclear membrane of cardiomyocytes and skeletal muscle cells [39, 40]. During muscle differentiation modeled in cell culture using the myogenic C2C12 cell line, the mature nuclear ring forms concomitant with development and cell specification [33]. In proliferating cells like myoblasts, there is comparatively lower expression of nesprin and lamin A/C. Once induced to differentiate, nesprin and lamin A/C are upregulated. At this point during differentiation foci of nesprin-1 and lamin A/C are seen within nuclei (Fig. 4). These foci are not clearly membrane-associated since they are internal where they may interact with intranuclear contents. Once mature myotubes have formed, the ring structure is evident at the nuclear rim and this mirrors what is seen in the mature cardiomyocyte or skeletal myofiber nucleus. This maturation of the nuclear rim occurs concomitant with cell commitment and determination. The lack of a suitable cell model for cardiomyocyte differentiation limits making this same observation in cardiomyocytes. But it is expected that cardiomyocytes follow a similar course.

Mutations in Nuclear Membrane Protein Genes Cause Heart and Skeletal Muscle Myopathy

Mutations in *LMNA*, the gene encoding lamins A and C, are a relatively common genetic cause of dilated cardiomyopathy, accounting for 5–8 % of all familial dilated cardiomyopathy [41, 42]. *LMNA* mutations may cause a "pure" dilated cardiomyopathy with four-chamber enlargement and systolic dysfunction. Congestive heart failure with *LMNA* mutations may necessitate cardiac transplantation. Cardiomyopathy-associated *LMNA* mutations may also cause cardiac conduction system disease that includes atrial fibrillation, sinus node dysfunction leading to bradycardia, slow conduction across the atrioventricular node, and ventricular tachyarrhythmias [43]. Cardiac conduction system disease may be the first clinical evidence of an *LMNA* mutation and may develop prior to the onset of a dilated and dysfunctional left ventricle [44]. In addition to causing dilated cardiomyopathy and conduction system disease, *LMNA* mutations also cause skeletal myopathy that can vary in presentation from congenital myopathy to later onset limb girdle muscular dystrophy [45, 46].

Emery Dreifuss Muscular Dystrophy (EDMD) was first described as an X-linked recessive disorder that affects males with muscle weakness, cardiac conduction system disease, and cardiomyopathy [47]. One of the more unique features of EDMD is the association of muscle contractures, or the appearance of tonically contracted muscle. These contractures do not derive so much from underlying muscle disease, but rather derive mainly from tendon hypercontracture since tendon releases surgery can relieve the symptoms. As such, the contractures associated with EDMD, typically affecting the Achilles and elbow tendons, arise from the role of nuclear membrane proteins at the myotendinous junction as well as in the tendons. This may reflect lamin A/C function in fibroblasts, a cellular component that may also be important for the heart given the major contribution of fibroblasts to its cellular composition. Mutations in the emerin gene on the X-chromosome were the first nuclear membrane protein gene implicated in cardiac and skeletal myopathy [48]. Emerin is a 34 kDa, single pass transmembrane protein that is concentrated on the inner nuclear membrane. Emerin harbors an LEM domain, so named for its presence in MAN1, emerin, and LAP [49]. LEM domains bind directly to BAF, a small protein that oligomerizes and binds directly to chromatin [50]. This link is one of the several between the inner nuclear membrane and DNA within the nucleus (see below). X-linked emerin mutations are loss of function as well as point mutations that may generate defective connections within the inner nuclear membrane [51]. Autosomal dominant EDMD linked to *LMNA* mutations was discovered because of the phenotypic overlap between X-linked EDMD.

Nesprin-1 and -2 gene mutations have also been found in patients with cardiomyopathy and muscle disease [40, 52]. To date, comparatively few nesprin (*SYNE1* and *SYNE2*) mutations have been defined in human disease when considering the number of distinct LMNA mutations. *LMNA* mutations have been found in a multitude of disorders including Dunnigan Partial lipodystrophy and Hutchinson

Gilford progeria [53]. These two disorders are mentioned since they have targeted *LMNA* mutations that affect specific regions of lamins A and C. For example, mutations that target *LMNA* exon 8 lead to lipodystrophy-like phenotypes [54] (Fig. 2). A mutation that alters *LMNA* splicing leading to an accumulation of unprocessed lamin A causes premature aging in progeria [55]. In contrast, the *LMNA* mutations linked to cardiomyopathy and muscle disease distribute along the length of the lamin A/C protein. Most human mutations, especially those leading to cardiomyopathy, are heterozygous dominant mutations, and the precise effect of these mutations on the mechanical roles of the LINC complex is challenging to study because of limited access to human tissues.

Mouse Models of Laminopathy

Mouse modeling has proved instructive in defining the importance of the nuclear membrane for cardiac function. Sullivan et al. first described mice with a deletion of *Lmna* exons 8, 9, and 10 [56]. Mice with a homozygous *Lmna* deletion exhibit growth retardation, reduced muscle mass, reduced adiposity, and cardiomyopathy typically dying at 8–10 weeks of age. The cardiac phenotype of these mice was characterized further noting that by 4–6 weeks of age *Lmna* null mice develop a dilated heart with prolonged PR and QRS intervals [57]. *Lmna* null cardiomyocytes also develop a progressively disrupted desmin network, another intermediate filament protein critical for cardiomyocyte integrity. Desmin mutations are associated with a similar phenotype to LMNA mutations in both humans and mice where they develop cardiomyopathy, cardiac conduction system defects, and skeletal myopathy [58]. Desmin myopathies, in contrast to LMNA-associated myopathies, are typically associated with intracytoplasmic accumulation of aggregates that are desmin-positive [59]. The interrelationship between the desmin filament network and the nuclear lamina is not fully established. However, the overlapping phenotypes suggest that these two networks are linked.

The nuclei from *Lmna* null mice are often grossly deformed, a feature often seen with dominant human *LMNA* mutations [60], and frequently display nonuniform patterning of other nuclear membrane proteins such as LAP2, lamin B, and nup153. Fibroblasts isolated from *Lmna* null mice responded to biaxially applied strain with far greater nuclear deformation than wildtype [61]. Furthermore, the cytoskeletal stiffness of *Lmna* null fibroblasts was significantly reduced compared to normal mouse fibroblasts [61]. This defective mechanotransduction from the plasma membrane through the cytoskeleton and the nucleoskeleton also produced gene expression changes in *iex-1* and *egr-1*, two genes known to be responsive to mechanical stimuli. Defective mechanotransduction was also associated with defective NF-κB signaling. In a similar set of experiments, compression of fibroblasts produced disruption of nuclear disruption far more easily in *Lmna* null compared to control [62]. Moreover, nuclear disruption was associated with spillage of nuclear contents into the cytoplasm consistent with loss of nuclear membrane integrity in *Lmna* null cells, and these features could be corrected with reintroduction of lamin A or lamin C [62].

The Lmna null cells, generated by deleting exons 8, 9, and 10, lack full length lamin A and lamin C and have markedly reduced nuclear stiffness. Deletion of exons 11 and 12 in mouse *Lmna* was used to produce mice that can still synthesize lamin C but not lamin A [63]. These lamin C only (LCO) have a near normal phenotype and fibroblasts from LCO mice have markedly improved, but still slightly reduced, nuclear stiffness. Fibroblasts deleted for lamin B have nearly normal nuclear stiffness but still display nuclear blebbing [64]. Nuclear blebbing is often seen in cultured fibroblasts from *Lmna* null mice and notably in dermal fibroblasts from patients carrying missense mutations in *LMNA* [65].

Signaling Defects from Nuclear Membrane Mediated Cardiomyopathy

Mice with missense *Lmna* mutations were generated by knocking in specific variants into the endogenous *Lmna* locus including H222P, N195K, and delK32 [66–68]. *Lmna* H222P homozygous mice have reduced survival, decreased mass, and cardiac defects. Notably, *Lmna* H222P mice display phenotype only when homozygous, unlike their human counterparts [66]. Males were more severely affected compared to females. By 6 months of age, *Lmna* H222P homozygous mice developed dilated hearts with markedly reduced fractional shortening. Lamin A/C was normally localized in the hearts of *Lmna* H222P mice, so the effect of H222P is by inducing abnormal filament formation at the nuclear membrane. An increase in TGFβ-signaling, phosphorylated SMAD2/3 was also noted in H222P hearts.

A profile of gene expression changes was performed from H222P homozygous hearts. Animals from 10 weeks of age were selected since there are no overt pathological defects at this age [69]. A number of pathways were altered including MAPK, IGF, and Wnt-related gene expression changes. Phospho-ERK, JNK, elk-1, and Bcl were all upregulated in hearts as well as in isolated cardiomyocytes. Together these findings support upregulation of the MAPK cascade as a consequence of a defective nuclear mechanotransduction. Strikingly, this same pathway was also observed as upregulated in emerin null mouse hearts supporting a link between nuclear membrane defects and the MAPK pathway [70]. Inhibition of this pathway with an ERK-specific inhibitor PD98059 limited the development of cardiomyopathy in *Lmna* H222P hearts demonstrating that this signaling pathway is pathogenic, at least in this specific *Lmna* mutation [71]. Furthermore, inhibiting the MAPK pathway after the onset of cardiomyopathy in H222P *Lmna* mice reversed aspects of the cardiomyopathy [72]. It is hypothesized that mutations that disrupt the normal assembly of the nuclear lamina lead to defective mechanotransduction that is manifest as these signaling defects. The precise molecular links between these signaling defects and the nuclear membrane are not fully established. In addition to these signaling changes, there are clear morphological defects in the cardiomyocyte nuclei in *Lmna* H222P mutant hearts such that the cardiomyocyte nuclei are detectably elongated. Treatment with MAPK inhibitors results in shortening of the elongated nuclei.

Lmna heterozygous (*Lmna*$^{+/-}$) mice develop cardiomyopathy at an older age [73, 74]. This later onset disease may be more molecularly and phenotypically consistent with what is seen in human patients. *Lmna*$^{+/-}$ mice have increased ERK signaling when subjected to transaortic constriction. Early treatment with the β adrenergic receptor blocker carvedilol reduced the echocardiographic parameters in *Lmna*$^{+/-}$ mice with reversal of the increase in LV diameter and fractional shortening. These data are consistent with β adrenergic blockade limiting the mechanotransduction defects arising from the defective nuclear membrane. Interestingly, the calcium channel sensitizer SCH00013, a positive inotropic agent, also improved survival and heart function in this same model [75]. This result is perhaps paradoxical since increased inotropy would be expected to enhance mechanical forces on cardiac nuclei.

The *Lmna* N195K variant was also engineered as a point mutation in mice. Like the H222P variant, homozygous N195K mice develop cardiomyopathy [67]. These mice also develop cardiac conduction system disease seen with an increase in PR interval. There was also an increase in ventricular ectopic beats. This mutant also displayed abnormal desmin staining and also mislocalization of gap junction proteins connexin 40 and 43. Heterozygous mice did not show these same changes.

Nesprin Mouse Models

Mice engineered to lack nesprin-1 or -2 survive while mice lacking both nesprin-1 and -2 have perinatal lethality from abnormal diaphragm muscle innervation [76]. This observation indicates that the highly related nesprin-1 and nesprin-2 have the capacity to compensate for each other. Consistent with this, mice with a deletion of nesprin-1's KASH domain retain expression of the truncated nesprin-1α where it is presumed to act as a dominant negative and prevent the ability of nesprin-2 to assume nesprin-1's function [39]. Loss of nesprin-1's KASH domain leads to cardiomyopathy and cardiac conduction system defects that affect both the atria and ventricles. Nesprin-deleted mice share some features in common with *Lmna* null mice with reduced weight gain and skeletal muscle involvement [77]. Like *Lmna* null mice, the phenotype was described from mice with a homozygous mutation in nesprin-1. The deletion of nesprin-1's KASH domain is anticipated to alter giant nesprin-1 as well as the shorter nesprin-1α. A mutation that disrupted only the larger giant nesprin-1 induced little phenotype suggesting that the myopathic feature of nesprin-1 KASH deletion derives from loss of the smaller, muscle-enriched nesprin-1α. The signaling defects associated with disruption of nesprin may overlap with what occurs from disrupting *Lmna*. Nesprin-2 has been shown to tether MAPK1 and MAPK2 [78]. Nesprin-1α has also been shown to localize the A kinase anchoring protein enriched in skeletal and cardiac muscle known as MAKAP [79]. Together, these data identify a molecular connection between the nuclear lamina and signaling components. It has been in debate the degree to which these signaling components are found within the nucleus, but these data point to direct mechanisms by which the LINC complex directly scaffolds signaling components in both LMNA- and nesprin-associated cardiomyopathy. Whether these are mechanosensitive pathways, especially in the case of nesprin, requires additional study.

Mechanosensitive Gene Expression

Mechanotransduction in the heart results in acute cellular responses that transmit from force producing elements in the sarcomere to the sarcolemma and its surrounding matrix but also to the nucleus. Mechanotransduction triggers a number of signaling cascades but also transmits signals to the nucleus resulting in gene expression changes. In addition to gene expression changes, mechanotransduction may also be associated with epigenetic regulation of gene expression through chromatin modification and/or chromatin regulation by the nuclear membrane. Therefore, the network of myofilaments, Z bands, intermediate filaments, and the nuclear cytoskeleton provides an opportunity for extracellular, cytoplasmic, and nuclear bidirectional communication and integration.

Cardiac hypertrophy is associated with changes in gene expression including the expression of immediate early genes such as fos, jun, c-myc, and Egr-1 [80]. Expression of early genes can still occur in isolated cells even after treatment with actin- and microtubule- disrupting compounds, suggesting an additional complexity of the communication network [81]. Bloom et al. suggested that it is desmin/lamin that mediates mechanosensing gene expression in the myocardium [82]. In these experiments, rat hearts were stretched varying degrees by adjusting perfusion pressures from 30 to 90 mmHg which resulted in varying sarcomere lengths ranging from 1.4 to 2.6 μm. This change in sarcomere length was sufficient to induce changes in distribution and positioning of these intermediate filaments around and within the nucleus.

The nuclear membrane can receive signals through the LINC complex and transmit this information to nucleus and directly to chromatin. Chromatin refers to the DNA in its higher order structure complexes with histones and compressed. Heterochromatin is more densely packed than euchromatin, and euchromatin represents a more open configuration associated with active gene transcription. Euchromatin is more commonly found in the nuclear interior while heterochromatin is concentrated at the nuclear periphery adjacent to the inner nuclear membrane. Individual chromosomes reside within chromosome territories within the nucleus. Whole chromosome territories or subregions of chromosomes may alter their position within three-dimensional space of the nucleus shifting position concomitant with changes in gene expression [83, 84]. Chromosome territories are also known to shift during proliferation, differentiation, quiescence, or senescence [65, 85]. Chromatin interaction with the nuclear lamina markedly changes during terminal differentiation [86]. Consistent with this, *LMNA* mutant cells have altered chromosome territories [65]. LMNA mutations associated with cardiomyopathy produce changes in gene expression that also associate with altered chromosome territories [87] thereby linking the lamina to the regulation of gene expression.

Lamin A/C and its binding partners directly bind DNA, histones, transcription factors, and chromatin [88]. Lamin-associated domains (LADs) are *cis*-acting DNA sequences that mediate the binding of DNA to the nuclear lamina [89]. Gene expression is then regulated by bringing chromatin in contact with the nuclear membrane, and this repositioning may be highly important to differentiation and even the maintenance of the differentiated state. During adipogenesis, several genes are relocalized to the nuclear interior correlating with the upregulation of gene expression [90]. How chromosome positioning directly regulates gene expression is not well understood, and this could reflect movement of chromosomes or regions of chromosomes to nuclear domains containing proteins that subsequently act on those sequences, either to repress or to promote gene expression. SC-35 domains, defined as being positive for the SC-35 antigen, co-localize with splicing components and poly (A) RNA. Myocyte-specific genes co-localize with SC-35 domains in terminally differentiated muscle, but not in myoblasts [91]. In this model, genes important for cell specification move to SC-35 domains to allow for fast and efficient transcription [91]. This is just one intranuclear marker and there may be others important for gene expression or RNA processing. The full role of the nuclear membrane in regulating these events is only beginning to be explored, and the degree to which this regulation is mechanosensitive needs further investigation.

Conclusions

The cardiomyocyte undergoes marked deformation with each beat and transmits force from the myofilaments both to the plasma membrane and to the nuclei within each cell. The connections between the cytoskeleton and the nucleus in the cardiomyocyte include those present in many cell types but also include specialized linkers that often involve intermediate filaments such as desmin and lamin A/C. Desmin is found throughout the sarcoplasm interweaving between myofilaments and creating a latticework that crosses from the subcortical cytoskeleton, adjacent to Z bands, and to the nuclear membrane. The lamina, composed mainly of the intermediate filament proteins lamins A and C, provides a subnuclear membrane scaffolding important for many nuclear functions including the regulation of gene expression, nuclear to cytoplasmic transport, and potentially RNA splicing.

Two intermediate filament proteins, lamin A/C and desmin, are targets for inherited myopathy. In most cases, the mutations in *LMNA* and *DES*, the genes encoding these proteins, have autosomal dominant mutations indicating that the intermediate filament networks assemble but not normally. More subtle perturbation of these intermediate filament networks likely produces cells with abnormal mechanotransduction properties. The LINC complex, which includes the nesprins and SUN proteins, is not unique to the striated muscle cells. However, there may be specialized components of the LINC network in striated muscle cells to accommodate the mechanical forces associated with repetitive contraction.

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Biophysical Forces Modulate the Costamere and Z-Disc for Sarcomere Remodeling in Heart Failure

Allen M. Samarel, Yevgeniya Koshman, Erik R. Swanson, and Brenda Russell

Introduction

Cardiac remodeling at the macroscopic level occurs as the heart fails and is accompanied by changes in size, shape, and performance of its cardiomyocytes. Therefore, an understanding of the mechanisms by which cardiomyocytes sense and respond to biomechanical stress is of prime importance to the development of a molecular basis of heart failure. The cardiomyocyte experiences changes in stress and strain throughout every cardiac cycle, which is generated both internally by contractile proteins and externally through cell–cell and cell–matrix interactions. Cells within the heart are constantly remodeling through processes of gene transcription, protein translation, posttranslational modification, and the assembly of complex organelles. Because the heart is constantly challenged mechanically, it relies upon the near instantaneous posttranslational modifications of existing proteins to sense and respond rapidly to acute external stressors. Transcription and translation takes longer but are important to the overall response to chronic stress and strain. However, the mechanisms by which cardiomyocytes sense and respond to chronic mechanical strains remain largely unknown. Our team and many others are testing hypotheses that mechanical forces drive the growth of heart cells in healthy exercise but become maladaptive in disease. In this chapter we review how the biophysical forces in normal cardiomyocytes drive sarcomere remodeling in heart failure via two key structural components—namely the costamere at the cell membrane that is connected to the Z-disc of the myofibril. Each of these

A.M. Samarel, M.D. (✉) • Y. Koshman, Ph.D.
The Cardiovascular Institute, Stritch School of Medicine, Loyola University Chicago, Building 110, Room 5222, 2160 South First Avenue, Maywood, IL 60153, USA
e-mail: asamare@lumc.edu

E.R. Swanson, M.S. • B. Russell, Ph.D.
The Department of Physiology and Biophysics, University of Illinois—Chicago, Chicago, IL 60612, USA

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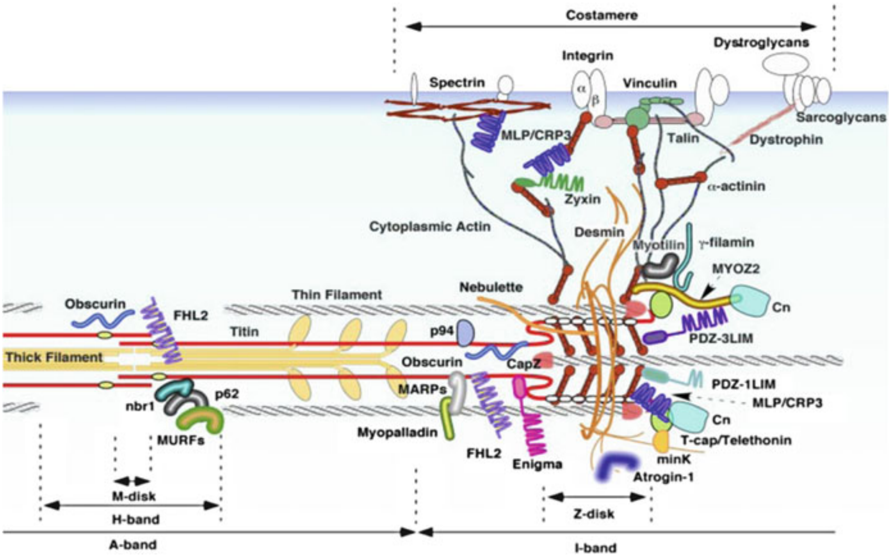

Fig. 1 Cytoskeletal protein complexes within the cardiomyocyte costamere and Z-disc. Structural and signaling proteins within the costamere and Z-disc are depicted. Many of these molecules have been implicated in either mechanosensing or in sarcomere assembly. *MYOZ2* myozenin 2, *Cn* calcineurin, *PDZ-3LIM* one-PDZ and three-LIM domain protein, *PDZ-1LIM* one-PDZ and one-LIM domain protein, *MLP/CRP3* muscle-specific LIM protein/cysteine-rich protein 3, *FHL2* four-and-a-half LIM protein 2, *MAPRs* muscle ankyrin repeat proteins, *MURFs* muscle-specific ring-finger proteins. Reprinted from Hoshijima et al. [10] with permission

structural complexes has numerous proteins, many of which sense mechanical forces and are also involved in filament assembly. A diagram of the costamere and Z-disc indicates many of the key proteins (Fig. 1). In this chapter, we review how the costamere and specific components of the Z-disc may accomplish both functions of stress detection and sarcomere remodeling during adaptation to hemodynamic overload.

Biophysics of Cardiomyocyte Mechanotransduction

Mechanotransduction is the process by which load-bearing cells sense physical forces, transduce the forces into biochemical signals, and generate adaptive or maladaptive responses that lead to alterations in cell structure and function. Mechanical forces regulate gain and loss of adhesion, membrane and cytoskeletal stretch, and cellular compression due to changes in pressure. Mechanical perturbations then activate intracellular signal transduction pathways that have profound effects on cellular phenotype. Mechanotransduction in the heart affects the beat-to-beat regulation of cardiac performance, but also profoundly affects the growth, phenotype, and survival of cardiomyocytes [1]. Conversely, injury to the cardiomyocyte can affect the structural integrity of components of the mechanosensory apparatus, and impair force generation, growth regulation, and cell survival [2–6]. Mechanical strain physically deforms a protein, and changes the affinity of binding sites, which alters the association between signaling molecules and their effectors [7]. Strain to a cell alters the external physical properties of the matrix molecules, which propagate to the internal networks in a secondary wave of mechano-regulated outside-in and subsequent internal cell signal changes. In many cell types, these processes are best studied at the single molecule level. One example is fibronectin, a crucial extracellular matrix (ECM) protein, but internal proteins like p130Cas and talin also respond to strain [8]. The sequences of events sensing stress and strain are similar for the protein complexes of the costamere and Z-disc in muscle. A mechanical perturbation deforms one or more proteins in the sensor complex, thus triggering changes in binding partners enabling posttranslational modifications. In some cases, specific proteins are released and translocate to the nucleus to affect transcription.

Cardiomyocytes rely on several intracellular components to sense mechanical load and convert mechanical stimuli into biochemical events that cause sarcomere remodeling during heart failure. The mechanosensors include integrins and other membrane-associated proteins that link the ECM to the cytoskeleton, protein components within the myofilaments and Z-discs, and stretch-activated ion channels. As described in a number of recently published reviews [1, 9–11], information regarding each component's role in modulating sarcomere addition and remodeling, and their complex interactions in stress detection and cytoskeletal assembly remain limited. In fact, it is likely that multiple mechanosensors are primarily responsible for sarcomere remodeling in response to increased wall stress.

Let us consider how forces are transmitted in muscle. Muscle cells are unique in that they both respond to externally applied mechanical forces, as well as generate large internal loads that are transmitted to adjacent cells and their surrounding ECM. Externally, forces that are generated outside the cardiomyocytes are transmitted from the ECM to the interior through costameres, the general cell mechanosensor of the focal adhesion complex [12]. The anisotropic geometry of the cardiomyocyte with its longitudinal and lateral structures may allow for distinct pathways of force recognition and transmittance [13–15]. When a sarcomere is lengthened by strain, both the costameric complex and the Z-disc are extended in the longitudinal direction (Fig. 2). However, what is not generally appreciated is that normal cell shortening causes a widening of the cell, thus providing a stretch in the transverse direction which increases the distance between the thick and thin filaments (Fig. 2). Under passive tension, the filaments are closer together and the Z-disc in cross section has a small lattice. With active tension as the sarcomeres contract, the filaments move further apart and the Z-lattice becomes basket weave in pattern (Fig. 3) [16]. Cardiac muscle is usually under tension, so the basket weave pattern is generally seen. The repetitive features in the sarcomere have permitted this detailed analysis of protein complexes to be studied, which is not possible for the focal adhesion complex. Nonetheless, separate directional pathways are implicated by static transverse and longitudinal loading to activate

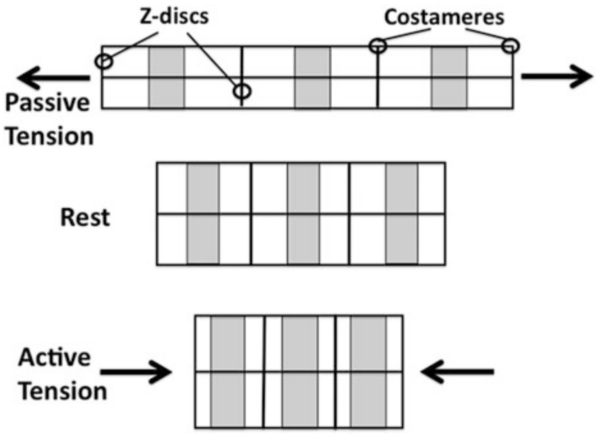

Fig. 2 Force is distributed externally from the costameres and internally throughout the myocyte by the Z-discs. Both the longitudinal and transverse directions are deformed by cell lengthening with passive tension or during contraction by active tension, as shown by directions of *arrows*, respectively. Width of cell decreases with lengthening and increases with shortening. A-band (*grey*) length stays constant; I-band length varies (*white*) with shortening. Dimensions are exaggerated for clarity

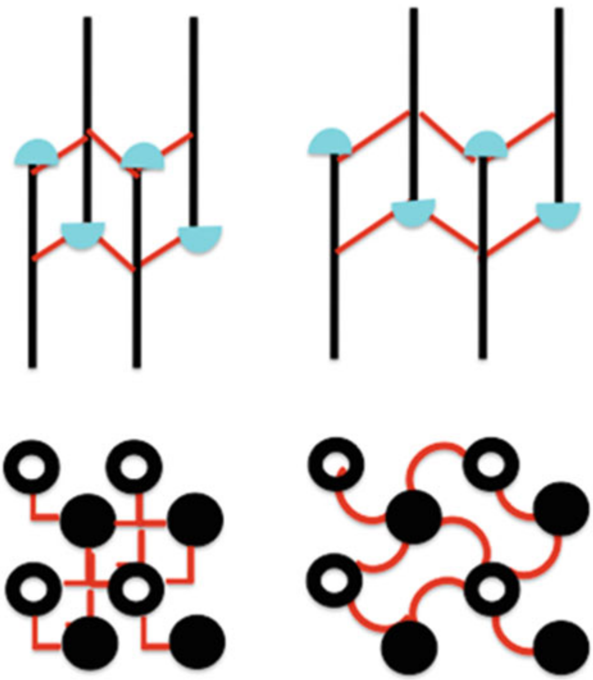

Fig. 3 Z-disc lattice is deformed with tension. *Upper panels* show a longitudinal section and *lower panels* in the transverse plane through the Z-disc. *Left side* is under passive tension (rest); right active tension (contraction). Thin filaments (*black*); to distinguish polarity the *open circles* enter Z-disc from below and closed from above. α-actinin (*red*) has small square lattice with passive but basket weave with active tension. CapZ (*blue*) the α/β heterodimer caps the barbed end of the thin filament. Thin filament lattice spacing is 24 nm at rest. Redrawn after Goldstein et al. [16]

stress in muscle for costameric proteins, such as the activation of the ERK cascade [17], or different levels of phosphorylation of focal adhesion kinase (FAK) [18] with transverse vs. longitudinal strain.

Costameres in Cardiomyocyte Force Transmission and Remodeling

Peripheral myofibrils are physically attached to the sarcolemmal membrane by costameres, which are components of the subsarcolemmal cytoskeleton (Fig. 1). This structure was first identified by Pardo and colleagues [19] as vinculin-positive, circumferentially oriented bands that mechanically couple the sarcolemma and its invaginations to the contractile machinery of the cardiomyocyte during fiber lengthening and shortening. As outlined below, this structure is critically important for both force transmission and sarcomere remodeling during adaptation to hemodynamic overload.

Costameres Are Important Sites for Cell Attachment to the Extracellular Matrix

Attachment of myofibrils at costameres resembles the attachment sites of the subcortical actin cytoskeleton of nonmuscle cells to the plasma membrane at focal adhesions. Indeed, costameres are now considered striated muscle-specific extensions of focal adhesions, where they form an interface between the intracellular and extracellular environment. Like their focal adhesion counterparts in nonmuscle cells, costameres are also critically important adhesive structures that serve to physically attach cardiomyocytes to their surrounding ECM. Thus, they form a stress-tolerant linkage between the cardiac ECM and the myofibrillar apparatus of adjacent muscle cells. As described below, force generated by one cardiomyocyte can then be transmitted laterally to adjacent cells via attachment to the ECM, allowing for coordinate contraction and relaxation of the functional syncytium.

Costameres Are Sites for Outside-In and Inside-Out Force Transmission

In addition to their role in cell attachment, Danowski and colleagues first demonstrated that costameres were sites where contractile forces generated within the cardiomyocyte are directly transmitted to the surrounding ECM [20]. They showed that cultured adult rat ventricular myocytes maintained on a flexible, silicone membrane generated pleat-like wrinkles of the surrounding ECM each time the firmly attached cells contracted. The pleats were spaced 1.8–2.0 μm apart, coinciding with the distribution of α-actinin-containing Z-discs, and sites of close approximation of the cell membrane to the silicone substratum as determined by interference-reflection microscopy. Costameres are also sites where longitudinal displacement of the ECM is transmitted directly to the contractile machinery of the cell. A 10 % static, linear stretch of aligned neonatal rat ventricular myocytes maintained on laminin-coated, microtextured silicone membranes resulted in an immediate, uniform increase in sarcomere length of ~10 % throughout the entire length of the longitudinally oriented, rod-shaped cell [21]. In this case, cell attachment via circumferentially organized costameres alone was sufficient to transmit the externally applied longitudinal strain directly to the underlying myofibrils, indicating that both externally applied and intrinsically generated mechanical loads are transmitted through costameres both outside-in and inside-out.

Integrins Attach the Cardiomyocyte Cytoskeleton to the Cardiac ECM

ECM attachment at costameres is accomplished by specific, integral membrane components within two major protein complexes: the dystrophin-glycoprotein complex containing α-dystroglycan that binds laminin-2, perlecan, and other ECM proteins in the cardiomyocyte basement membrane [22], and β_1-integrins, that bind laminin, fibronectin, fibrillar collagens, and a variety of other extracellular proteins to the cardiac ECM [23]. Membrane-associated proteins on the cytoplasmic face of both types of adhesion complexes collectively form a linkage to the myofibrillar apparatus via protein–protein interactions that ultimately terminate at the Z-disc of peripheral myofibrils [24]. These costamere-associated linker proteins provide both structural and signaling roles in initiating and maintaining sarcomeric organization and remodeling during hemodynamic stress [1]. Disruption of either protein complex produces left ventricular dysfunction, and combined defects act synergistically to reduce cardiac function more than disruption of either adhesive complex alone [25].

Integrin receptors are heterodimeric transmembrane proteins that are responsible for initiating cell–matrix attachment by adherens junctions in a variety of cell types. In cardiomyocytes, integrins are not randomly distributed on the cell surface, but rather are found embedded within the sarcolemmal membrane directly adjacent to costameres [26]. Thus, the ECM-integrin-costameric protein network can be viewed as a highly specialized form of lateral adherens junction, in which cell-to-matrix attachment is mediated by direct interaction of cardiomyocyte integrins with specific ECM protein sequences within the cardiac interstitium. The physical interaction between integrin cytoplasmic domains and adaptor proteins within the cytoskeleton generate a submembrane adhesion plaque that is critical for transmitting mechanical force between the ECM and the actin cytoskeleton. Furthermore, the cytoplasmic domains of integrin subunits play a direct role in these connections, as several cytoskeletal adaptor proteins can link integrins directly to actin filaments [27]. With the inclusion of additional protein–protein interactions, it is apparent that integrins provide a critical transmembrane linkage between the cardiac ECM and the actin-based cardiomyocyte cytoskeleton.

All integrin receptors consist of noncovalently associated α and β subunits that combine to form a single receptor for various ECM components. At least 18 α and 8 β subunits have been identified in mammals, and they combine to form at least 24 different paired integrin receptors in various cell types [28]. However, myocardial integrins are relatively restricted to integrins of the β_1-type, including the β_{1D} splice variant isoform, which is the predominant integrin expressed in the postnatal heart [29]. In contrast, various α subunits are expressed throughout cardiomyocyte development, with α1, α3, and α5 subunits identified in immature cardiomyocytes, whereas α6 and α7 isoforms predominate in adult ventricular myocytes [30, 31]. Nevertheless, a single integrin receptor is capable of binding to several different ECM proteins, whereas a single ECM ligand can engage several different integrin heterodimers. The promiscuous nature of ECM attachment allows for subtle differences in ECM composition to occur during development and in response to biomechanical overload.

β_1-Integrin Cytoplasmic Domains Form Cytoskeletal Attachments Through Talin Dimers

Unlike growth factor receptors, the cytoplasmic tails of integrin α and β chains possess no intrinsic catalytic activity. However, they interact with other cytoskeletal proteins to transmit extrinsically applied and internally generated mechanical force. A major binding partner of the β_1-integrin cytoplasmic domain is the cytoskeletal protein talin, which is a rod-shaped, multidomain protein involved in bidirectional activation of integrins [32]. Cell-surface integrins can exist in either low- or high-affinity states, and cellular modulation of integrin affinity is in part accomplished by reversible binding of the N-terminal, globular head domain of talin to the C-terminal, β_1-integrin cytoplasmic tail [33]. Cardiomyocytes express predominantly talin-2 and integrin β_{1D} isoforms, which have the highest binding affinities of various β-integrin cytoplasmic domains, suggesting that integrin engagement to the cardiac ECM favors a mechanically strong, activated integrin binding conformation [34].

The mammalian genome contains two genes for talin encoding two structurally similar proteins (talin-1 and talin-2) that share 74 % sequence identity [35]. The specific function of each talin isoform in mechanotransduction and sarcomere remodeling is not known, but it appears that the talin-2 isoform plays a unique role in muscle development and disease. Localization of talin-2 is restricted to costameres and intercalated discs in striated muscle [36], but talin-2 appears dispensable in the presence of talin-1 for normal costamere and sarcomere assembly.

However, deletion of both genes produced a severe defect in sarcomere assembly in striated muscle, with profound defects in the assembly of adhesion complexes and sarcomeres by cultured myoblasts isolated from double knockout embryos [37]. The failure of normal sarcomere development in these immature muscle cells also highlights the importance of talin (and perhaps other cytoskeletal linker proteins) in myofibrillar assembly.

Cardiomyocyte adhesion to ECM proteins via β_1-integrins causes the recruitment of talin dimers to the cytoplasmic face, leading to integrin transition to their high affinity state. This process is an important, early step in "outside-in" signaling during cell attachment. Conversely, talin activation by a number of intracellular signaling pathways causes the physical displacement of α-integrin subunits, thereby allowing for high-affinity ECM engagement during "inside-out" signaling [34, 38]. Intracellular talin binding alone is sufficient to alter the conformation of integrin extracellular domains and promote their attachment to ECM proteins [39]. The recruitment of talin involves the Src-dependent tyrosine phosphorylation of the β-integrin tail, and its recognition by the talin head region [40]. Thus, intracellular stimuli that cause Src-dependent integrin phosphorylation promote the activation of integrins via talin recruitment to costameres, and stimulate costamere formation during inside-out signaling. Subsequent recruitment of additional cytoplasmic linker proteins (such as paxillin and vinculin) to the costamere may also be required for Z-disc assembly and premyofibril formation during myofibrillogenesis [41, 42].

Focal Adhesion Proteins Are Critical Regulators of Costamere Assembly

Vinculin is another cytoskeletal linker protein that is recruited to focal adhesions and costameres during integrin engagement and clustering [43]. It is a cytoskeletal adaptor protein consisting of three functional domains: an N-terminal head, a flexible, proline-rich hinge region, and a C-terminal tail domain. Intramolecular association between the head and tail domains constrains vinculin in an inactive conformation, but vinculin unfolds during activation, thereby allowing vinculin to interact with a variety of other cytoskeletal proteins, including α-actinin and paxillin [44]. As vinculin unfolding and activation is driven by talin binding to the head region of the molecule, and activated vinculin binds α-actinin, then talin binding to the β_1-integrin cytoplasmic tail thereby indirectly provides a physical linkage to the actin-based cytoskeleton.

Vinculin localization to costameres is accomplished by two distinct mechanisms. As indicated above, the head region binds to talin during integrin activation, whereas the C-terminal tail region can bind to paxillin, another cytoskeletal adaptor protein that is targeted to focal adhesions and costameres via its LIM3 domain [45]. The C-terminal region of vinculin also contains a four-helix bundle that is structurally similar to the focal adhesion targeting (FAT) sequence of

FAK [46]. The FAT domain of FAK binds to paxillin during integrin engagement and clustering and targets the protein kinase to the growing subsarcolemmal adhesion plaque. Thus, the combinatorial interactions of all four proteins (talin, vinculin, paxillin, and FAK) suggest both structural and signaling roles for each in costamere formation and turnover [44]. Based on structural studies of focal adhesions in nonmuscle cells, it is likely that individual costameric proteins are also organized both horizontally and vertically into specific layers responsible for signaling, force transduction, and cytoskeletal attachment [47].

Costameres Are Components of the Mechanosensory Apparatus of Cardiomyocytes

Attachment is clearly one way in which costameres contribute to mechanotransduction, but there is also substantial evidence to indicate that mechanical forces (generated by passive stretch and active tension development) are "sensed" by costameres, or their focal adhesion counterparts in cultured cardiomyocytes. Biochemical signals are then transmitted internally, leading to sarcomere assembly and altered gene expression characteristic of cardiomyocyte hypertrophy. Stretch-induced deformation of cardiomyocyte integrins triggers the recruitment and activation of several signaling kinases (such as FAK, proline-rich tyrosine kinase 2 (PYK2), Src, Rho kinase (ROCK), and ERKs) to the cytoplasmic face of the adhesion complex, where they participate in downstream signaling to the nucleus and other organelles [12]. Results obtained in mechanically stressed, cultured cardiomyocytes complement elegant studies performed in pressure-overloaded, intact myocardium [48–52], and also support the close interaction between the ECM-integrin-cytoskeletal complex and growth factor receptor signaling during cardiomyocyte hypertrophy and sarcomere remodeling [53–59].

FAK and Cardiomyocyte Mechanotransduction

Exactly how components of the focal adhesion complex sense mechanical stimuli remains unclear. Seminal observations by Ingber and colleagues [60] using a magnetic twisting device to transfer force directly from integrins to the local cytoskeleton suggest that mechanical deformation of one or more adhesion plaque proteins is the proximal step in an intracellular signaling cascade that leads to global cytoskeletal rearrangements and mechanotransduction at multiple, distant sites within the cell. As integrin subunits are devoid of any catalytic activity, transmission of mechanical signals from integrins to the cytoskeleton requires the "stress activation" of one or more signaling molecules capable of transmitting biochemical signals to the internal cellular environment. Talin localization during integrin engagement and clustering may initiate outside-in signaling, but talin has no

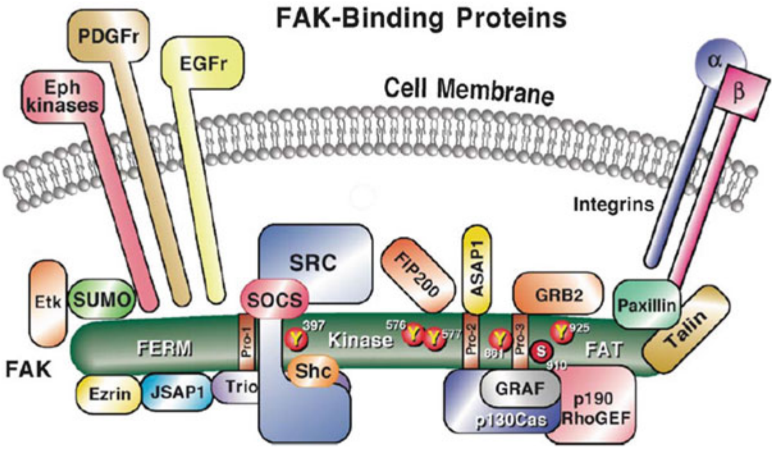

Fig. 4 FAK binding partners in cardiomyocytes mechanotransduction. The N-terminal, autoinhibitory FAK FERM domain is important for signal integration from growth factor receptors such as the Eph-family, EGF and PDGF receptor protein tyrosine kinases, and the cytoplasmic Etk protein tyrosine kinase. The FAK C-terminal focal adhesion targeting (FAT) domain binds the cytoskeletal adaptor proteins paxillin and talin, and mediates FAK localization to integrin-enriched focal adhesions and costameres. The FAT domain and proline-rich (PR) regions bind molecules involved in Rho-GTPase activation and deactivation. Numerous tyrosine and serine phosphorylation sites on FAK are also depicted. *JSAP1* scaffolding protein of the JNK kinase pathway, *ASAP1* 130-kDa phosphatidylinositol 4,5-biphosphate (PIP_2)-dependent Arf1 GTPase-activating protein (GAP), *GRB2* growth factor receptor-bound protein 2, *GRAF* GAP for Rho associated with FAK, *SOCS* suppressors of cytokine signaling, *SUMO* small ubiquitin-like modifier protein. Reprinted from Schlaepfer et al. [61] with permission

intrinsic catalytic activity capable of relaying biochemical signals into the cell interior. However, secondary recruitment and activation of protein tyrosine and serine/threonine kinases to the cytoplasmic adhesion plaque may accomplish this function. FAK is clearly one candidate enzyme that is responsible for integrin-mediated mechanotransduction within cardiomyocyte focal adhesions and costameres. As indicated above, FAK is a nonreceptor protein tyrosine kinase that functions as an “activatable scaffold” [61] in integrin-dependent signal transduction (Fig. 4). An autoinhibitory FERM domain, located within the N-terminal region of FAK, associates with the plasma membrane via its interaction with several different growth factor receptors. The C-terminal region of FAK comprises the FAT domain, which binds directly to paxillin and talin, which in turn bind to the cytoplasmic tail of β_1-integrins at sites of integrin clustering. Once localized, FAK phosphorylates itself at a single tyrosine residue (Y_{397}). This autophosphorylation site serves as a high-affinity binding domain (pYAEI motif) for the SH2 domain of Src-family protein tyrosine kinases [62] (Fig. 5). Once bound to FAK, active Src then phosphorylates FAK at residues Y_{576} and Y_{577} within its catalytic domain (which augments FAK kinase activity toward exogenous substrates), and at Y_{861} and Y_{925} near its C-terminus [63]. The Y_{861} phosphorylation site promotes the

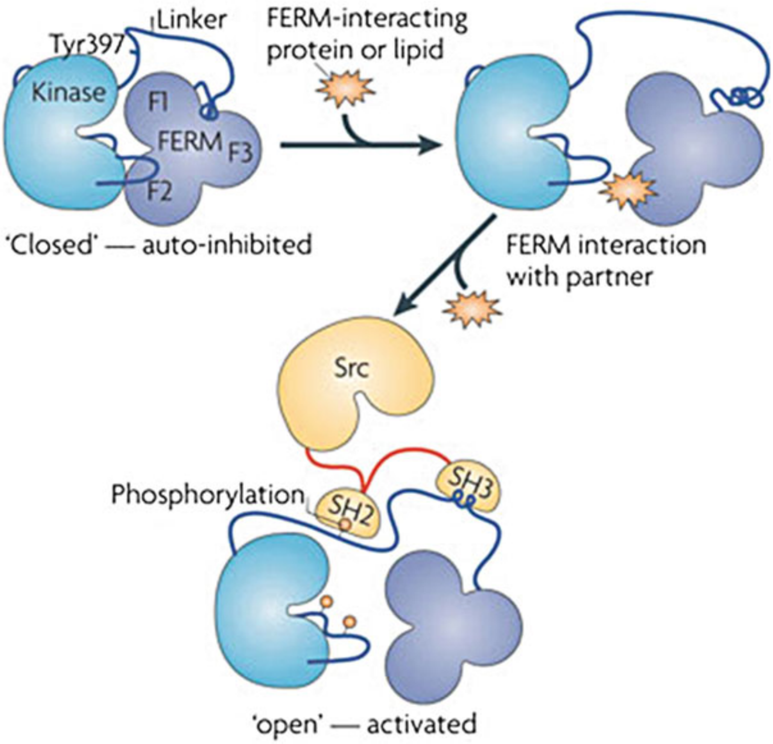

Fig. 5 Model for the autoactivation of focal adhesion kinase. The "clover leaf" structure of the autoinhibitory FERM domain of FAK is depicted. The FERM domain binds directly to the kinase C-lobe when FAK is auto-inhibited, impeding access to the active site and protecting FAK's activation loop from phosphorylation by Src. FAK is activated when it is released from the auto-inhibited "closed" state by the binding of a protein or lipid partner to its FERM domain. Binding of partners to regions in the F1 or F2 subdomains of the FERM domain is likely to "open" the protein, permitting auto-phosphorylation and Src binding. This leads to Src-mediated Tyr phosphorylation of the acceptor Tyr residues Y_{576} and Y_{577} in the kinase loop of FAK and full catalytic activation, and Src-mediated Tyr phosphorylation at Y_{861} and Y_{925} to promote binding of p130Cas and GRB2, respectively. Reprinted from Frame et al. [63] with permission

binding of p130Cas to FAK [64]. The Y_{925} phosphorylation site promotes the binding of Grb2 to FAK, and other adaptor proteins and kinases containing SH2 domains. The FAK-Src complex also phosphorylates paxillin, p130Cas, and other cytoskeletal proteins involved in costamere and cytoskeletal assembly.

In addition to multiple tyrosine phosphorylation sites, FAK contains several serine residues (S_{722}, S_{843}, S_{846}, and S_{910}) that undergo reversible phosphorylation in response to hypertrophic stimuli. These serine residues are in close proximity to critical protein–protein interaction sites within the C-terminal region of FAK, such as the binding site for p130Cas and the adjacent FAT domain. The functional role of FAK serine phosphorylation in cardiomyocytes is largely unknown, but one report indicates that serine (and tyrosine) phosphorylation of FAK increases dramatically in hypertensive rats, with different sites of phosphorylation appearing to regulate FAK subcellular localization [65]. Our group [66] recently demonstrated that endothelin-1 and other hypertrophic factors induced a time- and dose-dependent increase in FAK-S910 phosphorylation. Endothelin-induced FAK-S910 phosphorylation required endothelin Type A receptor-dependent activation of PKCδ and Src via parallel Raf-1→MEK1/2→ERK1/2 and MEK5→ERK5 signaling pathways. Replication-deficient adenoviruses expressing wild-type FAK and a non-phosphorylatable, S910A-FAK mutant were then used to examine the functional significance of FAK-S910 phosphorylation. Unlike wild-type FAK, S910A-FAK increased the half-life of GFP-tagged paxillin within costameres (as determined by total internal reflection fluorescence microscopy and fluorescence recovery after photobleaching) and increased the steady-state FAK-paxillin interaction (as determined by co-immunoprecipitation and Western blotting). These alterations resulted in reduced NRVM sarcomere reorganization and cell spreading. Finally, we found that FAK was serine-phosphorylated at multiple sites in non-failing, human left ventricular tissue, and FAK-S910 phosphorylation and ERK5 expression were both dramatically reduced in patients undergoing heart transplantation for end-stage dilated cardiomyopathy (DCM). These results suggest that reduced FAK-S910 phosphorylation may contribute to sarcomere disorganization that is frequently observed in these heart failure patients [66].

We and others have shown that FAK and the highly homologous protein tyrosine kinase PYK2 are both expressed in neonatal and adult cardiomyocytes, where they are activated in response to mechanical loading [12, 67, 68] and agonists (e.g., phenylephrine, angiotensin II, endothelin-1) that stimulate Gq-coupled receptors [53–59, 69]. Thus, FAKs, and other protein kinases bound to FAK and PYK2 during integrin clustering, can activate downstream signaling pathways that regulate integrin-mediated mechanotransduction at local as well as distant sites within the cell.

Further support for an important role for FAK in cardiomyocyte mechanotransduction has come from studies of global and cardiomyocyte-specific FAK knockout mice. Global FAK deletion led to lethality at embryonic day 8.5, and the mutant embryos displayed a profound defect in development of all mesodermal structures, including the heart and vasculature [70, 71]. Interestingly, the developmental defects found in FAK$^{-/-}$ embryos were phenotypically very similar in timing and phenotype to the morphological defects observed in fibronectin-null mice, suggesting an important relationship between fibronectin- and FAK-dependent signaling, especially with respect to development of the cardiovascular system [61]. In both cases, the developmental defects were attributed to the inability of mesodermal cells to migrate normally. Indeed, fibroblasts isolated from FAK$^{-/-}$ embryos displayed markedly reduced mobility and abnormally large focal adhesions, indicating a defect in focal adhesion turnover. However, cardiomyocyte-restricted FAK knockout mice have a variable cardiac phenotype depending on when during development FAK is deleted. Embryonic deletion of

FAK in cardiomyocytes caused perinatal lethality due to the presence of large ventricular septal defects and abnormalities in outflow tract alignment, indicating again that FAK predominantly regulates mammalian cardiomyocyte migration during early cardiac development [72]. However, cardiomyocyte FAK deletion during later prenatal development was not associated with any congenital heart defects, but led to spontaneous DCM in aged animals [73], and a blunted hypertrophic response to angiotensin II infusion [73] or transverse aortic constriction [74] in the adult heart. The inability to respond normally to hypertrophic stimuli supported earlier cell culture studies that demonstrated impaired hypertrophic responses of cultured cardiomyocytes overexpressing FAK-related nonkinase (FRNK, a naturally occurring inhibitor of FAK), Y397F-FAK (a FAK autophosphorylation mutant), FAK antisense RNA, or just the FAK-FAT domain [12, 21, 54, 56–58, 75].

Other Protein Kinases Involved in Cardiomyocyte Mechanotransduction

In addition to FAK, cardiomyocytes express a structurally related kinase known as PYK2 (also known as cell adhesion kinase-β (CAK-β), related adhesion focal tyrosine kinase (RAFTK), or cell adhesion tyrosine kinase (CADTK)) [76]. PYK2 is a Ca^{2+}-dependent nonreceptor protein tyrosine kinase that undergoes bimolecular transphosphorylation [77] in response to integrin engagement, increased intracellular Ca^{2+}, and activation of PKCs in many cell types, including cardiomyocytes [69, 78–83]. Although PYK2 is predominantly localized to the cytoplasm [69], a minor component of the enzyme co-localizes with paxillin in focal adhesions of cultured neonatal rat ventricular myocytes [83]. Like FAK, PYK2 acts as an important scaffolding protein, and transduces signals from G-protein-coupled receptors to downstream MAPK signaling pathways depending upon which signaling kinases and adaptor proteins bind to the phosphorylated enzyme [84, 85]. PYK2 has also been shown to link a variety of stressful stimuli, including Ca^{2+} overload, UV irradiation, and TNF-α treatment to MAPK activation in several cell types [86]. Recently, Hirotani et al. [82] demonstrated that PYK2 is an essential signaling component in endothelin- and phenylephrine-induced cardiomyocyte hypertrophy, perhaps acting via the Ca^{2+}- and/or PKC-dependent activation of Rac1.

Bayer et al. [51] have demonstrated that PYK2 expression and phosphorylation were significantly increased in adult rat ventricular myocytes in vivo in response to acute left ventricular pressure overload. Similarly, Melendez et al. [78] showed that PYK2 expression and phosphorylation were increased in a mouse model of DCM, but its exact role in these conditions has not been elucidated. Nevertheless, recent studies have confirmed that PYK2 is an important upstream regulator of the stress-activated protein kinases ($p38^{MAPK}$ and JNK1/2) in cardiomyocytes [81, 83]. Inhibition of PYK2 in vivo (by direct gene transfer of its C-terminal FAT domain) reduced activation of the fetal gene program and reduced LV remodeling in a rat model of myocardial infarction [87]. Thus PYK2 activation has been implicated in hypertrophic gene expression changes during pathological cardiomyocyte hypertrophy [83] and in the induction of apoptosis [81].

Integrin-linked kinase (ILK) is a third protein kinase that may be involved in integrin-dependent mechanotransduction in cardiomyocytes. ILK has a sequence homology to serine-threonine protein kinases, but its kinase domain is nonfunctional, thus indicating that ILK is a pseudokinase [88, 89]. However, ILK's pseudokinase domain binds tightly to α-parvin and the complex can directly bind to the cytoplasmic tail of β_1-integrins as well as other focal adhesion adaptor proteins [90, 91]. One of these interacting proteins, PINCH1, is essential to early embryonic development, but appears dispensable when its expression is specifically reduced in cardiomyocytes [92]. However, targeted ablation of ILK in cardiomyocytes caused a rapidly progressive, DCM [93]. The ILK-PINCH-parvin complex is involved in regulating Akt activity and cell survival signaling in many cell types, and despite its lack of kinase activity, ILK appears to serve these roles in cardiomyocytes. ILK is an important upstream regulator of Akt phosphorylation at S_{473} [94], which is essential for Akt activity and may explain ILK's ability to suppress apoptosis [95]. ILK also interacts with thymosin β4, an actin-binding peptide that stimulates cardiomyocyte and endothelial cell migration. ILK activation by expression of thymosin β4 led to activation of Akt and improved cardiomyocyte cell survival following coronary artery ligation, further implicating ILK and the focal adhesion complex in integrin-dependent cell survival signaling [96]. Nevertheless, it remains unclear whether ILK undergoes translocation and activation in response to mechanical loading of cardiomyocytes in a manner similar to FAK and PYK2.

There is also some evidence to indicate that PKCε, the major novel PKC isoenzyme expressed in cardiomyocytes, is directly involved in integrin-dependent mechanotransduction. Three families of PKCs have been identified to date, containing a total of 11 isoenzymes. The "classical" isoforms (α, βI, βII, and γ) are regulated by calcium, diacylglycerol (DAG), and phosphatidylserine; the "novel" isoforms (δ, ε, η, ϕ, and μ) are regulated by DAG and phosphatidylserine; and the "atypical" isoforms (ζ and λ) only require phosphatidylserine for activation. The major PKC phorbol ester-sensitive isoenzymes found in adult cardiomyocytes are PKCα, PKCβ, PKCδ, and PKCε. Using standard immunofluorescent microscopy, Disatnik et al. [97] first localized PKCε in a striated pattern within myofibrillar structures of cultured neonatal rat cardiomyocytes following stimulation with norepinephrine or phorbol myristate acetate. Subsequently, Huang et al. [98] demonstrated that PKCε translocated in response to arachidonic acid treatment of adult rat ventricular myocytes to a region adjacent to the Z-line where actin filaments are anchored, and where transverse tubules are closely apposed to the myofilaments. This site of translocation was specific for PKCε, as PKCδ, the other novel PKC expressed in rat ventricular myocytes, translocated to the nucleus in response to arachidonic acid. Borg et al. [99] then showed that PKCε localized to the cytoplasmic side of the sarcolemma directly adjacent to the Z-disc, and Heidkamp et al. [59] demonstrated that the kinase co-localized with FAK in typical focal adhesions in cultured neonatal cardiomyocytes. These morphological results are complemented by biochemical data demonstrating that PKCε forms functional signaling complexes with PYK2 [100] and Src-family protein kinases [101–103] in tissue homogenates of left ventricular myocardium from PKCε-overexpressing mice. PKCε, in turn, is involved in the endothelin-induced activation of both FAK [59] and PYK2 [80], via signaling pathways that may regulate local changes in the actin cytoskeleton [104]. Thus, there is ample evidence to indicate that at least a portion of cardiomyocyte PKCε is found in costameres and focal adhesions, where it may regulate focal adhesion and costamere formation [105] and sarcomeric assembly [21] in response to mechanical loading and growth factor stimulation.

One way that PKCε may localize to costameres and focal adhesions is via binding to RACK1 (Receptor for Activated C-Kinase-1). RACK1 is a seven WD-domain-containing protein that binds to the cytoplasmic tail of β-integrins [106], and anchors PKC isoenzymes [107], Src family protein kinases [108], and other proteins to focal adhesions. Although originally described as a selective receptor for activated, Ca^{2+}-dependent PKCs [109, 110], RACK1 also binds active PKCε, and increases focal adhesion formation, integrin clustering, and lamellipodia formation in human glioma cells. These responses are quite similar to those produced by overexpression of constitutively active PKCε in cultured neonatal cardiomyocytes [105]. Once localized, PKCε can phosphorylate a number of membrane-anchored and cytoskeletal proteins and participate in the activation of Rac1, which is required for cell spreading and cytoskeletal assembly [111]. Alternatively, active PKCε binds directly to the Z-disc [112] via an interaction with a RACK2-like protein [113], thus creating a situation in which PKCε may locally shuttle between costameres and Z-discs to regulate contractile function in response to changing hemodynamic loads.

It remains unknown exactly how PKCε is locally activated in response to mechanical loading. Vuori and Ruoslahti [114] showed that PKC activity in the cell membrane fraction transiently increases preceding cell spreading on fibronectin but not on polylysine, and PKC activation is required for FAK activation in response to cell spreading on fibronectin. These results would suggest that a membrane phospholipase within integrin-dependent cell attachment sites provides a local source of DAG sufficient to activate PKCε. Phospholipase C (PLC)-γ can bind to the Y_{397} phosphorylation site of FAK [115], and Ruwhof et al. [116] have shown that PLC (but not PLD) activity rapidly increases in neonatal cardiomyocytes in response to cyclic stretch, suggesting that PKC activation may be both upstream and downstream of FAK activation in response to mechanical loading.

Costameres Are Sites for the Earliest Steps in New Sarcomere Assembly

In addition to their important role in cell attachment, costameres may be an important site for the initial events in new sarcomere formation [41]. In cultured cardiomyocytes, costameres reorganize to form adhesive structures that are similar to typical focal adhesions found in nonmuscle cells [117], and their formation precedes the assembly of newly synthesized myofibrillar proteins into sarcomeres [118]. Because of the similarities between costamere formation in cardiomyocytes and focal adhesion formation in nonmuscle cells, we and others have proposed that FAK, and other FAKs play an important role in both processes [1]. Cardiomyocytes isolated from mice with tissue-specific deletion of FAK showed increased length but not width, and displayed disorganized myofibrils with increased nonmyofibrillar space filled with swollen mitochondria [73]. FAK deletion also causes DCM with aging and produces eccentric, rather than concentric, LV hypertrophy with angiotensin II infusion or transverse aortic coarctation, suggesting an intrinsic abnormality in sarcomere assembly [73, 74, 119, 120]. Overexpression of FRNK, or "knocking-down" FAK by siRNA prevented normal costamerogenesis and myofibrillogenesis during skeletal muscle differentiation [121]. Furthermore, overexpression of FRNK in cardiomyocytes also prevented the endothelin-induced increase in total protein/DNA, and the assembly of newly synthesized myofibrillar proteins into sarcomeres [54]. As discussed above, we recently proposed that FAK serine phosphorylation is critical for regulating the conformation of the FAK-FAT domain and, therefore, its interaction with other focal adhesion proteins required for new sarcomere addition [66]. Stabilizing the open conformation of the FAT domain may secondarily decrease FAK-paxillin interaction and promote its exit from newly forming costameres, thereby enhancing the vinculin–paxillin interaction [122, 123]. These events should strengthen the costamere and promote sarcomere formation and reorganization [37, 124] Thus, these findings suggest that FAK, and other proteins reversibly bound to FAK during costamere formation, are required for the normal assembly of sarcomeres in response to both biomechanical and neurohormonal stimuli that induce cardiomyocyte hypertrophy.

FAK, and it C-terminal binding partners p130Cas and paxillin, all co-localize to costameres of cultured neonatal rat ventricular myocytes, where they participate in sarcomere assembly induced by endothelin-1 [54, 57]. Displacement of FAK from these structures (by overexpressing FRNK, or just the FAK-FAT domain) prevented endothelin-induced assembly of newly synthesized contractile proteins into sarcomeres, and coincidently prevented the phosphorylation of paxillin [54] and p130Cas [57] by the FAK/Src complex. Conversely, preventing p130Cas binding to the C-terminal region of FAK (by overexpressing FAK residues 638–841, containing only the proline-rich, C-terminal p130Cas binding site) also substantially reduced endothelin-induced sarcomeric assembly, indicating a critical role for p130Cas binding and phosphorylation by FAK/Src in this process [57]. FAK also reduced steady-state adhesive force by recruiting vinculin to focal adhesions. FAK knockdown increased vinculin recruitment to focal adhesions, resulting in cells more firmly attached to their ECM [43]. Indeed, these cell culture results are consistent with studies of both paxillin [125] and p130Cas [126] knockout mice, which demonstrated early embryonic lethality due to severe defects in the development of the cardiovascular system. In the case of paxillin, the overall phenotype closely resembled that observed in both fibronectin- and FAK-knockout mice. In the case of p130Cas-deficient embryos (which died at a somewhat later developmental stage), cardiomyocytes displayed disorganized myofibrils and disrupted Z-discs, indicating important structural and mechanochemical signaling functions for these adaptor proteins.

Z-Disc Structure in Mechanotransduction and Filament Assembly

Internally, cardiomyocytes generate force through their contractile filaments that are anchored together by an abundance of proteins organized at the Z-disc (Fig. 1). The Z-disc anchors parallel, actin containing thin-filaments, which were observed over 30 years ago by electron microscopy to be bundled by struts assumed to be α-actinin [127]. Interestingly, cell tension extensively deforms this Z-disc lattice complex [16]. Many other proteins have subsequently been shown to form the architectural complexity of the Z-disc, which functions not only as a mechanical joint but also as a signaling complex for mechanotransduction containing proteins such as titin-T-cap, as reviewed in [128–130]. Another signaling protein is the muscle LIM protein (MLP), also known as cysteine rich protein 3 (CSRP3, CRP3). It is a mechanosensor found at the Z-disc with interactions to histone deacetylases (HDAC4) and acetylases (PCAF) and at the costamere where it interacts with ILK, zyxin, α1-spectrin, and α-actinin [131]. MLP in mechanically stimulated neonatal rat myocytes can shuttle to the nucleus where it is involved in regulating the hypertrophic gene program. Furthermore, MLP subcellular localization is sensitive to directional strain. Using microtopography, aligned cardiomyocytes were strained either transversely or longitudinally to determine nuclear translocation and myofibrillar distribution. MLP did not translocate in response to uniaxial, but only to biaxial strain. With transverse strain, MLP aligned along the fiber axis; i.e., perpendicular to the axis. But after longitudinal strain, MLP was more striated [132].

Hypertrophy in response to biophysical forces is achieved by increasing the number of thick and thin filaments in the myocytes, of which the actin filaments are thought to be assembled first into premyofibrils and the myosin thick filaments are spliced in later [133]. Since actin is one of the oldest and most conserved proteins in all living cells, there is an abundance of information about actin filament nucleation, assembly, and severing. All actin filament assembly requires numerous partnering proteins, with specialized variants found in the heart, such as CapZ, formin, and cipher. It seems likely that the filaments are built to serve the functional work being demanded at a subcellular region and that local mechanical conditions ultimately regulate how many filaments are assembled or degraded and where this occurs inside the cell. Nature is efficient in this regard, since muscle is a very high consumer of energy. Strain to the Z-disc modifies the function of the F-actin capping protein (CapZ) via mechanotransduction signaling pathways, such as those involving PKCε [97, 129, 134]. Interestingly, in transgenic models of CapZ downregulation, PKC-dependent regulation of myofilament function is abolished, and activated PKCε and PKCβ binding to the myofilament is diminished [135]. In neonatal myocytes, the additional sarcomeres are added after strain at the Z-disc [136], and this process is also regulated by PKCε [21]. Furthermore, dynamics of actin capping by CapZ is increased by hypertrophic stimuli, and regulated by PKCε activity [137]. These processes are discussed further below.

CapZ Structure and Function

Because of its major role in actin cytoskeletal remodeling, CapZ needs further discussion. CapZ was first discovered in the 1960s by Maruyama and colleagues and was called β-actinin [138–140]. An increasing concentration of CapZ decreased the length of F-actin polymers after sonication and prevented polymerization of G-actin. A fast recovery to the original state could be slowed or prevented by addition of CapZ at various time points [141]. CapZ was finally purified to homogeneity in 1980 which led to the discovery that it is composed of two major polypeptides approximately 30 kDa in size that pair to cap the barbed end of actin filaments [142]. CapZ is a heterodimer composed of an α and a β subunit. Vertebrates contain three α isoforms that are encoded from three individual genes, and three β isoforms that are generated through alternative splicing. The α1 and α2 isoforms are found throughout many tissues though their expression varies widely, and the α3 isoform is specific to the testis. In cardiac tissue, the ratio of α1 to α2 is approximately 1.2:1 [143]. The different biochemical, cellular, and functional roles of each α isoform continues to be elucidated.

The CapZ β1 and β2 isoforms are also found throughout various tissues, and similarly to the α3 subunit, the β3 isoform is specific to the testis. Because each β isoform is generated through alternative splicing, they contain great sequence similarity. The β1 and β2 isoforms vary only in their COOH-terminal ends (β1—31 amino acids, β2—26 amino acids) and β3 is identical to β2 with the addition of 29 amino acids at the NH_2 terminal end [144–146]. Although the protein sequences are extremely similar, the β1 and β2 isoforms have distinct functions and biochemical roles. The evidence for their differing roles includes high conservation among vertebrate species, tissue specificity, and well-defined subcellular location [145]. The β1 isoform has been shown to be highly expressed in muscle tissue with a ratio of β1 to β2 approximately 2:1, whereas the β2 isoform is predominately expressed in non-muscle tissue [143, 145, 147]. Furthermore, the β1 isoform localizes to the Z-disc in striated muscle, whereas the β2 isoform localizes to the intercalated discs and plasma membrane [145]. Overexpression of the β1 isoform in cardiac tissue causes disruptions in the intercalated discs, and overexpression of the β2 isoform causes severe malformations of the myofibril architecture [147].

The CapZ α/β complex is shaped liked a mushroom [148]. Both subunits have similar secondary structure and are arranged such that the molecule has a pseudo-twofold axis of rotational symmetry [148]. The COOH-terminal ends of both of the α and β subunits have amphiphilic α-helices which bind actin on the hydrophobic side [148–150]. The hydrophilic side of the COOH-terminal ends has isoform-specific charge distributions and may be involved in isoform-specific recognition of target ligands [148]. Thus, the tentacles present on CapZ are hypothesized to allow binding of both actin and a specific target protein. The separation of the two C-terminal ends ensures that each tentacle binds a single actin monomer at the end of filamentous actin (F-actin). This property explains the inability of CapZ to bind monomeric actin (G-actin) and its strong association with F-actin. Furthermore, studies have shown that when CapZ is only bound to F-actin by its β-tentacle, the molecule is able to "wobble" and thus exposes additional actin and target binding sites to other molecules [151]. Although it was initially believed that the β tentacle was the only mobile region on CapZ [152], new data suggest that CapZ is intrinsically flexible allowing it to interact with the barbed-end of actin in both a high- and low-affinity state [153].

The abundance of PKC anchoring proteins at the cardiac Z-disc indicates a significant role for both the Z-disc and CapZ in PKC-dependent signaling pathways. Wild-type CapZ binds F-actin with sub-nanomolar affinity (~0.1 nM) and it has been shown through multiple studies that the C-terminal domain is necessary for high-affinity actin binding [150, 154]. Any modifications or mutations that affect the C-terminal ends may greatly reduce the affinity of CapZ to F-actin [154]. CapZ was believed to bind to actin in a two-step model whereby electrostatic interactions with the α subunit dictate the on-rate and hydrophobic interactions of actin with the β subunit dictate the off-rate [155]. Based on affinity and X-ray crystallography studies, an improved mechanism by which CapZ binds actin is as follows: (1) basic residues on the α tentacle interact electrostatically with the barbed end of actin, (2) a conformational change of CapZ to a high-affinity form occurs, and (3) supportive binding of the β tentacle strengthens the association [154]. Thus, a factor or mechanism that disturbs any of the binding or unbinding steps may inhibit the capping ability of CapZ or promote uncapping. It takes nearly 10 min to translocate PKCε to the Z-disc, suggesting intramolecular rearrangement of its binding partners and surfaces at the Z-disc [112]. It has also been suggested that the regulatory binding region of PKCε is obscured until stimulation [156]. The treatment of cardiomyocytes with the hypertrophic cytokine endothelin-1 or the α_1-adrenergic receptor agonist phenylephrine caused an alteration in CapZ dynamics through a PKC- and PIP2-dependent pathway that decreased the affinity of CapZ to F-actin [137]. Recent work from our group is focused on mechanical stimulation and CapZ dynamics [157]. The alteration of CapZ dynamics and posttranslational profile in response to mechanical strain is dependent upon both the subunit isoform of CapZβ and the activity of PKCε. The dynamics data suggest that CapZβ is only able to respond to mechanical strain in a PKCε-dependent manner, and the proteomic data corroborates this finding by showing all modifications of CapZ are abrogated when cells are infected with an adenovirus expressing dominant-negative PKCε. The mechanism through which strain and PKCε are integrated on CapZ remains unknown but could be due to RACK binding, HDAC binding, or other unknown interactions [113, 158–161]. Ultimately, the increase in the actin uncapping rate may be adaptive in that it disrupts the structure of the Z-disc allowing new actin filaments to polymerize or insert, which leads to a global remodeling that decreases the localized load, and therefore strain, at the Z-disc. This feedback loop would support clinical findings of cardiomyopathies in situations of increased load and constitutively active PKCε overexpression in transgenic animals [105, 162, 163].

CapZ is also regulated by the phospholipid signaling pathway, with protein dynamics affected by PIP2 for neonatal myocytes undergoing hypertrophy in culture [137]. PIP2 is the most abundant of the phosphoinositides known to bind cellular proteins, though it accounts for approximately 1 % of lipid in the plasma membrane of a typical mammalian cell [164]. It has been reported that PIP2 might regulate the calcium-insensitive CapZ [142, 165–168]. PIP2 has been assumed to be the binding partner of CapZ and yet this lipid dissociates the CapZ-actin complex [169, 170]. PIP2 binding of CapZ results in a reduction in binding affinity of the COOH-terminal extensions of the CapZ dimer subunits for the actin filament [137, 171]. Addition of PIP2 to capped actin filaments in a polymerization reaction leads to an increase in the polymerization rate consistent with complete and rapid uncapping [151, 172, 173]. According to the computational analysis, the C-terminal half of CapZ β-subunit could contribute to lipid interaction/insertion [174]. CapZ may bind up to four PIP2 molecules, through direct hydrogen-bonding interactions with the binding sites [174]. Furthermore, treatment of cardiac myofilaments (in which only the CapZ β1-subunit is present) with PIP2 extracts CapZ and depresses myofilament tension generation [175]. PIP2 pathways increase uncapping when myocytes are growing [137].

Other Z-Disc Proteins Involved in Cardiac Remodeling and Hypertrophy

Much has been learned about important proteins relevant to the biophysics of heart failure from studies of humans with DCM, which is characterized primarily by left ventricular dilation and systolic dysfunction. This literature has been extensively reviewed elsewhere [176–179]. The recognition that genetic defects may play pivotal roles in the pathogenesis of DCM, especially familial DCM, has received increasing attention during the past decade. Mutations in multiple cytoskeletal and sarcomeric genes (dystrophin, vinculin, desmin, titin, actin, β-myosin heavy chain (MHC), troponin T, and so on) have been linked to the pathogenesis of familial DCM in both human and mouse models.

Mutations in Z-line alternatively spliced PDZ-motif protein (ZASP), a human orthologue of Cypher, have been identified in patients with isolated non-compaction of the left ventricular myocardium (INLVM), DCM, hypertrophic cardiomyopathy (HCM), as well as skeletal myopathy [180–182]. Cypher is a member of the PDZ-LIM domain family and complexes directly with Z-line-associated proteins, such as α-actinin (actinin-2), thus playing a critical role in muscle ultrastructure and function by maintaining Z-disc integrity. Global Cypher-null mice are postnatal-lethal with severe defects in striated muscle, including a congenital form of DCM [183]. In addition to its developmental roles, Cypher also plays a critical role in the adult heart, as cardiac-specific deletion in mice causes a severe form of DCM resulting in premature adult lethality [184]. Cardiac and skeletal muscle each contain two long isoforms, which have three C-terminal LIM domains and may have a signaling role in addition to a structural role at the Z-disc, and one short isoform without LIM domains, which is primarily localized to the Z-disc. Cypher has two major isoforms. Cypher short (CypherS) isoforms (2c, 2s) are barely detectable during embryogenesis but are strikingly induced postnatally in both cardiac and skeletal muscles. In contrast, Cypher long (CypherL) isoforms (1c, 1s, 3c, 3s) exhibit consistent expression patterns from embryonic stages to adulthood. CypherL and CypherS isoforms contain a common PDZ domain, which recognizes the C-terminus of α-actinin, calsarcin-1, and myotilin. In humans, a ZASP mutation (R268C) that affects only short isoforms is associated with skeletal myopathies, while ZASP mutations (I352M, D626N, T350I D366N, Y468S, Q519P, P615L) that affect only long isoforms are primarily associated with cardiomyopathies, some of which affect specific signaling pathways such as those activating PKCs (PKCα, β, and ε). Selective deletion of long but not short Cypher isoforms leads to late-onset DCM with PKC activation [184]. The long isoform of Cypher directly interacts with PKC isoforms (α, β, ζ, γ, and ε) via their LIM domains and can be phosphorylated by PKCβ1. As discussed above, PKC signaling plays a critical role in the development of cardiac hypertrophy, and in vivo models of pressure overload-induced hypertrophy and human heart failure are associated with increased activity of a number of PKC isoenzymes. Studies in patients with Cypher/ZASP mutations identified a particular gain-of-function mutation within the Cypher/ZASP third LIM domain (D626N) that is causative for DCM and enhances the affinity of PKCs for Cypher/ZASP. The identities of all the binding partners of PKCs at the Z-disc remain unknown, though some have suggested Cypher-1 and enigma homologue protein, PDZ domain containing proteins that also bind α-actinin, several RACKs, and F-actin [159, 185–187].

Formin is another protein involved in actin capping and filament assembly. The localization of the cardiac-specific formin, FHOD3, appears to vary with filament maintenance or during rapid filament assembly that is correlated with mechanical work the cell is able to do [188–191]. The Ehler group proposes that FHOD3 is at the Z-disc in the resting state but is found from the adjacent of Z-disc through the overlap region of the thick and thin filament during growth [189]. Precedent for this is found in drosophila muscle, where an actin-associated protein (SALS) also localizes closer to the pointed end of the thin filament during development of the body wall muscles, but re-localizes to Z-disc in the adult [192]. However, there is still some dispute about this explanation, in which a Japanese group claimed that FHOD3 mainly localizes to a region distant from the Z-disc in either the developing or resting stage. This is probably because of the antibodies directed to different regions of FHOD3. The antibodies of the Japanese group are against 650–802,873–974 and C-20, but the Ehler antibody recognizes 1–339, the N-terminal region of FHOD3. FH2 is the assumed catalytic domain of formin, which is much closer to C-terminus (~1,100–1,483), and associates with F-actin at the barbed end [188]. Perhaps, altered protein posttranslational modification results in different accessibility of the different antibodies along the length of the filament. More research is needed to understand how formin regulates actin assembly induced by mechanical stimulation.

The formin family of proteins stimulates actin assembly directly at the barbed end both in vivo and in vitro [193]. Formins are relatively large (100–200 kDa) multi-domain proteins, compared to CapZ (32–33 kDa). Formins usually dimerize to be functional with two FH2 domains forming a donut-like structure that wraps around the actin filament and controls its processive elongation. The FH1 domain recruits profilin-bound actin and drives rapid actin assembly from the profilin-actin pool [194]. Importantly, many formins can be autoinhibited. The mechanism of autoinhibition is most studied in the diaphanous-related formins (DRFs) where interaction between the diaphanous autoregulatory domain (DAD) and the diaphanous inhibitory domain (DID) is sufficient for autoinhibition [195]. FH1/FH2 domain-containing proteins, FHOD1 and FHOD3, are sometimes classified as DRFs, with FHOD1 first discovered in the spleen [196] and FHOD3 in heart, kidney, and brain [197]. Two isoforms of FHOD3 exist, and the heart contains only the larger one [189, 198]. FHOD1 is phosphorylated at three specific sites within C-terminal DAD by the ROCK [199], which releases the autoinhibition of FHOD1 and leads to F-actin stress fiber formation in endothelial cells [188]. Using an actin polymerization assay in vitro, formin blunted the capping property [200] but, as of yet, there is no evidence showing these proteins directly interact [201].

Conclusions with Respect to the Biophysics of the Failing Heart

Individual myocytes in the human ventricle remodel their sarcomeres over time in response to the mechanical forces experienced. This chapter reviews how biophysical forces modulate the costamere and Z-disc for sarcomere remodeling in heart failure. At each subcellular location, it is the sum of the internally generated force of the contractile filaments and the externally imposed by the stresses and strains of the other working myocytes. Mechanotransduction converts these local forces into chemical signals for myocyte assembly to begin. Since, in the failing heart, the local forces vary from one region of the ventricle to another, sarcomere remodeling also varies from cell to cell resulting in populations of myocytes in one region becoming longer or stronger over time. It is this slow remodeling process that eventually becomes maladaptive exacerbating rather than resolving the ability of the heart to pump blood effectively.

The biophysics of cardiomyocyte mechanotransduction is discussed in detail for the two major structures that drive sarcomere remodeling in heart failure, namely the costamere and the Z-disc. The muscle is highly anisotropic so biophysical force transmission depends on both the longitudinal or transverse direction of the force as well as the balance between outside-in and inside-out force generation. For the costamere, the earliest steps in new sarcomere assembly are described in terms of the attachment to the ECM and local force transmission. The most important molecules in the mechanotransduction and assembly processes of the costamere are the integrins, talin dimers, focal adhesion proteins, and other protein kinases. The forces are transmitted from the costamere internally to the Z-disc permitting an extensive amplification of filament assembly throughout the width and length of the myocyte. Therefore, the specialized structure and proteins of the Z-disc are discussed, especially the actin capping protein CapZ.

Despite the vast amount of knowledge of the multi-protein complexes of the costamere and Z-disc, there is currently no clinical strategy to reduce or prevent the maladaptive cardiac remodeling that occurs at the subcellular level of each myocyte in the heart. Most of these same local responses of the myocyte to force are advantageous in remodeling to exercise making it challenging to determine the differences between the two in order to redirect a failing heart down a healthier path.

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Heart Failure: The Final Frontier for Biophysics in Cardiovascular Medicine?

Luis F. Santana

Excitation Transcription Coupling, the New Frontier in HF Therapeutics

Alteration in how the cardiomyocyte manages intracellular calcium is not only important acutely in alteration of the cardiac action potential leading to potentially arrhythmogenic changes within the myocardium, but long-term, global changes in intracellular calcium have been linked to hypertrophy and heart failure [1–3]. Recent work implicates the NFATc3 transcription factor as a key player that translates these changes in intracellular calcium into changes in gene expression, ion current remodeling, and ultimately reshaping of the cardiac action potential via reduction in repolarizing Kv currents. As early as 48 h post MI, this reduction in repolarizing K+ currents leads to an increase in action potential duration (APD), QT interval prolongation, and thereby increases the probability for developing potentially life-threatening arrhythmias [4]. Under more chronic conditions, this reshaping of the cardiac action potential leads to a global increase in intracellular calcium via an increase in the open probability of the LTCC due to prolongation of phase 2 of the cardiac action potential and ultimately activation of genes leading to hypertrophy and HF. Data suggest that the initiating event for these changes in intracellular calcium is the increase in β-AR stimulation seen with the catecholamine surge during acute MI or decompensated HF [5, 6].

With the exception of acutely decompensated HF, it is clear that beta-blockers are important in improving clinical outcomes following infarction. Numerous randomized control trials have shown that the use of beta-blockers in HF not only leads to subjective improvement of NYHA class, but leads to an increase in survival and decreased hospitalizations [7–9]. Brophy et al. in a 2001 meta-analysis that included 22 trials involving more than 10,000 patients with left ventricular ejection fraction (LVEF) $<$35–45 % noted that beta-blockers significantly reduced 1-year

L.F. Santana, Ph.D. (✉)
Department of Physiology & Biophysics, University of Washington, Seattle, WA 98195, USA

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mortality (OR 0.65; 95 % CI 0.53–80) and estimated that the use of beta-blockers saved 3.8 lives per 100 patients treated in 1 year. In the same study the use of beta-blockers significantly reduced hospitalizations for heart failure (odds ratio 0.64, 95 % CI 0.53–0.79) with an absolute benefit of four fewer hospitalizations in the first year per 100 patients treated. Based on these findings, the authors concluded "beta-blocker therapy is associated with clinically meaningful reductions in mortality and morbidity in patients with stable congestive heart failure and should be routinely offered to all patients similar to those included in the trials" [7]. Major society guidelines including ACC/AHA (2005 guidelines), Heart Failure Society of America (HFSA, 2006 guidelines), and the 2006 European Society of Cardiology (ESC, 2006 guidelines) recommend the use of beta-blockers in conjunction with ACE inhibitors regardless of NYHA class [10–14].

Despite this preponderance of evidence of the utility of beta-blockers to improve clinical outcomes, we are only recently beginning to understand this benefit at the molecular level. Increases in PKA activity, via activation of the βAR can illicit dramatic changes in calcium handling within the cardiomyocyte. These changes include an increase in I_{ca} via phosphorylation of the LTCC, increased SR calcium release as observed with increased calcium transients, and the quantal release of calcium from the SR, known as calcium sparks both as a result of phosphorylation of the RYR. Multiple studies have implicated the Ca^{2+}-activated phosphatase, calcineurin (Cn), as a pivotal player, in translating these changes in intracellular calcium to changes in gene expression via the activation of NFATc3 [2–4]. On activation, Cn dephosphorylates NFATc3, allowing for its translocation to the nucleus where it modulates the transcription of multiple genes within the cardiomyocyte including the voltage-gated potassium channels Kv 1.2, 2.1, 4.2, 4.3, as well as the Kv4 accessory protein, KChIP2 that acts as a molecular chaperone to increase surface expression of Kv 4 proteins. Together these channels and accessory proteins comprise the molecular entities that underlie the repolarizing Kv currents I_{to} (Kv 4.2, 4.3, and KChIP) and I_{sust} (Kv 1.5 and 2.1) that shape phase 2 and 3 of the cardiac action potential. A reduction in the expression of these channel proteins occurs as rapidly as 48 h after infarction, leading to elongation of the cardiac action potential, which in itself leads to an increased influx of intracellular calcium via increasing the activity of the LTCC and increasing SR calcium load [4, 15, 16]. Ultimately, these changes perpetuate the changes in intracellular calcium signaling establishing an intracellular environment that promotes sustained activation of Cn/NFAT, the genes responsible for hypertrophy and ultimately the HF phenotype. The use of beta-blockers, in a mouse model of infarction was shown to prevent these changes in intracellular calcium and thereby prevent activation of the Cn/NFAT signaling pathway [4]. Importantly, in the same study, the use of beta-blockers also prevented the down regulation of repolarizing Kv currents and at a molecular level, preventing the down regulation of Kv 1.5, 2.1, 4.2, and 4.3 mRNA and protein [4]. Adding more weight to his hypothesis, pharmacological inhibition of NFAT (with CsA or FK506) or the use of NFAT null animals in similar experiments prevented the down regulation of Kv transcript and protein, and thereby prevented pathological ion current remodeling [4]. Conversely, overexpression of a constitutively active form of

NFAT was shown induced reduction in Kv currents, channel proteins, and transcript similar to that seen after infarction. These findings provide a mechanism for the cardioprotective effects of beta-blockers, both acutely (ion current remodeling) and chronically (HF).

Refining of the Model

The cell sees changes in cytosolic [Ca] that varies from beat to beat with contraction of the cardiomyocyte; however, there are clearly some changes in intracellular calcium that lead to variations on genes expression. Numerous groups have worked to gain a better understanding of this signal that leads to the pathological changes repolarizing Kv currents and hypertrophy/HF. Studies have shown that in the mammalian heart there is a striking difference in the amplitude of I_{to} between cells isolated from the epicardium and the endocardium of the left ventricle [17, 18] with I_{to} being larger in epicardium [17, 19, 20]. This transmural gradient in I_{to} function is important for normal ventricular repolarization [19, 21, 22]. Calcium signaling also differs between the endocardium and epicardium with in the left ventricle. Dilly et al., described difference in both diastolic and systolic [Ca(2+)](i) which was higher in paced endocardial cells in comparison to their epicardial counterparts [15]. They noted that while differences in the action potential waveform could account for some of these differences in intracellular calcium, the amplitude of the $[Ca^{2+}]_i$ transient itself was larger. This study went on to describe spontaneous Ca^{2+} spark activity that was almost threefold higher in the endocardium and that expression of the ryanodine receptor type 2 (RyR2) was nearly twofold higher in endocardium in comparison to the epicardium. Additionally, they observed that efflux of calcium during the cardiac action potential via activity of the Na^+–Ca^{2+} exchanger was reduced in the endocardium. Interestingly, this study did not show a difference in the trigger of CICR, showing no difference between L-type calcium current between the endocardium and epicardium. The authors proposed that transmural differences in AP waveform, SR Ca^{2+} release, and Na^+–Ca^{2+} exchanger function together underlie differences in intracellular calcium across the left ventricular free wall.

Based on these observations and the recently established role of the Cn/NFATc3 signaling pathway in down regulation of repolarizing Kv currents after MI, Rossow et al. proposed that this pathway played a role in establishing the I_{to} gradient under normal physiological conditions, due to variations in calcium signaling across the left ventricle [16]. In support of this, this study found that both Cn and NFAT activity were higher in the endocardium when compared to the epicardium using a luciferase reporter mouse to compare NFAT activity between the two cell types. The study found that differential expression of I_{to} between the cells isolated from the epicardium and endocardium resulted from NFATc3-dependent regional differences in *only* Kv4.2 expression. Providing further evidence of the pivotal role of the Cn/NFAT pathway in establishing this gradient, NFAT null animals showed no difference in I_{to} expression between the endocardium and epicardium. The study went on to describe a calcium-sensitive threshold for down regulation of other proteins contributing to AP repolarization via the outward Kv current, including Kv 4.3, Kv 1.2, Kv 1.5, and KChiP, all of which like Kv 4.2, contain multiple NFAT binding elements within their 5' UTR. While Kv 4.2 was the only gene that showed regional differences in expression under normal physiological conditions, Kv 4.3, Kv 1.2, Kv 1.5, and KChiP all showed down regulation after MI [4]. The mechanism underlying the differential expression of the RYR between the endocardium and epicardium is unknown, but it is reasonable to speculate that it too may be regulated via regional differences in gene expression regulated by this gradient in intracellular calcium similar to its Kv counterparts. In these studies evidence suggests that increased calcium leads to increased activity of Cn/NFAT which leads to down regulation of Kv 4.2 expression, this leads to reduction in the repolarizing Kv current and increased APD and further increases in $[Ca^{2+}]_i$, establishing the transmural gradient under normal physiological conditions. Further up-regulation of this pathway under pathological conditions such as post MI or decompensated HF, driven by a dramatic increase in catecholamines, leads to further increases in intracellular calcium and further down regulation of the molecular entities that compose these currents. These changes promote resetting of intracellular $[Ca^{2+}]_i$ to perpetuate these changes once established leading to HF and hypertrophy.

Despite the preponderance of evidence in support of this model, the source of calcium responsible for pathological hypertrophy has not been clearly defined. The role of the LTCC in CICR and contraction has been well described. These channels are predominantly localized in the T-tubules, in close apposition to the ryanodine receptor composing the fundamental signaling complex for CICR. However, recent evidence suggests that *this* calcium signal may not be the etiology of pathologic changes within the myocyte that lead to hypertrophy. In addition to the Cn-NFAT signaling pathway described above, other calcium signaling pathways including CaM Kinase and PKC have been implicated in pathological hypertrophy (ref). Mounting evidence suggests that in the intact heart simple increases in diastolic Ca^{2+} alone may not account for activation of these signaling pathways, as such the source of the calcium signal that is responsible for the initiation of this cascade of events has yet to be definitively established. Potential sources include the LTCC [23, 24], T-type calcium channel [25, 26], and TRP channels [27].

Makarewich et al. in a recent publication have proposed an intriguing model that describes two disparate populations of L-type calcium channels responsible for what they have dubbed as "contractile calcium" and small population of LTCCs within caveolae responsible for "hypertrophic calcium" [28]. This subpopulation of LTCC was previously described by Nichols et al. [29], which are stabilized by scaffolding protein cavelolin-3, the predominant caveolin in heart, forming a microdomain for localized calcium signaling. In this study the authors used a novel LTCC inhibitor, REM, which they targeted to cav-3 containing membranes, selectively targeting the LTCC with in the caveolae microdomains. The authors were able to selectively inhibit this subpopulation of LTCCs without altering whole

L-type calcium currents or affecting contractility. Interestingly, the selective inhibition of this population of calcium channels prevented the Ca^{2+}-induced translocation of NFAT, presumably by disrupting the calcium signaling complex required for activating of this transcription factor.

Further underscoring the importance of localized calcium signaling within discrete microdomains within the cardiomyocyte, in the study by Nichols et al. describing this subpopulation of LTCCs associated with the scaffolding protein AKAP 150 (also called AKAP150/79), this scaffolding protein was shown to also target adenylyl cyclase, PKA, and calcineurin to caveolin-3 enriched membranes within the t-tubule forming a microdomain for calcium signaling. In this study the authors used cardiomyocytes isolated from wild type and AKAP null mice to investigate the role of this signaling complex in β-adrenergic signaling. The study found that β-adrenergic augmentation of calcium transients and the phosphorylation of substrates involved in calcium handling were disrupted in AKAP5 knockout cardiomyocytes. The authors found that under normal conditions, only the caveolin 3-associated Cav1.2 channels are phosphorylated by PKA in response to sympathetic stimulation in wild-type heart. However, with loss of this signaling complex in AKAP5 null hearts, adenylyl cyclase 5/6 no longer associated with caveolin 3 in the T-tubules, and noncaveolin 3-associated calcium channels become phosphorylated after β-adrenergic stimulation. Functionally, the disruption of this complex leads to the loss of increase in the calcium transient normally seen in wild-type animals in response to β-adrenergic signaling. The authors went on to show that this signaling microdomain orchestrated by AKAP5 was also essential for the PKA-dependent phosphorylation of ryanodine receptors and phospholamban. These findings demonstrate that AKAP5 anchoring protein is essential in organizing a signaling complex that is associated with caveolin-3 within the t-tubules and is essential for sympathetic modulation of the calcium transient in the heart. These observations of spatially localized calcium signaling microdomains acting on functionally disparate populations of calcium channels raises the exciting possibility of a novel therapeutic targets for treatment if HF and hypertrophy that does not effect contractility, a tool that would be useful during decompensated HF when traditionally the use of beta-blockers has been contraindicated due to the negative inotropic effects.

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Arrhythmogenesis, Heart Failure, and the Biophysics of Z-Band Protein Networks

M. Vatta and R. John Solaro

Introduction

Cardiac function is determined by exquisite and finely tuned electromechanical activity of each myocyte, which in partnership with neighboring cells achieves a synchronized contraction and relaxation of the ventricular chambers to attain proper blood flow into systemic circulation. The complex cellular scaffold of each cardiomyocytes, the cytoskeleton (see also Chaps. 6 and 10), has been recently shown to be tightly associated with proteins forming channels, pores, and pumps that allow ions to move in and out the cellular barrier, and across different cells to provide the appropriate electrical stimulus throughout the heart. Here we consider some recent understanding of the connection between a subset of the cytoskeleton, the Z-band, and representative ion channels involved in the development of cardiac failure and arrhythmias.

Structure of the Normal Cardiac Muscle

Cardiac muscle fibers are comprised of separate cellular units (myocytes) connected in a functional syncytium [1], whereas skeletal muscles form a true syncytium with the fusion of multiple peripherally nucleated myocytes into fibers. Cardiomyocytes contain one or two central nuclei and they are rod-shaped cells that

M. Vatta (✉)
Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, USA
e-mail: mvatta@iu.edu

R. John Solaro
Department of Physiology and Biophysics, Center for Cardiovascular Research, University of Illinois at Chicago, Chicago, IL 60611, USA

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bifurcate and recombine to form a complex three-dimensional network, joined at each end to adjacent myocytes at the intercalated disc, a specialized structure of the cell membrane. The intercalated disc contains gap junctions, with connexins being the major components, and mechanical junctions, comprised of adherens junctions containing N-cadherin, catenins and vinculin, and desmosomes, which are formed by desmin, desmoplakin, desmocollin, and desmoglein. The cardiomyocyte cell membrane, also called sarcolemma, contains several transmembrane proteins such as adrenergic receptors, structural glycoproteins, and the major ion channels involved in the cell membrane potential change, which is at the origin of the cardiomyocytes depolarization and repolarization pattern [2]. Beneath the sarcolemma, each cardiac cell contains bundles of longitudinally arranged myofibrils, which form the core of the contractile apparatus.

The Contractile Apparatus

The myofibrils contain repeating constituents called sarcomeres, which are the elemental contractile unit of the striated muscle and represent well-organized cytoskeletal structures responsible for force generation and transmission. Sarcomeres contain intertwined thin (actin) and thick (myosin) filaments (Fig. 1) that give the muscle its characteristic striated appearance [3, 4]. The thick filaments are composed primarily of myosin but additionally contain myosin-binding proteins C, H, and X. The thin filaments are composed of cardiac actin, α-tropomyosin (α-TM), and troponins T, I, and C (cTnT, cTnI, cTnC). Myofibrils also contain a third filament formed by the giant filamentous protein titin, which extends from the Z-band to the M-line and acts as a molecular spring during contraction and relaxation of cardiac muscle. The Z-band at the borders of the sarcomere is formed by a network of intertwined proteins that preserve myofilament organization by cross-linking antiparallel titin and thin filaments from adjacent sarcomeres. This antiparallel organization is mainly assembled by the Z-band protein α-actinin, a pivotal component of the sarcomere, which along with other proteins in the Z-band such as nebulette, telethonin/T-cap, capZ, MLP, Ankrd1/CARP, myopalladin, myotilin, Z-band Alternatively Spliced PDZ motif protein (ZASP), filamin, and FATZ, provides the structure of the Z-band and molecular coupling between sarcomere contraction and relaxation components [3–6].

The Connection Between Sarcomere and Sarcolemma

The sarcomere is anchored to the sarcolemma and the extracellular matrix (ECM) through a complex network of proteins, thus providing structural support for subcellular structures and transmitting mechanical and chemical signals within and between cells (Fig. 1). Among the components of this complex protein network

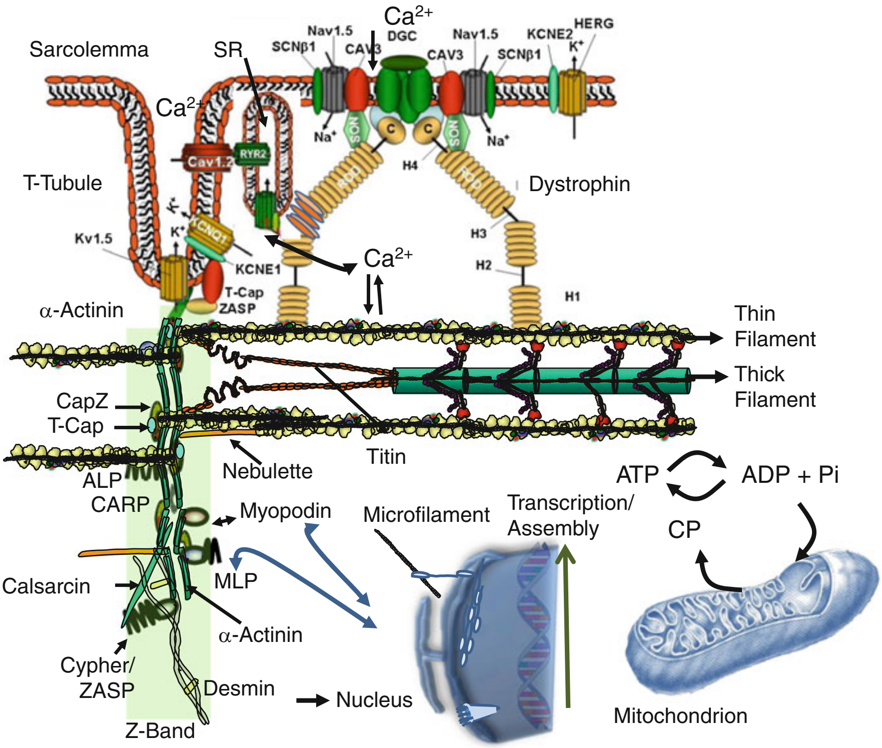

Fig. 1 Schematic illustration of the network linking Z-band and thin and thick filament proteins to cellular elements controlling electro-chemical coupling, energetics, and transcription. As described in the text, the figure depicts a cytoskeletal network whereby t-tubular, sarcomlemmal, and sarcoplasmic reticulum (SR) channels are under the influence of mechanical strains on dystrophin and the myofilaments. Dystrophin complex proteins also connect the extracellular matrix. The Z-band is shown as a structural element as a well as a hub of signaling via kinase/ phosphatase docking and regulation as well as docking of transcription factors that shuttle back and forth to the nucleus. Desmin filaments surround the Z-band, allowing for connections to the nucleus, longitudinal connections to adjacent Z-bands and lateral connections to sub-sarcolemmal costameres (not shown). The protein network forms a means of outside in and inside out mechanical and chemical communication between cellular elements

connecting the sarcomere to other cellular compartments there are intermediate filaments (IFs), microfilaments, and microtubules [7–9]. The IFs form a three-dimensional scaffold made mainly by desmin filaments surrounding the Z-band, allowing for longitudinal connections to adjacent Z-bands and lateral connections to sub-sarcolemmal costameres [8, 10]. Costameres are subsarcolemmal domains forming a intermittent, grid-like pattern, which flank the Z-bands and overlaps the I band (see Chap. 7). Costameres are molecular structures connecting cytoskeletal networks linking sarcomere to sarcolemma, thus providing cell shape maintenance and integration of pathways involved in mechanical force transduction. Costameres contain three principal components: the focal adhesion-type complex, the spectrin-based complex, and the dystrophin/dystrophin-associated protein complex (DAPC) [10, 11]. The focal adhesion-type complex, formed by cytoplasmic proteins such as vinculin, talin, tensin, paxillin, zyxin, associates with the cytoskeletal actin filaments and with the transmembrane proteins α-, β-dystroglycan, α-, β-, γ-, δ-sarcoglycans, dystrobrevin, and syntrophin, all components of the dystrophin-associated glycoprotein complex (DGC). The C-terminus of dystrophin links to β-dystroglycan (Fig. 1), which in turn interacts with α-dystroglycan to link to the ECM via α-2-laminin [10, 11]. The N-terminus of dystrophin interacts with actin as the main site, although a secondary actin-binding site resides in the rod-domain of dystrophin, where the spectrin-like repeats 11–17 of dystrophin appear to bind actin independently of its amino-terminal domain [12, 13]. The actin-binding sites present at the amino-terminal and rod domains of dystrophin produce a stronger association with the actin filaments, thus making a more effective mechanical stabilization of the sarcolemma and ensuring to maintain the integrity of the cell membrane during muscle contraction [13].

In addition to its structural role, dystrophin, along with syntrophin, has been shown to associate and modulate the activity of the voltage-gated sodium channel Nav1.5, which is coded by the *SCN5A* gene mapping to 3p21 [14–16]. Besides dystrophin and syntrophin, Nav1.5 has been shown to associate with several other proteins, such as β-spectrin, ankyrin, caveolin-3, calmodulin (CaM), and N-cadherin among others [17]. In fact, although the sodium channel's main function is to provide a regulated conductance of Na ions into the cardiomyocytes during the action potential, Nav1.5 is part of a large multiprotein complex associating with specific cytoskeletal, regulatory, trafficking, cell adhesion, and gap junction proteins, some of which can affect its biological and/or biophysical properties [17].

In addition to the sarcolemma, Nav1.5 is also known to associate with the sarcomeric Z-band, representing an important connection between these subcellular compartments. The spectrin-like repeats of α-actinin-2, a major component of the sarcomeric Z-band, provide a physical interaction with the cytoplasmic loop connecting domains III and IV of Nav1.5 [18]. Although α-actinin-2 does not appear to affect the kinetics of the sodium channel, it increases sodium channel density on the surface of the cell membrane [18]. Thus, α-actinin-2 not only anchors Nav1.5 to the sarcolemma, but also provides, along with dystrophin and the DGC, an additional connection to the actin cytoskeleton [18]. Besides α-actinin-2, another member of the Z-band, the small protein telethonin/T-Cap, has been reported to physically interact with Nav1.5 [19]. Interestingly, telethonin/T-Cap, not only binds α-actinin-2, but is also able to bind and modulate the *KNCE1*-coded minK, the main beta subunit of the *KCNQ1*-coded alpha subunit constituting the slow delayed rectifier I_{Ks} potassium channel [20].

The role of α-actinin-2 in generating the major scaffold of the Z-band is well known. This structure provides anchoring to most of the proteins localizing at the Z-band such as the *LDB3*-encoding ZASP, a major component of the Z-band in both skeletal and cardiac muscle [21]. ZASP binds α-actinin-2 through the

N-terminal PDZ [postsynaptic density 95 (PSD95), disklarge (Dlg), and zona occludens-1 (ZO-1) proteins] domain and the ZASP-like motif (ZM), which has a role in targeting α-actinin-2 to the Z-band and in stabilizing its conformation to support the Z-band integrity [22]. We have already shown that ZASP is also part of a macromolecular complex able to stabilize and modulate Nav1.5 function [23]. In addition, we have recently demonstrated that ZASP also binds telethonin/T-Cap, which, as mentioned above, has been previously shown to physically interacts with Nav1.5 via forming protein complex with telethonin/T-Cap [18, 24].

Therefore, primary or secondary disruption of the protein continuum from the nucleus to the sarcomere, the sarcolemma, up to the ECM could potentially cause a cascade of events leading to both structural and electrical remodeling, resulting in heart failure and arrhythmias.

Ion Channels and the Normal Cardiac Action Potential

The cardiac action potential is generated by in and out movement of ions through various ion channels, which are pore-forming proteins embedded in the sarcolemma of the cardiomyocytes [1, 2]. The kinetics of cardiac depolarization and repolarization is a key function in cardiac cells, and any alteration caused by disease or drugs may lead to susceptibility to fatal arrhythmias. The heart does not require an external electrical stimulation from the nervous system to initiate a spontaneous depolarization, and this feature is called automaticity. Normally, specialized cells localized at the sinoatrial node (SAN), the so-called pacemaker of the heart, have ion channels on the sarcolemma with reduced permeability to potassium (K^+) but allowing a passive transfer of calcium ions, thus developing a net charge. At the molecular level, the automaticity is determined by the activity of channels belonging to the family of the Hyperpolarization-activated, Cyclic Nucleotide-gated (HCN) pacemaker channels, which comprises four isoforms (HCN1–4) and lead to the generation of the so-called funny current (I_f) [25]. The HCN1–4 isoforms, coded by separate genes, generate subunits that can assemble as homotetramers and/or heterotetramers to form functional channels. Each subunit follows the classical structure of other voltage-gated channels comprised by six membrane-spanning domains (S1–S6), with a putative voltage sensor (S4) and a pore-forming (P) region between the S5 and S6 segments [25]. The HCN4 isoform appears to be the major component of the pacemaker channels in the SAN, while HCN3 is absent from SAN and HCN2 is the prevalent subunit expressed in atria and ventricles [25]. The HCNs are poorly selective cation channels, able to conduct both potassium and sodium ions, which increase their conductance as the membrane hyperpolarized. The activity of these channels in the SAN cells causes the membrane potential to slowly depolarize, thus preparing the cells for the following action potential.

Other specialized cells capable of automaticity are those localized at the atrioventricular (AV) node, although they provide cardiac contraction at lower rate than

SA cells, while also ventricular cardiomyocytes may spontaneously start contraction, but at even lower rate than AV node cells and without perfect synchrony.

The standard model to depict cardiomyocytes depolarization uses the action potential occurring in the ventricular myocytes, in which five phases (numbered 0–4) normally repeat themselves in the same sequence over and over. This sequence involves the influx and efflux of ions from the cardiac cell and the propagation of the electrical stimulus to the neighboring cells. *Phase 4* represents the resting membrane potential of non-stimulated ventricular cardiomyocytes. The normal resting membrane potential in the ventricular myocardium is about −85 to −95 mV. If the resting membrane potential level is not properly reinstated, the cardiomyocytes may fail to undergo another excitation wave, and cardiac conduction may be delayed, increasing the risk for arrhythmias.

Phase 0 represents the rapid depolarization phase, which is governed by the opening of the fast voltage-gated Na^+ channels causing a rapid increase in the membrane conductance to Na^+ (G_{Na}) and thus a rapid influx of Na^+ ions (I_{Na}) into the cell. The Na^+ channels are generally closed at resting membrane potential and the excitation wave will cause their opening leading to a massive and rapid influx of Na^+ ions, while a more positive membrane potential will cause some Na^+ channels to be in an inactivated state insensitive to opening, thus causing a reduced response to excitation of the cell membrane and subsequent sodium current. The alpha subunit of the cardiac sodium channel Nav1.5 coded by the *SCN5A* gene (Table 1) is the main ventricular sodium channel isoform, which governs the *Phase 0* of the cardiac action potential is Nav1.5 [2]. Other channels of the Nav1.x family such as the neuronal Nav1.1 (*SCN1A*), Nav1.3 (*SCN3A*), and Nav1.6 (*SCN6A*) isoforms are also expressed in ventricular cardiomyocytes although at lower levels [26].

Phase 1 of the action potential comprises the inactivation of the Na^+ channels and the concomitant activation of transient outward of K^+ and Cl^- flux regulated by the transient outward repolarizing currents I_{to1} and I_{to2} respectively. In particular, the cardiac transient outward potassium current (I_{to1}), which has a fast (I_{to1f}) and a slow (I_{to1s}) kinetic variant in the heart, provides the most significant contribution to the small deflection of the action potential, also known as *Phase 1* repolarization notch, while I_{to2} appears to have a more marginal role [27]. The transient current I_{to1} is generated by a Ca^{2+}-dependent channel formed by the assembly of the *KCND2*-coded (Kv4.2) and *KCND3*-coded (Kv4.3) subunits comprising the fast (I_{to1f}) current, while the *KCNA4*-coded (Kv1.4) and *KCNA7*-coded (Kv1.7) subunits comprising the slow (I_{to1s}) current [28]. Although I_{to1} is a very small current, the high expression and sarcolemmal density of the Kv4.2/Kv4.3 channels in the right epicardial cardiomyocytes causes a more pronounced *Phase 1* notch in epicardial cells compared to mid-myocardial and endocardial myocytes [29]. Interestingly, the transient current I_{to1} can also be modulated by the product of *KCNE2*, a beta subunit of the rapid delayed rectifier K^+ current (I_{Kr}) that is active during *Phase 3* of the action potential [30]. What is the significance of this apparent "crosstalk" between *Phase 1* and *3* remains unknown.

Table 1 Genes coding for major ion channels modulating the cardiac action potential

Gene	Locus	Protein	Gene product	Main current	Action potential phase
SCN5A	3p21	Nav1.5	Sodium channel, voltage-gated, type V, alpha subunit	I_{Na}	0
SCN4B	11q23.3	SCN4B	Sodium channel, voltage-gated, type IV, beta subunit	I_{Na}	0
KCND3	1p13.3	Kv4.3	Potassium voltage-gated channel, Shal-related subfamily, member 3	I_{to}	1
KCND2	7q31.31	Kv4.2	Potassium voltage-gated channel, Shal-related subfamily, member 2	I_{to}	1
KCNQ1	11p15.5	Kv7.1	Potassium channel, voltage-gated, KQT-like subfamily, member 1	I_{Ks}	2
KCNE1	21q22.12	Isk/β1	Potassium channel, voltage-gated, Isk-related subfamily, member 1	I_{Ks}	2
CACNA1C	12p13.3	Cav1.2	Calcium channel, volatage-dependent L-type, alpha-1C subunit	$I_{Ca,L}$	2,3
KCNH2	7q36.1	Kv11.1	Potassium channel, voltage-gated, Subfamily H, member 2	I_{Kr}	3
KCNE2	21q21.12	MiRP1/β2	Potassium channel, voltage-gated, KQT-like subfamily, member 2	I_{Kr}	3
KCNJ2	17q24.3	Kir2.1	Potassium channel, inwardly rectifying, subfamily J, member 2	I_{K1}	3,4
HCN4	15q24.1	HCN4	Hyperpolarization-activated, cyclic nucleotide-gated potassium channel 4	I_f	4

Phase 2 represents the "plateau" phase of the cardiac action potential, which is sustained by a concomitant inward movement of Ca^{2+} (I_{Ca}) through L-type calcium channels and outward movement of K^+ through the slow delayed rectifier potassium channels, I_{Ks}. The sodium-calcium exchanger current, $I_{Na,Ca}$ and the sodium/potassium pump current, $I_{Na,K}$ also play minor roles during *Phase 2*. The calcium channel consists of a complex of alpha-1, alpha-2/delta, beta, and gamma subunits in a 1:1:1:1 ratio. In the heart, the *CACNA1C*-coded α1C subunit Cav1.2 is the main α subunit mediating the L-type calcium currents, which leads to a transient increased in intracellular calcium that induced further calcium release from the endoplasmic reticulum (ER) reservoir via ryanodine receptors channel [2]. The slow delayed rectifier potassium I_{Ks} current is generated by the *KCNQ1*-coded Kv7.1 α subunit and by the *KCNE1*-coded β subunit minK/Isk [2].

Phase 3 represents the rapid repolarization phase of the action potential in which the L-type Ca^{2+} channels close, while the slow delayed rectifier (I_{Ks}) K^+ channels remains open, thus ensuring a net outward current, leading to a negative membrane potential that causes other K^+ channels, such as those generating the rapid delayed rectifier K^+ current (I_{Kr}) and the inwardly rectifying K^+ current (I_{K1}) to open [2]. The rapid delayed rectifier I_{Kr} current is generated by the *KCNH2*-coded Kv11.1 α subunit and by the *KCNE2*-coded β subunit MiRP1, while the inwardly rectifying I_{K1} current is generated by the *KCNJ2*-coded Kir2.1 subunit [2]. The sustained and increased net K^+ outward flux leads to the cell repolarization, which completes when the delayed rectifier K^+ channels close after the membrane potential is restored to about −80 to −85 mV.

Phase 4 is the stage in which the resting membrane potential is reestablished and the cell prepares itself for the following external electrical stimulus, typically from an adjacent cell. This phase of the action potential is associated with the cardiac diastole. In *Phase 4* the *KCNJ2*-coded Kir2.1 (I_{K1}) channels remain open, thus contributing to reinstate the resting membrane potential.

Sarcomeric Mutations and Heart Failure

Heart failure (HF) is among the major causes of death in the United States, with approximately five million cases nationwide, including 500,000 new cases and more than 800,000 hospitalizations each year. (http://www.cdc.gov/dhdsp/data_statistics/fact_sheets/fs_heart_failure.htm). Myocardial dysfunction in the end-stage failing heart is very often associated with increasing susceptibility to ventricular tachycardia (VT) and ventricular fibrillation (VF), both of which are common causes of sudden cardiac death (SCD). Although the majority of cases with congestive heart failure are subsequent to coronary artery diseases (CAD), a substantial percentage of subjects that present with primary cardiomyopathies and develop HF due to a genetic etiology.

Primary cardiomyopathies represent a major cause of morbidity and mortality in both children and adults and they are classified in multiple forms with distinct clinical, structural, morphological, and functional presentations. These forms include dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), left ventricular non-compaction (LVNC), arrhythmogenic right ventricular cardiomyopathy (ARVC), and restrictive cardiomyopathy (RCM).

Electric Dysfunction in Heart Failure

Myocardial remodeling is the hallmark of HF irrespective of the underlying etiology such as ischemic cardiomyopathy (ICM) or DCM and is characterized by alterations in baseline ECG, which include the prolongation of the QT interval, as well as QT dispersion, ST-segment elevation, and T-wave abnormalities, especially during exercise. In particular, severe left ventricular dilation, which may occur in DCM can present with left bundle branch block (LBBB), whereas right bundle branch block (RBBB) is more characteristic of right ventricular failure. In heart failure, both LBBB and RBBB have been associated with conduction disturbances such as AV block. All these HF-associated ECG alterations can be appreciated in subjects with primary arrhythmogenic syndromes such as Long-QT syndrome

(LQTS), Brugada syndrome (BRS), or catecholaminergic polymorphic ventricular tachycardia (CPVT), diseases that are not normally associated with obvious morphological and structural changes, such as myocardial remodeling [29].

Notably, treatments aimed at halting or reverting the cardiac remodeling, such as beta blockers, left ventricular assist devices (LVAD), and resynchronization therapies, have been at least partially successful in reducing the rate or severity of arrhythmias in heart failure patients.

In large epidemiological studies, such as the REMATCH study, the employment of LVAD significantly improved survival rate and the quality of life compared to optimal medical management [30], although controversial results have also been reported with an increased rate of new-onset monomorphic ventricular tachycardia (MVT) in patients treated with an axial-flow HeartMate LVAD while no effect was observed on the development of polymorphic ventricular tachycardia (PVT)/ventricular fibrillation (VF).

Contractile Dysfunction in Mutated Sarcomeric Proteins

Mutations in genes encoding sarcomeric proteins play a critical role in most cardiomyopathies. Mutations in the same genes have been identified to cause various forms of cardiomyopathies.

Among the various forms of primary cardiomyopathies DCM and HCM are the most common. DCM is characterized by an enlarged left ventricular chamber, left ventricular wall thinning, and systolic dysfunction [31]. Currently, approximately 33 genes have been identified to cause DCM in isolation, demonstrating a high level of genetic heterogeneity (Table 2), and many private mutations have been observed in unrelated subjects suggesting a high level of allelic heterogeneity. Recently, an important contribution to the genetics of cardiomyopathies came from a study that analyzed patients with DCM diagnosis for mutations in the *TTN* gene mapping to 2q31 and coding the giant filamentous sarcomeric protein titin. Titin has been known since 1999 to be associated with DCM due to the brilliant seminal work of Dr. Benjamin L. Siu (1960–2012) and other investigators [32, 33]. Due to the enormous size of the TTN gene, comprehensive mutational analysis became possible only with the application of next generation sequencing (NGS) technique [34]. At the beginning of 2012, right after the premature passing of Dr. Siu, the group of Dr. Seidman, along with an international collaborative effort, published an article in which *TTN* truncating mutations were identified in approximately 25 % of familial cases of idiopathic DCM and in 18 % of sporadic cases, making *TTN* the most common gene mutated in DCM followed by *LMNA* (5 %), *MYH7* (4 %), and *TNNT2* (3 %) [34]. In addition, to titin, many other sarcomeric proteins have been identified to cause DCM (Table 2).

Another form of primary myocardial disease is HCM, a disease of the sarcomere, which with a prevalence of 1/500, not only is among the most common genetic disorders but also represents the most frequent cause of SCD in young athletes in the

Table 2 Genes associated with DCM, HCM and their allelic disorders

Gene	OMIM[a]	Locus	Gene name[b]	Associated phenotypes[b]
ABCC9	601439	12p12.1	ATP-binding cassette, subfamily C, member 9	DCM
ACTC1	102540	15q14	Actin, alpha, cardiac muscle 1	DCM HCM RCM
ACTN2	102573	1q42–q43	Actinin, alpha 2	DCM HCM
BAG3	603883	10q25.2–q26.2	BCL2-associated athanogene 3	MFM DCM
CSRP3	600824	11p15.1	Cysteine- and glycine-rich protein 3-cardiac LIM protein	DCM HCM
DES	125660	2q35	Desmin	MFM DCM
DMD	300377	Xp21.2	Dystrophin	DMD BMD DCM
DSG2	125671	18q12.1	Desmoglein 2	ARVC DCM
DSP	125647	6p24	Desmoplakin	ARVC DCM
EMD	300384	Xq28	Emerin	EDMD DCM
EYA4	603550	6q23–q24	Eyes absent homolog 4	DFNA DCM
FKTN	607440	9q31	Fukutin	FCMD DCM
HSPB7	610692	1p36.13	Heat shock 27 kDa protein family, member 7	DCM
LAMP2	309060	Xq24	Lysosomal-associated membrane protein 2	DD HCM DCM
LDB3	605906	10q22–q23	LIM domain binding 3	DCM ± LVNC MFM
LMNA	150330	1q21.2	Lamin A/C	DCM HCM
MYBPC3	600958	11p11.2	Myosin-binding protein-C, cardiac	DCM HCM
MYH6	160710	14q12	Myosin, heavy chain 6, cardiac muscle, alpha	DCM HCM RCM
MYH7	160760	14q12	Myosin, heavy chain 7, cardiac muscle, beta	HCM

(continued)

Table 2 (continued)

Gene	OMIM[a]	Locus	Gene name[b]	Associated phenotypes[b]
MYL2	160781	12q23–q24	Myosin, light chain 2, regulatory, cardiac, slow	HCM
MYL3	160790	3p21.3–21.2	Myosin, light chain 3, alkali; ventricular, skeletal, slow	DCM
MYPN	608517	10q21.1	Myopalladin	DCM
NEBL	605491	10p13–p12	Nebulette	DCM
				HCM
NEXN	613121	1p31.1	Nexilin	DCM
PLN	172405	6q22.1	Phospholamban	HCM ± WPW
				FCNCG
PRKAG2	602743	7q36.1	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit	AD
				DCM
				FAI
PSEN1	104311	14q24.3	Presenilin 1	AD-4
				DCM
PSEN2	600759	1q31–q42	Presenilin 2	DCM
RBM20	613171	10q25.2	RNA binding motif protein 20	BRS
				LQTS
				DCM ± CD
SCN5A	600163	3p21	Sodium channel, voltage-gated, type V, alpha subunit	LGMD
				DCM
SGCD	601411	5q33–q34	Sarcoglycan, delta	BTS
				LVNC
				DCM
TAZ	300394	Xq28	Tafazzin	LGMD
				HCM
				DCM
TCAP	604488	17q12	Titin-cap-telethonin	DCM
TMPO	188380	12q22	Thymopoietin	DCM
				HCM
TNNC1	191040	3p21.1	Troponin C type 1	DCM
				HCM
				RCM
TNNI3	191044	19q13.4	Troponin I type 3	DCM
				HCM
				RCM
TNNT2	191045	1q32	Troponin T type 2	DCM
				HCM
TPM1	191010	15q22.1	Tropomyosin 1	TMD
				LGMD
				EOMFC
				DCM
				HCM
				HMERF

(continued)

Table 2 (continued)

Gene	OMIM[a]	Locus	Gene name[b]	Associated phenotypes[b]
TTN	188840	2q31	Titin	DCM HCM
VCL	193065	10q22.1–q23	Vinculin	

AD Alzheimer disease, *ARVC* arrhythmogenic right ventricular cardiomyopathy, *BMD* Becker muscular dystrophy, *BRS* Brugada syndrome, *CD* conduction defects, *CMT2B1* Charcot-Marie-tooth disease type 2B1, *CPVT* catecholaminergic polymorphic ventricular tachycardia, *DCM* dilated cardiomyopathy, *DD* Danon disease, *DFNA* autosomal dominant late-onset progressive nonsyndromic deafness, *DMD* Duchenne muscular dystrophy, *EDMD* Emery–Dreifuss muscular dystrophy, *EOMFC* early-onset myopathy with fatal cardiomyopathy, *FAI* familial acne inverse, *FCMD* Fukuyama congenital muscular dystrophy, *FCNCG* fatal congenital nonlysosomal cardiac glycogenosis, *FPLD2* familial partial lipodystrophy of the dunnigan type, *HCM* hypertrophic cardiomyopathy, *HGPS* Hutchinson-Gilford progeria syndrome, *HMERF* myopathy with early respiratory muscle involvement, *LGMD* limb-girdle muscular dystrophy, *LQTS* long-QT syndrome, *LVNC* left ventricular non-compaction, *ND* Naxos disease, *MADA* mandibuloacral dysplasia type A with partial lipodystrophy, *MFM* myofibrillar myopathy, *RCM* restrictive cardiomyopathy, *TMD* tibial muscular dystrophy, tardive, *WPW* Wolff–Parkinson–White syndrome
The symbol ± indicates that a phenotype can be associated or not with other clinical presentations
[a]*OMIM* Online Mendelian inheritance in man (http://www.ncbi.nlm.nih.gov/omim)
[b]Genetic home references (http://ghr.nlm.nih.gov/)

United States [35]. HCM is characterized by a pathological thickening of the left ventricular myocardium and the interventricular septum not explainable by aortic stenosis or hypertension. HCM usually presents with myocyte disarray, while left ventricular outflow tract obstruction may or may not occur [35]. The interventricular septal hypertrophy usually appears asymmetric but focal areas of septal hypertrophy or concentric hypertrophy have also been reported [35]. Symptoms in HCM may or may not present early in life and many patients can be asymptomatic or present syncope, non-resuscitated sudden death, dyspnea, diaphoresis, chest pain, palpitations, or arrhythmias. The age of onset of HCM-related symptoms varies from infancy to adulthood, although the majority appears in adolescence.

Genetic defects have been identified in most HCM cases and currently, approximately 21 genes, mostly encoding for sarcomeric proteins and mainly involved in force generation, have been identified to cause HCM (Table 2), and many of them are allelic to DCM. Also HCM is characterized by significant genetic and allelic heterogeneity and variable expressivity, and genetic testing performed in the four most common sarcomeric genes, β-myosin heavy chain (*MYH7*), myosin-binding protein-C (*MYBPC3*), cardiac troponin T (*TNNT2*), and cardiac troponin I (*TNNI3*), detect the main culprit in approximately 80 % of all familial HCM cases and in about 40 % of sporadic and idiopathic cases of HCM. Mutations in sarcomeric proteins causing HCM may give rise of variable degrees of severity; i.e., apparently, mutations in *MYH7* are associated with earlier disease presentation, and in some cases, a more severe phenotype, while subjects with *MYBPC3* mutations develop HCM later, while *TNNT2* mutations are associated with a high incidence of SCD. Although the majority of HCM is considered to be a monogenic disorder inherited as an autosomal dominant pattern, double heterozygote mutations, and some cases of autosomal recessive inheritance have been described and appear to be associated with earlier-onset and a more dramatic phenotype.

Mutated Sarcomeric Proteins and Arrhythmias

As discussed in the previous paragraphs, genetic mutations in sarcomeric proteins have been associated with the development of heart failure. The mechanism of myocardial remodeling, mechanical failure, and pump dysfunction have been for the most part elucidated (John, you should add some of your papers). However, contractile dysfunction resulting from primary genetic mutations can frequently be associated with the development of electrical imbalance leading to cardiac arrhythmias. There is evidence that HCM linked to mutations in some sarcomeric proteins alters Ca-binding to cTnC in association with enhanced myofilament sensitivity to Ca^{2+}. The relation between these primary changes in myofilament regulation appears to involve two arrythmogenic mechanisms. One is straightforward and related to hypertrophy/remodeling, which provides a substrate for arrhythmias. Evidence for this mechanism has been reported [36] in which HCM linked cTnT mutations led to Ca^{2+} sensitization. This change in myofilament response to Ca^{2+} induced significant modifications in action potential shape and beat to beat variability in duration with relative short effective refractory periods. At fast heart rates, there was also an increased dispersion of ventricular conduction velocities. These findings led to the hypothesis that myofilament Ca^{2+} sensitization leads to an arrhythmogenic substrate. Interestingly, the data also demonstrated that the greater the influence of the various cTnT mutations on myofilament response to Ca^{2+}, the greater the propensity toward triggered arrythymias. A second mechanism linking myofilament Ca-sensitivity and Ca-binding to arrhythmias has been developed in work reported from the ter Keurs laboratory [37]. These studies provide evidence that nonuniformities of contraction of myocardium, for example a quick release of a myocardial weak segment by relaxation of a strong segment during ischemia, induces a release of Ca^{2+}, which may lead to delayed after depolarization with extrusion of the Ca^{2+} Na/Ca exchanger thereby.

Loss-of-function mutations of sodium channel (Nav1.5) have been recognized as a key mechanism associated with cardiac arrhythmias [2]. Recent studies demonstrated that, in addition to primary genetic defects disrupting Nav1.5 function, sodium current could be affected by defects in non-ion channel proteins or channel interacting proteins (ChIPs) that affect the function of various ion channels, leading to secondary channel dysfunction [38, 39].

In section "The Connection Between Sarcomere and Sarcolemma" we have described the relationship between Nav1.5 and the sarcomere. Thus, it appears intuitive that disruption of specific sarcomeric proteins could directly influence the kinetics of the sodium channel and or its metabolism.

In fact, mutation in the small titin-capping protein of the Z-band telethonin/T-Cap, have been shown to alter Nav1.5 function [19]. Therefore, it is not surprising that mutations in ZASP, which we recently demonstrated to bind to telethonin/T-Cap [24], could lead to cardiomyopathy and ion channels disturbances in human subjects [23, 24, 40]. The ZASP mutation p.D117N, previously identified in DCM/LVNC and conduction defects and affecting mainly the short cardiac isoform of ZASP [40], could in fact decrease the conduction velocity (CV) of I_{Na} by 27 % [24]. We have similarly previously reported a cardiac-specific transgenic murine model of DCM caused by the ZASP mutation p.S196L identified in human DCM and affecting another ZASP isoform highly expressed in human hearts [40]. Mice expressing ZASP4-S196L presented with cardiac conduction defects and atrioventricular block at 3-months of age before developing myocardial structural remodeling and ventricular dysfunction, which occurred at 10 months of age [23]. Interestingly, electrophysiological studies in cardiomyocytes isolated from ZASP4-S196L mice hearts demonstrated the attenuation of L-type Ca^{2+} currents and a rightward shift of voltage dependency of Nav1.5, a different mechanism from the electrical remodeling generated by ZASP1-D117N [23, 24]. In fact, pull-down assays using ZASP4 detected both Nav1.5 and Cav1.2, which are components of a macromolecular complex localizing at the striation of the sarcomere [23]. Also the complete genetic ablation of the murine ZASP homolog *cypher* disrupts the cytoarchitecture causing the development of severe cardiomyopathy and skeletal myopathy [41], while the cardiac-specific ablation of *cypher* led to DCM and SCD [42].

Conclusions and Clinical Perspective

In this chapter we have briefly reviewed the current knowledge about the relationship the structure and function of the sarcomeric Z-band with particular emphasis on pathophysiology of mutations in the Z-band proteins identified in human subjects with heart failure and arrhythmias. The recent discoveries of a direct effect of Z-band alterations in mechanical and electrical dysfunction prompt a shift in the clinical approach of arrhythmias in these patients. In fact, the current therapeutic approaches may appear insufficient and sometimes paradoxically deleterious in managing certain individuals affected by genetically determined heart failure. This issue raises further awareness about the importance of detailed genetic investigation in all patients for which a monogenic or oligogenic component is suspected. Current technologies along with specific experience in clinical molecular genetics may provide a particularly useful tool for a precise clinical and molecular diagnosis and so to a more tailored treatment for the benefit of each patient affected by such a disease.

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Biophysics of Titin in Cardiac Health and Disease

Brian R. Anderson and Henk L. Granzier

Introduction

Titin is a gigantic multifunctional filamentous protein that spans from the Z-disk to the M-band region of the cardiac sarcomere. The elastic I-band region of titin generates passive force during sarcomere stretch that plays important roles in maintaining the structural organization of the sarcomere and that contributes greatly to diastolic stiffness. Recent work has shown that to match hemodynamic demands the mechanical properties of titin can be finely tuned through differential expression of titin isoforms and phosphorylation of titin's spring-like elements. Titin may also play a role in mechanically sensing sarcomere length changes due to its placement in the sarcomere and extensible, force-bearing I-band region. The precise ways in which titin behaves as a mechanosensor is not well established, but rapid progress is being made in our understanding of the various proteins involved in signaling pathways that interact with titin. Recent work also revealed mutations in the titin gene (TTN) as a major source of familial cardiomyopathies, including mutations in titin's spring region linked to arrhythmogenic right ventricular dysplasia and mutations in titin's A-band region responsible for ~30 % of dilated cardiomyopathy (DCM) cases. This chapter discusses the mechanical properties of titin and the role titin plays in cardiac health and disease.

B.R. Anderson
Department of Physics, University of Arizona, Tucson, AZ 85724, USA

Department of Physiology, Molecular Cardiovascular Research Program, Sarver Heart Center, University of Arizona, Tucson, AZ 85724, USA

H.L. Granzier (✉)
Department of Physiology, Molecular Cardiovascular Research Program, Sarver Heart Center, University of Arizona, Tucson, AZ 85724, USA

MRB 340, 1656 E Mabel St., Tucson, AZ 85721, USA
e-mail: granzier@email.arizona.edu

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The Giant Protein Titin

Titin spans the entire half sarcomere (Fig. 1a) and is embedded in the Z-disk at its N-terminus and in the M-band at its C-terminus [1]. The rest of titin is divided between its elastic I-band region and its thick filament-binding A-band region [2]. The elastic I-band region of titin consists of three distinct elements (Fig. 1b): (1) serially linked immunoglobulin(Ig)-like domains, (2) the spring-like PEVK (containing a high percentage of proline, glutamic acid, valine, and lysine residues), and (3) the spring-like N2B element [3]. These three elements largely determine the titin-based passive force that develops when sarcomere length increases (Fig. 1c) [4]. This force is important for centering the thick filament in the sarcomere [5] and for defining diastolic stiffness [6]. This region also contributes to viscosity in cardiac myocytes via PEVK-actin interaction and possibly titin–titin interaction [7].

A fundamental determinant of titin-based stiffness is titin isoform composition. Titin is the largest protein known in nature and is encoded by a single gene [3],

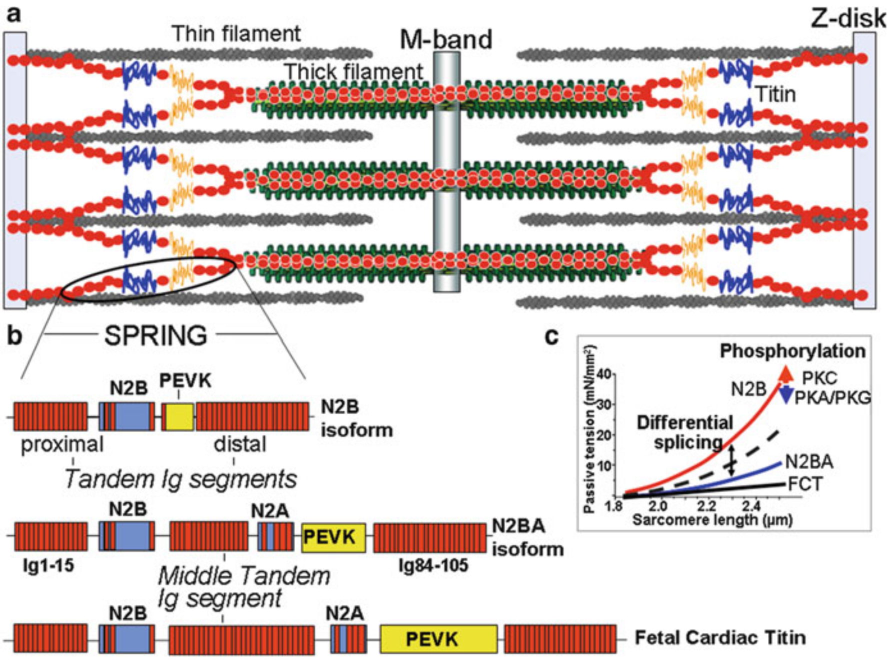

Fig. 1 (**a**) Schematic of the sarcomere. Titin spans from the Z-disk to the M-band and contains a spring-like region that generates passive force in stretched sarcomeres. (**b**) The three cardiac titin isoforms express different elastic I-band regions. The adult N2BA isoform and FCT isoform classes contain the N2A element, a middle tandem Ig segment, and a longer PEVK sequence compared to the adult N2B isoform. (**c**) Titin-based passive tension levels are determined by titin isoform composition and the phosphorylation status of the spring-like elements. The size of titin's I-band region is inversely proportional to its molecular stiffness, and phosphorylation events can either increase or decrease titin stiffness

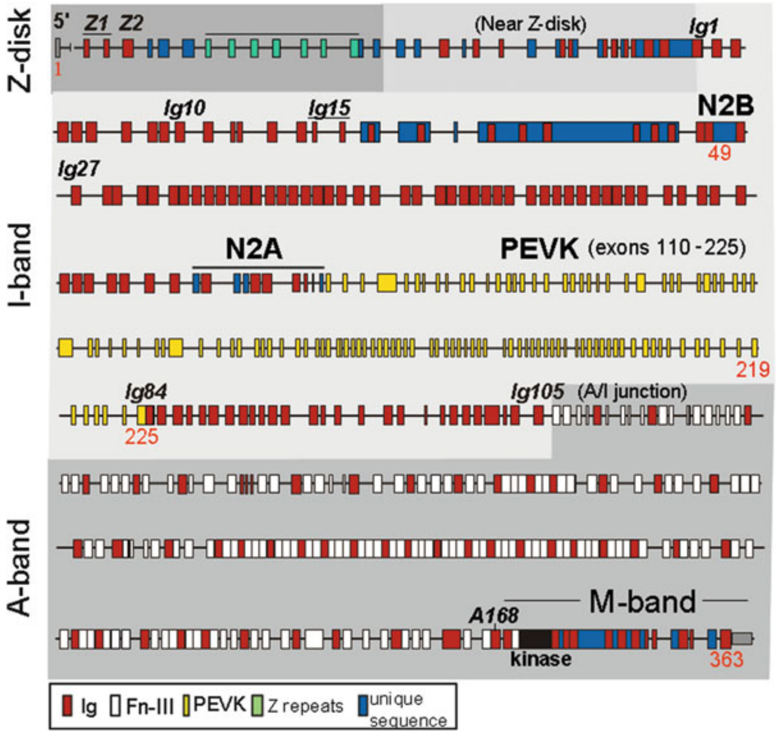

Fig. 2 The exon structure of the human titin gene (based on [3]). Each exon is represented by a box. Approximately 220 of titin's 363 exons are found in the I-band region. Titin's I-band region comprises immunoglobulin(Ig)-like domains, the PEVK element, and unique sequences. The A-band region of titin is bound to the thick filament and contains Ig and fibronectin-III (Fn-III) domains

but variable mRNA splice pathways result in distinct titin isoform classes. The human TTN contains 363 exons (Fig. 2) that code for 38,138 amino acids (4.2 MDa), although this full-length gene product is not known to be expressed in any muscle type. In cardiac muscle three classes of titin isoforms are present: adult N2BA, adult N2B, and the fetal cardiac titin (FCT) isoforms [8, 9]. Differential splicing of the I-band region of the TTN results in these three titin isoform classes; the rest of the gene (Z-disk, A-band, M-band localized regions) is largely identical. For this reason, it is common for titin isoforms to be identified by their elastic I-band regions. The I-band region of titin contains ~220 exons [3] that code for unique sequences, PEVK segments, and Ig domains. Exon 49 (~2,780 nucleotides) codes for the so-called N2B element that is found in all cardiac titin isoforms. This N2B element contains a 572 residue unique sequence (N2B-Us) flanked by Ig24/25 at its N-terminus and Ig26 and its C-terminus. In addition to behaving as a large molecular spring, the N2B-Us is a substrate for various kinases that affect its mechanical properties [10, 11]. Therefore, the N2B-Us plays crucial roles in changing titin-based passive tension levels that may be physiologically relevant.

While the N2B element is found in both adult cardiac titin isoforms, the N2A element is found in the N2BA isoform (hence its name) and FCT isoforms (Fig. 1b). Similar to the N2B element, N2A contains Ig domains and unique sequence. The N2A region is encoded by exons 102–109 and contains four Ig domains and a ~125 residue unique sequence. The N2A element is relatively understudied, although it is a binding site for the protease calpain-3 [12] and proteins involved in signaling pathways (see section "Titin-Based Signaling"). The remaining unique sequences in the I-band region of titin belong to three novel exons (Novex I, II, and III) that are not present in the conventional titin isoforms [3].

Like the N2B-Us, the PEVK region of titin also behaves as a molecular spring that can be phosphorylated to quickly change the spring-like properties of titin. The PEVK sequence is encoded by 114 exons although most of these exons are spliced out in the cardiac titin isoforms. For example, only seven PEVK exons are found in the N2B titin isoform while the PEVK region of N2BA titin contains several additional exons [13].

The last component of titin's extensible I-band region is the serially linked immunoglobulin(Ig)-like domains. These domains are structurally very similar to each other and natively exist as a stable, folded β-barrel [14, 15]. The N2B titin isoform contains a proximal tandem Ig segment (Ig1–15) and a distal tandem Ig segment (Ig84–105). These segments can be visualized as "beads on a string." Each folded Ig domain has a diameter of 4–5 nm separated by short peptide linkers [16]. The N2BA titin isoform contains the proximal and distal tandem segments as well as a middle tandem Ig segment that contains a variable number of domains [17]. The additional Ig domains and PEVK sequence and the inclusion of the N2A element make the N2BA titin isoform larger and more compliant than the N2B isoform (~3.3 MDa vs. 2.97 MDa). The FCT class of titin isoforms (3.5–3.6 MDa, [8]) contains a larger middle tandem Ig segment than the N2BA isoform and the largest PEVK sequence of all the cardiac titin isoforms [9, 18]. An exciting new discovery regarding titin splicing is that the TTN is spliced by RNA binding motif protein 20 (Rbm20) [19]. Mutations in Rbm20 result in exceptionally large titin molecules [19] and have been linked to DCM [20, 21]. Because of the intimate relationship between the size of titin's I-band region and titin-based passive tension (see below), with larger elastic I-band regions corresponding to lower passive tension, the titin isoform expression ratio in the heart is a crucial determinant of titin stiffness. The shorter, stiffer N2B isoform accounts for 80 % of mouse titin and 60 % of human titin [22]. Differences in titin isoform expression account for cardiac passive tension variability between species (more N2B is found in small mammals that have higher heart rates [22]), and the titin isoform ratio changes in diseased states and gives rise to alterations in passive stiffness. In order to understand the interplay between pathology and titin isoform expression it is important to discuss how titin's I-band region mechanically behaves in stretched muscle.

Extension of Titin's I-Band Region

The amount of force borne by titin's I-band region depends on sarcomere length. Since the thin and thick filaments stretch only a small amount [23, 24], changes in sarcomere length (SL) directly correspond with the end-to-end length of titin's extensible I-band region. As the SL increases above slack SL, the three elements of I-band titin extend and develop a passive restoring force that resists extension increases. However, the tandem Ig domains, PEVK, and unique sequences do not extend equally (Fig. 3). This was determined by electron microscopy performed on stretched mouse cardiac myocytes that were stained with antibodies to epitopes that flank the distinct spring-like elements of titin's I-band region [25]. Measuring the distances between epitopes as a function of SL showed that the tandem Ig domains extend at low SL ranges and that the N2B-Us and PEVK segments extend as the tandem Ig domains become taut. This behavior (early tandem Ig extension followed by PEVK and N2B unique sequence uncoiling) has also been shown in rat ventricle (mostly N2B), cow ventricle (equal N2B and N2BA), and cow atrium (mostly N2BA) [26]. The sum of the extensions of each titin I-band element is equal to the SL minus the unchanging length contributions of the thick filament and portion of titin bound to the thin filament. This fact is exemplified in a mouse model in which the N2B element of titin's I-band region is deleted and the remaining elements (tandem Ig, PEVK) compensate for this loss by extending further [27]. The increased extension of the remaining elastic elements of titin also resulted in higher passive tension [27], a phenomenon that is well described by the wormlike chain (WLC) equation: $F = \frac{k_B T}{L_p}\left(\frac{z}{L_c} + \frac{1}{4(1-z/L_c)^2} - \frac{1}{4}\right)$. This equation describes the force needed to extend a polymer as a function of persistence length (L_p) and fractional extension (z/L_c). Persistence length is a measure of bending rigidity and is inversely proportional to force, with a molecule of lower L_p (more flexible) requiring more

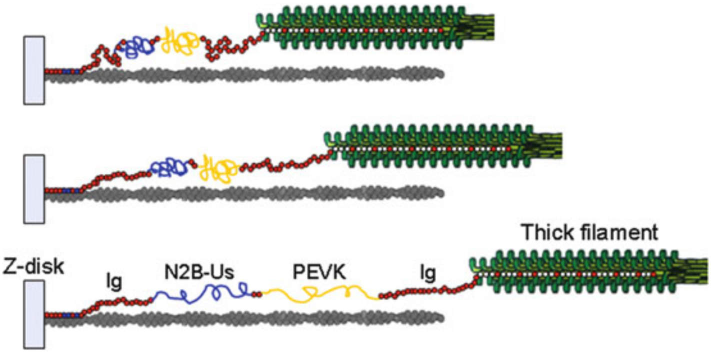

Fig. 3 The spring-like elements of titin's I-band region fractionally extend at different rates depending on sarcomere length. As the SL increases from slack, the proximal and distal Ig segments straighten out while the more flexible (entropically stiffer) N2B unique sequence and PEVK element remain compact. After the Ig segments become taut, the N2B-Us and PEVK extend

force to stretch. Fractional extension is the end-to-end length of the molecule (z) divided by the molecule's contour length (L_c), which is the distance traced along the molecular backbone. The force required to stretch a molecule depends nonlinearly on fractional extension, with significantly more force needed as its extension approaches its contour length. This last point explains why the N2B titin isoform is stiffer, i.e., requiring more force to stretch, than the N2BA titin isoform. At a given SL the extension of the I-band region of the N2B and N2BA titin isoforms (physical distance between the end of the thin filament-binding region of titin and beginning of the thick filament-binding region) is the same. However, since the N2B isoform has a shorter contour length (due to fewer Ig domains, a shorter PEVK segment, and absence of the N2A element), its fractional extension is greater. Therefore more force is needed to stretch the N2B titin isoform—it is stiffer because it is shorter. For this reason, changes in titin isoform expression change myocardial stiffness [22]. To gain a more quantitative understanding of how the N2B and N2BA isoforms differ, it is useful to model titin's I-band region as springs connected in series.

The magnitude of titin-based passive tension is a combined effect of the elements of titin's I-band region. The resistance of each element to stretch must be known in order for an accurate estimation of the entire region's stretch response. For example, consider three ideal springs ($F = -kx$) connected in series, where k is the spring constant, x is the displacement from equilibrium, and F is force. Each spring is initially at equilibrium and the length of the spring system is $x_1^0 + x_2^0 + x_3^0 = x_{\text{total}}^0$, where the zero superscript indicates equilibrium, i.e., no potential energy stored in the spring. If an external force stretches the system, force balance dictates that the tension in each spring is the same and the displacement of each spring from equilibrium is equal to F/k_i, where k_i is the spring constant of spring i. The sum of each spring's displacement from equilibrium equals the total displacement of the system from equilibrium: $\sum_i \left(x_i^f - x_i^0\right) = x_{\text{total}}^f - x_{\text{total}}^0$. The contribution of a spring to the total displacement of the system depends on its stiffness, with a compliant spring extending further than a stiff spring under the same external force. In the sarcomere, ideal Hookean springs are replaced by the entropic spring elements of titin's I-band region and the equilibrium length of the spring system is defined as the slack sarcomere length (length where force is zero). The relationship between force and extension is approximated by the WLC equation, and force balance in the system still holds. Technically, force balance is only satisfied at equilibrium, but this idea is helpful for understanding titin-based passive tension as a function of SL. In mechanical studies length changes are imposed by a motor and the restoring force response is measured with a force transducer [28, 29]. At a given SL the extension of each spring-like titin element depends on the contour length and persistence length of each element, with the tandem Ig domains, PEVK element, and N2B-Us extending different lengths such that two constraints are satisfied: (1) equal tension throughout titin's I-band region and (2) the sum of spring displacements from equilibrium equals the current SL minus the slack SL. Satisfying these constraints for wormlike chains is not trivial, but to make things easier the wormlike chain can be inverted (approximately) such that extension is a function of L_c, L_p, and force (Eq. (6) in [30]). The extension of each titin I-band component can then be estimated as a function of force and summed, which results in the total extension of the I-band region of titin (which directly relates to SL) as function of force. Therefore, to model the force-extension characteristic of titin's I-band region, the individual properties of the distinct I-band elements need to be known. This process also allows predictions of changes in titin-based passive tension from single molecule studies of titin fragments. To directly measure the mechanical properties of titin's elastic elements, each component must be studied in isolation at the single molecule level.

Mechanical Properties of Titin's Elastic Elements

Tandem Ig Segments

The majority of titin's elastic I-band region is made up of the tandem immunoglobulin(Ig)-like domains. These domains have a characteristic β-barrel structure and have been classified as members of the intermediate I-set of the immunoglobulin family [31, 32]. The tandem Ig domains of titin's I-band region can be further classified by their position within the I-band (Fig. 1). The proximal Ig segment is immediately adjacent to the actin-binding region of titin and contains Ig1–15 (Ig1 begins the I-band region of titin) and the distal Ig segment contains Ig84–105; these two Ig segments are constitutively expressed and form the entirety of the serially linked Ig segments in the N2B titin isoform. To be clear, the cardiac titin isoforms also contain three Ig domains in the N2B element and one Ig domain immediately C-terminal to the N2B element. In addition, the N2BA titin isoform contains a middle proximal Ig segment of varying length [17]. Ig domains have stable tertiary structure and the atomic coordinates of many Ig domains have been determined [14, 32–34]. Although there are some primary sequence differences between the Ig domains [35], they all seem to form β-barrels. Molecular dynamics simulations [36, 37] and single molecule force spectroscopy [38–40] have studied the unfolding behavior of Ig domains in detail and found that the average force needed to unfold an Ig domain in titin's I-band region is between 150 and 300 pN at physiological pulling speeds [39–41]. Although there is some evidence for two-step unfolding of Ig domains [42], most evidence has shown that the hydrophobic core readily disassembles once the mechanical stability of an Ig domain is compromised due to rupture between terminal β-strand networks [36, 38]. It is unclear whether Ig unfolding takes place in vivo and if unfolding is physiologically relevant. The unfolding rate under zero force of various Ig domains has been estimated between 10^{-4} and 10^{-5} domains/s [38, 39]. Unfolding rate is force dependent and can be approximated by $\alpha(F) = \alpha(0)\exp(F \cdot \Delta x / k_B T)$ [43], where F is the external force acting on the domain and Δx is the distance along the reaction coordinate from the folded protein state to the transition barrier. The upper limit of the force experienced by the I-band region of titin is <5 pN as determined from muscle mechanics [29], thick filament density [23], and working SL data [44], and Δx has been estimated as ~0.3 nm [38, 39]. Using these values, estimates that 1 out of every 10,000–100,000 Ig domains unfold per second at the end-diastolic sarcomere length. This suggests that Ig domain unfolding is not common under physiological conditions. When an Ig domain does unfold from a stable β-barrel structure to an unfolded polypeptide random coil, the contour length of titin's I-band increases by ~30 nm (the average length of an amino acid is 0.38 nm [45] and Ig domains are approximately 90 residues). This increase in contour length relieves some of the tension in titin by reducing the fractional extension of the PEVK and N2B elements, although the reduction in passive tension that results from unfolding of one domain is small (estimated at <1 %).

Ig domains may play an important role in disease mechanisms. For example, a mutation linked to arrhythmogenic right ventricular dysplasia was recently found in the tenth Ig domain of the proximal tandem Ig segment [46]. This is the first time an Ig domain in titin's I-band region has been linked to cardiac disease, and nuclear magnetic resonance (NMR) and proteolysis assays showed that domains containing the disease-linked mutation undergo structural changes and are more prone to degradation [46]. The structural weakening of the Ig domain with the mutation was also confirmed at the single molecule level [47]. Also, numerous titin mutations that result in truncated titin molecules have been discovered in patients with DCM [48]. Most of the mutations were found in the thick filament binding, A-band portion of titin, with relatively few mutations found in titin's elastic I-band region. Under the assumption that random mutations are equally likely to occur at any spot along the TTN, the paucity of reported mutations in titin's I-band suggests that they are particularly detrimental and are less likely to be propagated within a population.

PEVK

Containing a high percentage of proline (P), glutamic acid (E), valine (V), and lysine (K) residues, the PEVK element is well described as an entropic spring. Circular dichroism experiments suggest that the PEVK element may contain polyproline II helices [49], although the propensity to form helical structures is most likely isoform dependent, with the PEVK region of the N2B titin isoform predicted to form helices at very low levels [50]. Most single molecule studies have focused on the constitutively expressed PEVK element that is the full PEVK region of the N2B titin isoform, although the N2BA titin isoform contains two polyE (E-dense regions of negative charge) and 20 PEVK repeat motifs (26–28 residues each) while the N2B titin isoform contains zero polyE regions and only five repeat motifs [51]. The PEVK region of the N2BA titin isoform is also much larger (~800 residues compared to 188 for N2B titin; the L_c of random coil polypeptides is

~0.38 nm/residue) which contributes to the larger size and increased compliance of the N2BA isoform. The high concentration of bulky proline residues and charge clusters suggests that the PEVK region does not contain large stable structures [2], and this idea is supported by single molecule stretch studies of the constitutively-expressed PEVK segment using atomic force microscopy (AFM). AFM force-extension traces do not show force peaks indicative of structural transitions when PEVK is stretched; instead, the PEVK element smoothly extends in a characteristic WLC fashion with a persistence length of ~1 nm [50, 52–54].

N2B Element

The N2B element is found in all cardiac titin isoforms but is absent in skeletal muscle. Encoded by a single exon, the human N2B element contains 926 amino acids that comprise three ~90 amino acid Ig domains (Ig24–26) interspersed by unique sequences. The largest continuous unique sequence in the N2B element is 572 residues and is located between Ig25 and Ig26; this large unique sequence is termed the N2B-Us and has been studied in detail at the single molecule level. Like the PEVK, the N2B-Us acts as an entropic spring. Using AFM, the L_p of the N2B-Us was found to be ~0.65 nm [41, 50, 52]. Thus the N2B-Us has a lower L_p than the PEVK, which, according to the WLC equation, makes the N2B-Us harder to stretch (although, physically, a lower L_p means increased local flexibility). The N2B-Us is not thought to contain stable tertiary structures because its force-extension trace is absent force peaks [41, 50, 52]. The presence of secondary structure in both the PEVK and N2B-Us cannot be excluded by AFM experiments since the force resolution is typically ~10 pN. Also like the PEVK, the structure of the N2B-Us is unknown. Because both molecules are thought to be largely intrinsically disordered, solving the structures is highly challenging. Ligand binding [41] and phosphorylation [11, 53, 55] change the mechanical properties of the PEVK and N2B-Us, but the uncertainty regarding the structures of these elastic regions precludes predicting how protein binding and posttranslational modifications (PTMs) alter the structure of these extensible proteins.

Titin Stiffness Modulation

The two primary known ways in which titin-based stiffness is altered are changes in titin isoform expression ratio and PTMs of titin's elastic I-band region. While these two mechanisms are distinct, their effects on passive tension are not independent since the effect of PTMs on titin stiffness is isoform dependent [56] due to the different degrees that titin isoforms fractionally extend as a function of sarcomere length.

Titin Isoform Composition

As detailed above and shown in Fig. 1, the N2B, N2BA, and FCT isoforms have different I-band region compositions which imparts them with different mechanical properties. Since the FCT isoforms have the largest I-band region, they are more compliant than the shorter adult titin isoforms at a given SL. According to the WLC equation, the force needed to stretch a polymer rapidly increases as the fractional extension approaches one, i.e., when the end-to-end length of the polymer (z) nears its contour length (L_c). Co-expression of titin isoforms occurs at the level of the half sarcomere [57], which means that each titin isoform has the same end-to-end length between their anchoring points in the thin and thick filament of the sarcomere at a given SL. Since the N2B and N2BA isoforms have a shorter L_c than the FCT isoform class, at a given SL stretch the N2B and N2BA isoforms are at a greater fractional extension and require more force to stretch. The compliant FCT isoforms are highly expressed during embryonic development and at birth but are replaced by the stiffer adult isoforms during the first weeks of postnatal development [8] as the newborn heart must quickly adapt to the increased hemodynamic loads required to sustain independent life. In the adult, the N2BA titin isoform is considered compliant compared to the shorter N2B isoform because the I-band region of N2BA titin is larger. The ratio of N2BA:N2B isoform expression therefore directly influences passive tension and is quite variable between species and certain disease states.

Animals with faster heart rates typically have stiffer myocardium and a lower N2BA:N2B ratio [22], which might be necessary to allow for rapid early diastolic filling and rapid setting of the end-diastolic volume that accompanies the shorter diastolic period at the fast heart rate. Of particular interest is how titin isoform composition changes in certain pathological states. For example, 2 weeks of pacing tachycardia induced DCM in dogs and was characterized by chamber dilation and increased chamber stiffness [58]. In this study, the N2BA:N2B ratio differed in the subendocardium and subepicardium between the control and paced hearts, although the gradient difference did not reach significance. A similar study that paced dogs for 4 weeks showed that the ratio of N2BA to N2B titin in the left ventricle was significantly reduced from 1.01 in controls to 0.80 in the paced group [59]. This up-regulation of the stiffer N2B isoform was also accompanied by increased titin-based passive tension. Pressure overload imposed on a hypertensive rat model showed a reduction in N2BA titin expression [60] and is consistent with hypertensive rat cardiomyocyte studies that found elevated levels of passive tension [61, 62].

Variable titin expression ratios have also been found in human patients with cardiac disease. Patients with coronary artery disease (CAD) have been shown to express increased levels of N2BA titin that was accompanied by decreased stiffness at the myofibril level [63]. This did not correspond to decreased left ventricular wall stiffness, however, and was explained by increased expression of collagen, an extracellular matrix (ECM) protein that plays an important role in passive tension, and desmin, a cytoskeletal protein that is thought to contribute to ventricular wall stiffness [64]. The change in titin isoform expression in human CAD patients has been suggested to be a compensatory mechanism to counteract the increased ECM-based stiffness associated with the disease [65], which contrasts the animal model studies mentioned above in which titin stiffness contributed to the pathology. However, human patients are usually treated only after chronic cardiac stress has already led to the pathological state, which makes it unclear whether changes in titin expression contribute to the pathology or respond to the pathology. Changes in titin isoform expression have also been found in patients with end-stage heart failure due to DCM where the compliant N2BA isoform was up-regulated and associated with decreased passive stiffness and increased chamber compliance [66]. The same study also suggested a physiological benefit of this change in titin expression via correlation between the titin isoform shift and improved exercise tolerance. This suggests that up-regulation of the more compliant N2BA titin isoform may be a beneficial compensatory adaptation [66].

Posttranslational Modifications

It is well known that PTMs of contractile and regulatory proteins greatly affect cardiac function, and recent research has shown that titin is also modified by various kinases that lead to changes in titin-based passive tension. The mechanical properties of the tandem Ig segments, PEVK sequence, and N2B element together determine titin-based passive tension, and therefore changes in the properties of these three spring-like elements affect titin stiffness and myocardial stiffness. Single molecule force spectroscopy experiments have studied the elastic properties of titin fragments in isolation and have discovered that kinase phosphorylation significantly alters the stiffness of the PEVK and N2B-Us. This allows for rapid adjustment of titin stiffness and quick adaptations of cardiac performance to meet hemodynamic needs.

PEVK Phosphorylation

Although AFM traces suggest that the PEVK element does not contain stable structures, it does not seem to be purely a random coil since phosphorylation of PEVK by protein kinase C (PKC) changes its physical properties. PKC is activated by the α1-adrenergic signaling pathway that is a key mediator of physiological and pathological adaptation. PKC is involved in numerous cellular processes including regulation of cystolic calcium (Ca^{2+}), myofilament Ca^{2+} sensitivity, and cardiac contractility [67]. It was found that PKCα, the predominant isozyme in the heart and a key player in contractile dysfunction and heart failure [68, 69], phosphorylates the PEVK element of titin and leads to increased passive tension [70]. This passive tension increase was reversed by introduction of the dephosphorylating agent protein phosphatase-1 (PP1), which suggests that PKCα phosphorylation directly affects titin stiffness. The primary sites of phosphorylation were found to be two highly conserved serine residues (S26 and S170) within the constitutive PEVK element. The PEVK sequence adjacent to these phosphorylation sites is also highly conserved across species [70], which suggests that the PEVK has some structure because pure random coils would likely permit primary sequence drift. Phosphorylation of these conserved serine residues reduces the L_p of the PEVK element (from ~1 to 0.67 nm) [53], which is consistent with the increased passive tension seen at the tissue level [70]. Mutation of these conserved serines to alanine diminished the PKC effect and also reduced the L_p of PEVK to ~0.55 nm in the absence of PKC phosphorylation [53]. This further supports the idea of PEVK structure because if it was a pure random coil the L_p would not be significantly altered by point mutations. The link between PKCα, PEVK phosphorylation, and passive tension was further established by a study that showed that PKCα had no effect on passive tension in mice that had the constitutive PEVK element genetically removed [71]. The combination of techniques including single molecule force measurements and novel mouse models has established that posttranslation modifications of titin via PKC directly influence titin-based passive tension. The mechanisms by which phosphorylation and mutation change the PEVK's mechanical properties are unclear due to the limited information regarding its structure and require further study.

N2B-Us Phosphorylation

The N2B element of titin is also a kinase substrate that experiences changes in its mechanical properties following phosphorylation. Protein kinase A (PKA), which is stimulated by the β-adrenergic pathway, reduces passive tension in cardiac myocytes [10]. Phosphorylation assays and immunoelectron microscopy showed that PKA targets the large unique sequence in the N2B element (N2B-Us) [10]. The effect of PKA on passive tension is increased in the N2B titin isoform compared to N2BA, presumably due to the N2B isoform having a larger fractional extension at a given SL [56]. PKA also reduces passive tension in human cardiac fibers [72], with a more pronounced effect present when PP1 dephosphorylation was performed prior to PKA treatment, which shows that basal levels of phosphorylation play an important role in determining passive tension levels.

Similar to PKA, protein kinase G, which is cGMP-dependent, phosphorylates the N2B-Us and reduces passive tension, and the PKG phosphorylation site is also a residue targeted by PKA (Fig. 4) [11]. The effect of PKG on the passive tension of skinned fiber bundles from human donor hearts has been studied and a significant reduction in passive tension following PP1 treatment was found. Single molecule data suggests that PKG phosphorylation increases the L_p of the N2B-Us, which is consistent with the reduced passive tension measured in muscle mechanics experiments [11]. More work is needed to confirm this single molecule result due to the limited data collected in the initial study. Recently, the serine/threonine

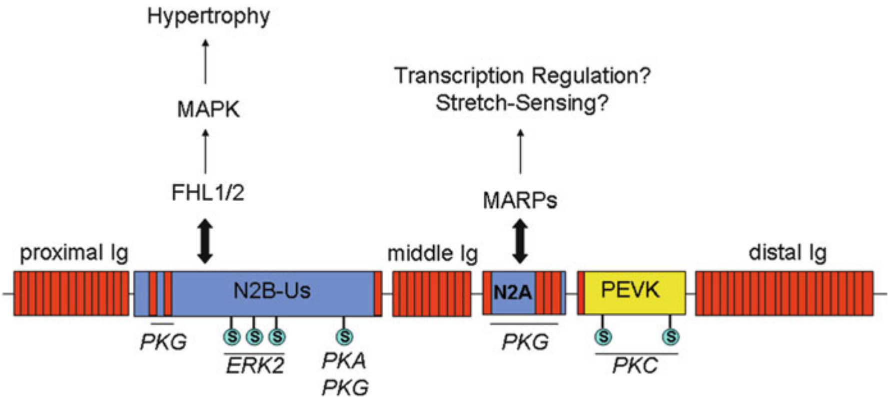

Fig. 4 Schematic of titin's elastic I-band region showing kinase phosphorylation sites and interactions between titin and proteins involved in signaling pathways. PKG phosphorylates the N2A element and the Ig24-unique sequence-Ig25 region of the N2B element, but site-specific information is unknown. The N2A and N2B unique sequences are the regions of titin most likely to be involved in cell signaling

kinase ERK2 has been shown to phosphorylate the N2B-Us at multiple residues [73], although the effect that this has on the mechanical properties of titin is unknown. ERK2 is involved in the MAPK pathway [74] (see section "Titin-Based Signaling"), a key player in hypertrophy signaling [75], which suggests interplay between the phosphorylation status of titin and the trophic state of the heart.

The mechanical properties of the N2B-Us can be altered by more than just phosphorylation status. For example, there are six cysteine residues in the human N2B-Us that have the potential to form disulfide bonds with one another depending on the oxidative state of the sarcomere. A disulfide bond would reduce the contour length of the N2B-Us and change its mechanical response to stretch. AFM experiments carried out in the presence and absence of the reducing agent DTT show that disulfide bonds can drastically decrease the L_c of the N2B-Us, which is predicted to significantly affect titin-based passive tension [76]. The effect of cysteine cross-linking on the mechanics of the N2B-Us was also shown at the tissue level where oxidative stress increased passive tension and hysteresis in wildtype tissue but had an attenuated effect in tissue from a mouse model where the entire N2B element was removed [29]. The study between oxidative conditions and changes in passive tension is especially interesting considering that oxidative stress is elevated in heart failure patients and has been correlated with myocardial dysfunction [77].

Since titin-based stiffness at the tissue level is determined by titin isoform composition and the phosphorylation state of titin's elastic I-band, with different kinases affecting titin elasticity is disparate ways, comprehensive studies of titin isoform expression and phosphorylation status is ideal for determining the mechanisms through which titin stiffness changes during acute and chronic stress.

Titin-Based Viscosity

Studying purified fragments of titin's elastic I-band region at the single molecule level is an ideal way to determine the properties of the tandem Ig, PEVK, and N2B elements in isolation. However, the cell is a protein-dense environment and interactions between the large myofilaments of the sarcomere can lead to intracellular viscosity, which increases the force needed to stretch the muscle since viscosity, which is a frictional force, opposes motion. Whenever sarcomere length is changing, viscosity is present and must be overcome [78].

Due to the high density and close proximity of actin, myosin, and titin in the sarcomere, it is not surprising that viscous interactions occur between these myofilaments. The main source of titin-based viscosity in the cell arises from interaction between the PEVK element of titin and the actin-based thin filaments. This protein–protein interaction opposes filament sliding and has been studied with single molecule spectroscopy [79], in vitro motility assays [54, 80], and myocyte mechanics [7, 80]. Integrative studies have also been performed on a mouse model in which the constitutive PEVK element of titin is deleted (exons 219–225) [7]. This study showed that removal of the PEVK element reduced viscosity by 60 % in myocytes, 50 % in muscle fibers, and 30–40 % in intact isolated hearts due to decreased PEVK-actin interaction. The affinity between PEVK and actin seems to be an electrostatic effect between actin, which is negatively charged [81], and PEVK. The constitutively-expressed PEVK element contains five basic (p*I* 9–10) PEVK repeat motifs that are each ~28 residues [51]. Therefore, at physiological pH levels the PEVK element has a net positive charge which may drive the electrostatic PEVK-actin interaction. This interaction is dependent on ionic strength [79, 80, 82] as well as lattice spacing [7], with increased ionic strength and increased lattice spacing both reducing PEVK-actin interaction. These experimental observations are consistent with the hypothesis that an electrostatic force drives PEVK-actin binding since Coulomb's Law of Electrostatics states that electrostatic force is proportional to the product of the two point charges (higher ionic strength effectively shields the PEVK from actin) and inversely proportional to the square of the distance between the two charges. The interaction between PEVK and actin has been well studied for the PEVK element of the N2B titin isoform, but the dynamics between actin and the additional PEVK sequence of the N2BA titin isoform are unknown.

Viscous force is speed dependent, and in cardiac cells the speed at which the myofilaments slide past each other is directly related to heart rate. For this reason, titin-based viscosity is predicted to be higher in small animals with high heart rates compared to humans. On the other hand, viscous forces may play a physiologically more relevant role in animals that experience a wider range of heart rates. For example, the human heart rate can triple during exercise while the murine heart rate changes only slightly. Although the magnitude of viscosity may be larger in small animals, it is not anticipated that this viscous force changes much as small animals are stressed. An important factor to consider when discussing viscosity is that viscosity is greatest after a cell or tissue has been at rest, i.e., not in dynamic equilibrium. Since the heart is always beating, viscosity experiments are most relevant if a physiological stretch protocol is administered, and indeed it has been shown that repeated stretches decrease the viscous contribution [4, 29], although a significant level of steady-state viscosity remains.

Titin-Based Signaling

How do changes in hemodynamic load lead to altered titin isoform expression? Does titin act as a mechanosensor? The answers to these intriguing questions likely lie in the elucidation of titin's involvement in signaling pathways. Since titin spans the entire half sarcomere and interacts with other proteins in the Z-disk, I-band, A-band, and M-band regions of the sarcomere, titin's role in stretch-sensing and signaling pathways may be very complex, but exciting results have already been realized.

Z-Disk Signaling

The protein-dense Z-disk region of the sarcomere mechanically connects adjacent sarcomeres and helps maintain the highly ordered myofilament lattice structure. The Z-disk undergoes strain when tension develops in the sarcomere as evidenced by changes in Z-disk thin filament lattice spacing as a function of active tension [83] and passive tension [23]. Two Ig domains at titin's N-terminus (Z1 and Z2, Fig. 2) embed titin in the Z-disk and strongly bind to T-cap (also called telethonin) and α-actinin. T-cap connects two titin molecules from the same half sarcomere in a sandwiched structure [84] via strong β-strand cross-linking [85, 86]. A possible mechanism for titin-based signaling involves T-cap's interaction with muscle LIM protein (MLP), a nuclear regulator of myogenic differentiation [87] that also promotes myogenesis [88]. Cardiac myocytes subject to stretch express the well-known stretch response markers brain natriuretic peptide and atrial natriuretic factor, although this response to stretch is absent in MLP deficient mice [89]. MLP mutations associated with mechanical stress signaling deficiency have been linked to hypertrophic cardiomyopathy (HCM) [89, 90], and mutations in T-cap have been linked to HCM and DCM with the pathological phenotype suggested to arise from compromised binding between T-cap, titin, and other Z-disk proteins [91, 92]. Many of the sarcomeric proteins localized in the protein-dense Z-disk of the sarcomere have been implicated in complex signaling pathways, and titin may play a key biomechanical stress sensor function.

I-Band Signaling

Except for PEVK interacting with the thin filament, the elastic I-band region of titin is thought to be unbound in the sarcomere. Because of this, the spring-like elements of titin's I-band directly bear the force that develops as sarcomere length changes. The N2B element found in all cardiac titin isoforms interacts with two members of the four-and-a-half-LIM domain protein family (Fig. 4), FHL1 [74] and FHL2 [93]. FHL1 interacts with the N2B element and is suggested to form a stretch-sensing complex downstream of G-protein-coupled receptor signaling [74]. This FHL1/N2B complex also associates with members of the MAPK signaling pathway, which are involved in hypertrophy signaling [75]. Mouse models have been used to determine the role that FHL1/N2B interaction plays in vivo. FHL1 knockout (KO) mice showed a decreased hypertrophic response and beneficial functional response to transverse aortic constriction (TAC)-induced pressure overload compared to control mice [74]. This suggests that FHL1 hypertrophy signaling responds to increased afterload, although it does not elucidate how load is being sensed. To investigate this question, mouse models missing segments of titin have been utilized. In the N2B KO mouse in which the entire N2B element has been removed, FHL2 levels were significantly reduced and cardiac atrophy was present [27]. On the other hand, when the constitutively-expressed PEVK sequence was removed (PEVK KO), which leads to increased strain placed on the rest of titin's I-band region including the N2B element, FHL1/2 were up-regulated and accompanied by cardiac hypertrophy [94]. It has been suggested that tension acting on the N2B element increases its strain and makes accessible FHL binding sites. Subsequent FHL binding may lead to assembly of a signaling complex that induces a hypertrophic response. This hypothesis is consistent with the elimination of FHL binding sites and atrophy found in the N2B KO as well as the proposed strain-induced increase of FHL binding sites and hypertrophy found in the PEVK KO.

The N2A element, which contains a ~90 residue unique sequence flanked by Ig80–83, has also been implicated in stretch-sensing pathways. The unique sequence between Ig80 and Ig81 binds to the cardiac ankyrin-repeat protein [95], diabetes-related ankyrin-repeat protein (DARP), and ankyrin-repeat domain-protein-2 (Ankrd2) [96]. These proteins belong to the muscle ankyrin-repeat protein family (MARPs), which relocate from the I-band of the sarcomere to the nucleus to regulate transcription following mechanical stress [97]. The N2A element has been proposed to form a stretch-sensing complex that involves MARPs, myopalladin, and calpain-3. In rat cardiomyocytes, externally applied stretch induced differential localization of CARP and DARP, including increased DARP levels at the intercalated disks [96]. Although not much is known about the structure of the unique sequence found in the N2A element, it is possible that it undergoes structural transitions while under strain that change its binding affinity for proteins involved in cellular pathways, similar to the proposed N2B/FHL binding mechanism.

M-Band Signaling

The region of titin bound to the thick filament near the middle of the sarcomere, the M-band region of titin, has also been implicated in signaling processes, although the strain experienced by the M-band region of titin is less than the I-band region of titin since titin is bound to the thick filament at the M-band and the thick filament is only slightly compliant [23]. The most studied domain of titin's M-band region is titin's lone catalytic domain, titin kinase (TK). TK has interested researchers because of its potential to act as a direct biological force sensor via stress-dependent phosphorylation. The crystal structure of TK has been solved [98] and shows that the C-terminal tail of TK blocks its catalytic site. This suggests that TK activity may be force dependent, with the C-terminal being displaced from its inhibitory location when external stress pulls on the termini of TK. Single molecule AFM experiments [99] and molecular dynamics simulations [100] have supported the idea that the regulatory C-terminal tail can be forcibly removed to allow solvent access to TK's ATP-binding site. It has been shown that TK can phosphorylate T-cap [98], but more evidence is needed to link TK function at the M-band with T-cap, which is embedded in the Z-disk. Nbr1 and p62, zinc-finger proteins that have been found to act as scaffolds for signalosome assembly [101], are TK substrates [102] which suggests that force-dependent TK activation may be involved in signaling. Deletion of TK results in cardiomyopathy and death in neonatal mice [103], and a conditional mouse model in which TK was removed in adult mice showed severe cardiac hypertrophy and congestive heart failure [104]. These studies show that TK may play a role in force-dependent signaling and cardiac adaptation.

Next to TK in the M-band are titin domains A168–A170 (two Ig domains and one fibronectin type 3 domain) (Fig. 2) that bind to MURF-1 [105, 106], a ubiquitin ligase that targets muscle proteins for degradation. The possible link between titin, MURF, and cellular response involves MURF-1 binding to glucocorticoid modulatory element binding protein-1 (GMEB-1), which regulates transcription [105]. The titin/MURF interaction may regulate myofibril turnover and the trophic state of the heart, although a MURF-1 KO mouse did not show a difference in the level of titin ubiquitination [107]. On the other hand, MURF-1/MURF-2 double KO mice develop extreme cardiac hypertrophy [108], which suggests that MURFs regulate myogenesis of the heart, although the role that titin plays in the process is unclear.

Summary and Future Direction

Since the discovery of titin over 35 years ago [109, 110], scientists have learned a great deal about the various roles that titin plays in striated muscle. Research has shown that titin is the elastic myofilament of the sarcomere that generates passive restoring forces that are crucial for sarcomere organization and determining myocardial stiffness. Evidence is accumulating that titin functions as mechanosensor that plays a role in hypertrophy singling. The three spring-like elements of titin's I-band region act together to determine titin-based passive tension, but specific phosphorylation events in a single spring-like element can significantly change how the collection behaves as a whole. Titin's involvement in cardiac dysfunction is also coming to light as disease-linked titin mutations are being discovered at an accelerating rate. Nonetheless, many aspects of titin remain unknown. For example, it is unclear how titin is properly assembled in the sarcomere, and the mechanisms responsible for this are likely complex due to titin's enormous size and extension. It has been shown that titin isoform expression ratios can change, but the mechanosensing machinery and cellular response pathways that breakdown titin bound in the sarcomere and integrate newly synthesized titin require further investigation. Novel breakthroughs in the titin field, such as the discovery of the Rbm20 titin splicing pathway [19], will continue to be realized as the extraordinary biology, chemistry, and physics of this giant protein continue to be revealed.

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Sarcomeres and the Biophysics of Heart Failure

Jillian N. Simon, Jil C. Tardiff, and Beata M. Wolska

Introduction

In heart failure the heart's impaired ability to function as a pump results in poor systemic perfusion that is unable to meet the body's metabolic demands. The reduced function of the heart is brought about by either impaired cardiac contractility, as is the case in systolic heart failure, or impaired cardiac relaxation and filling, as is the case in diastolic heart failure. In either state, however, the development of impaired pump function and, thus, heart failure can be ascribed to several factors, including alterations in protein expression and function, myocyte death, and changes in signaling pathways. Of these factors, changes in the function of the sarcomere play a significant role in the development of cardiac dysfunction underlying the development of heart failure. These changes in sarcomeric properties are the result of either a change in isoform expression, post-translational modification of the sarcomeric proteins, or gene mutations linked to hypertrophic or dilated cardiomyopathy. In this chapter we will discuss the basic structure and function of the sarcomere in the context of a healthy heart, as well as the maladaptive changes that occur within the sarcomere during heart failure. We will also touch upon the

J.N. Simon, Ph.D
Department of Physiology and Biophysics, University of Illinois at Chicago,
Chicago, IL, USA
e-mail: jsimon24@uic.edu

J.C. Tardiff, M.D., Ph.D.
University of Arizona, 1656 East Mabel Street, MRB 312, MS# 245217, Tucson,
AZ 85724-5217, USA
e-mail: jtardiff@email.arizona.edu

B.M. Wolska, Ph.D (✉)
Department of Medicine, Section of Cardiology and Department of Physiology
and Biophysics, Center for Cardiovascular Research, University of Illinois,
Chicago, IL, USA
e-mail: bwolska@uic.edu

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emerging studies of genetically linked cardiomyopathies as intrinsic modulators of sarcomere function and an alternate cause of heart failure. Our hope is to provide the reader with insight into how sarcomere structure and function are altered during heart failure and how these alterations contribute to disease pathology.

The Sarcomere's Function in Normal and Failing Heart

Basic Function of the Sarcomere

The myofilaments consist of highly organized thick and thin filament proteins that facilitate, in a calcium-dependent manner, contraction and relaxation of the sarcomere (Fig. 1). Through the hydrolysis of ATP and resulting cross-bridge cycling, the cardiac sarcomere eloquently coordinates the transduction of elevated intracellular calcium into the mechanical work of contraction and relaxation necessary for proper cardiac pump function. Moreover, this critical function of the sarcomere occurs with both high fidelity and plasticity to the ever-changing physiological environment over time.

The myofilaments consist of both thick and thin filament proteins that span the sarcomere. The hexameric myosin macromolecular complex is the major component of the thick filament, accounting for roughly 30 % of the sarcomeric mass [109]. Each individual complex of myosin consists of two globular heavy chains (containing S1 and S2 regions) and two pairs of light chains (essential light chain, MLC1, and regulatory light chain, MLC2), extending from the coiled-coil tail domain to the globular N-terminal head region. Within the "rod-like" structure of the coiled-coil tail domain, neighboring myosin molecules anneal to form the thick filament backbone. Within the bare zone, or M-line, myosin molecules overlap one

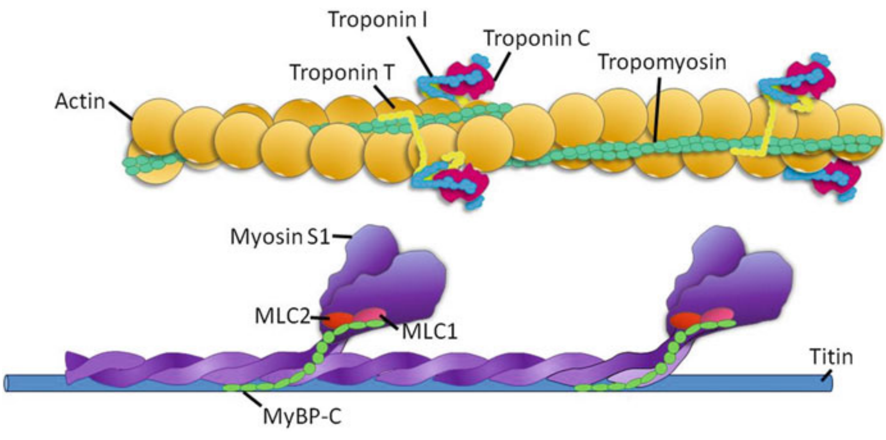

Fig. 1 Schematic representation of the thick and thin filaments of the cardiac sarcomere. *MLC* myosin light chain; *MyBP-C* myosin-binding protein-C

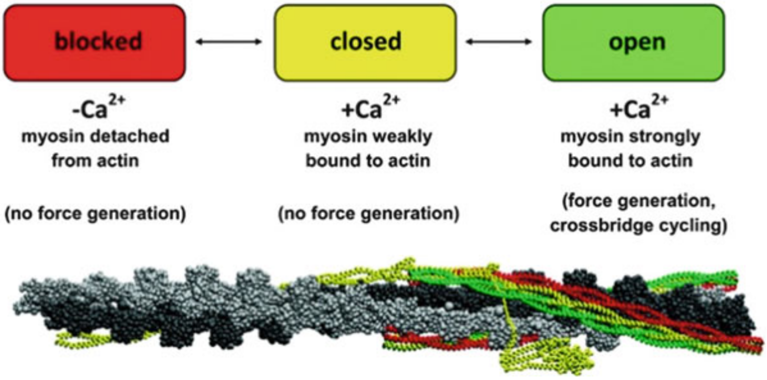

Fig. 2 The three states of tropomyosin (blocked, closed, and open) along the actin filament (modified from Manning et al. [56])

another in an antiparallel fashion, resulting in myosin heads that project from the backbone with opposite polarity. This unique organization allows for shortening of the sarcomere during cross-bridge cycling.

Further regulation of myosin structure and function occurs via the myosin-associated proteins, myosin-binding protein C (MyBP-C) and titin. MyBP-C collars the myosin head and acts to both tether the cross-bridge to the tail region, promoting greater order of the myosin heads along the thick filament [62], and connecting myosin to the cytoskeletal proteins through titin binding. As a consequence of binding to MyBP-C, myosin's kinetics is constrained and cross-bridge formation is extended [50, 103, 104]. Titin is a giant filamentous protein that extends from the Z-disk to the center of the sarcomere, where it interacts with myosin within the M line [31, 60]. Titin also provides elasticity and is further responsible for most of the passive tension within the sarcomere [29, 30].

Actin monomers, self-assembled into a filamentous structure, form the major portion of the thin filament and are critical for enhancing myosin ATPase activity during cross-bridge cycling. The functional unit of the thin filament comprises seven actin monomers, one tropomyosin (Tm) dimer, and one troponin complex that include the calcium-binding subunit, troponin C (TnC), the inhibitory subunit, troponin I (TnI), and the tropomyosin-binding subunit, troponin T (TnT). This multimeric structure allows for regulation of sarcomeric activation and cross-bridge cycling in a calcium-dependent manner. The Tn complex is crucial for binding calcium and allowing for the regulation of cross-bridge attachment, which it does by modulating Tm's position on the actin thin filament.

During diastole, intracellular calcium concentrations are low and the binding of calcium to the regulatory site on TnC is not favored. Furthermore, in this state the Tn complex acts to prevent actomyosin formation via Tn–Tm interactions that form an ordered structure along the actin filament. This structure promotes the positioning of Tm along the actin groove, resulting in either blockage of myosin binding to actin ("blocked" state) or weak cross-bridge attachment ("closed" state) [61]. As suggested by the three-state model of muscle contraction (Fig. 2), such positioning of Tm does not allow for significant force generation and the requirement of ATP for myosin binding and hydrolysis is minimal. It is also possible that the Tn complex itself may block the interaction between actin and myosin directly [111].

During systole, intracellular calcium levels rise promoting the binding of calcium to TnC. This binding induces a conformational change within the Tn complex resulting in strong binding of TnI, both the inhibitory region and the C-terminal domain, to TnC. As a result, TnI is released from actin and the interaction between TnT and tropomysin becomes significantly weaker [102]. This change in TnT–Tm interaction facilitates the movement of Tm into the "open" state. In this state, myosin-binding sites on actin are exposed allowing for the strong binding of myosin cross-bridges to actin, which greatly enhances actomyosin ATPase activity leading to cross-bridge cycling and force generation [21, 52, 61]. The formation of strong, force generating cross-bridges also promotes the binding of additional cross-bridges and enhances calcium binding to TnC [82]. This interdependence results in a relationship between calcium concentration and isometric force that is very steep and shows a highly cooperative character.

The interaction between the thick and thin filament is dependent on both the intracellular milieu, as well as the state of each protein within the sarcomere. Moreover, the functional interaction of these proteins involves multiple mechanisms, including allosteric, steric, and cooperative activation. It is this complex relationship within the sarcomere that makes the transition of the myofilaments from the relaxed state to the contractile (or activated) state highly sensitive to regulation at multiple points. Moreover, alterations in sarcomeric function can occur via regulation of practically any of the sarcomeric proteins. Of note, in the context of heart failure, chronic stress, indeed, causes alterations in a majority of the sarcomeric proteins, as will be discussed in subsequent sections of this chapter.

The Cross-Bridge Cycle and Dynamics

The cross-bridge cycle is the means by which the heart couples the hydrolysis of ATP to positive work production and produces force. It is driven by several thermodynamically favorable reactions. Cross-bridge cycling is dependent upon the ability of the myocyte to maintain sufficient levels of reactants (MgATP and H_2O) and products (MgADP, P_i, and H^+), thereby generating continual force production. A simplified schematic of the critical steps involved in cross-bridge cycling are shown in Fig. 3.

As mentioned in section "Basic Function of the Sarcomere," the myosin globular head domain contains an S1 region where both nucleotide binding and subsequent hydrolysis occurs (Fig. 3, Step 1). At this point, myosin remains bound to the hydrolysis products, MgADP and inorganic phosphate (P_i), with some myosin S1 heads binding weakly to actin. According to the three-state model of thin filament activation, this state of weakly bound myosin with a strongly bound nucleotide represents the "closed" state [65]. Further isomerization of the myosin head results

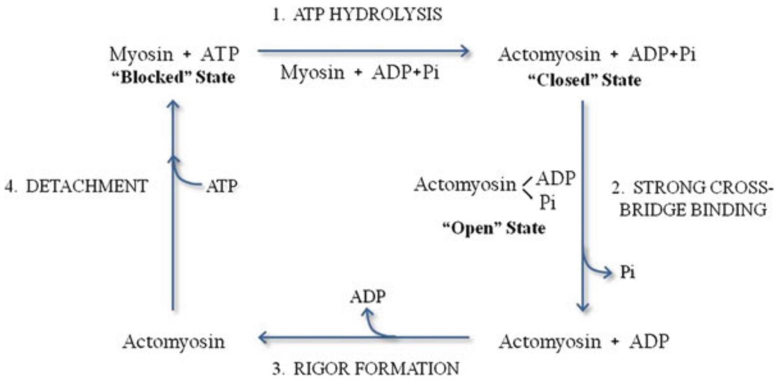

Fig. 3 A schematic of the cardiac cross-bridge cycle (adapted from Katz [46], *Physiology of the Heart*, Lippincott Williams & Wilkins)

in stronger binding to actin and a weakening of the associated nucleotide to form the "open" state. It is this transition from the closed to the open state that is regulated by the binding of calcium to TnC and subsequent movement of Tm away from myosin-binding sites along the actin thin filament [37, 66]. Following formation of the open state, inorganic phosphate is quickly released from the actomyosin complex (Fig. 3, Step 2). At this step along the cross-bridge cycle, the potential energy generated from ATP hydrolysis is transferred to the myosin "lever arm" and harnessed to produce the power stroke, which drives sliding of the thick and thin filament past one another [90, 112]. Under steady-state isometric force, ADP release from the actomyosin complex is rate-limiting and results in the formation of a rigor cross-bridge (Fig. 3, Step 3). At this point, given the high local concentration of MgATP, myosin undergoes rapid nucleotide binding followed by detachment of the myosin head from actin (Fig. 3, Step 4).

The precise biophysical changes that govern cross-bridge cycle dynamics have long been studied. For example, optical trapping technique allows for investigating and mathematically defining the force generated by a single myosin motor, the kinetics of the cross-bridge cycle, and how they change during different physiological and pathological conditions [112]. In these experiments, myosin undergoes transition from weak to strong actin binding and generation of a unitary force (F_{uni}) in a single step. This is proportional to the measured unitary displacement (d) (Fig. 4). In a normal cross-bridge cycle detachment of myosin from actin requires the release of ADP ($t_{-\mathrm{ADP}}$) and the binding of ATP ($t_{+\mathrm{ATP}}$). Thus, the duration of strong binding of the cross-bridges, or t_{on}, is a summation of $t_{-\mathrm{ADP}}$ and $t_{+\mathrm{ATP}}$. Moreover, once the length of time for a single cross-bridge cycle, t_{cycle} is obtained, the duty ratio (duty ratio $= t_{\mathrm{on}}/t_{\mathrm{cycle}}$) can also be determined. Kinetic measurements of d, t_{on}, and t_{cycle}, allow for determination of the average force (F_{ave}), which is proportional to the F_{uni} and the percentage of time myosin spends in strong binding state (i.e., the duty ratio). At the single molecule level myosin sliding velocity (V_{max}), measured using the in vitro motility assay, is proportional to d/t_{on} [44].

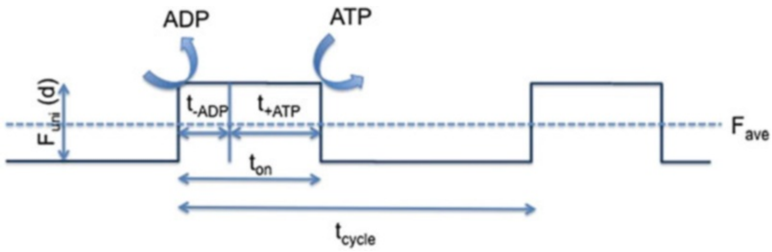

Fig. 4 A kinetic model of myosin unitary steps observed during the strong binding of cross-bridges to the actin thin filament. The upward deflection of the solid line reflects a weak-to-strong binding transition between myosin and actin during an optical trapping experiment, while the downward deflection of the line demonstrates dissociation of myosin from actin (modified from Tyska and Warshaw [112])

Interestingly, changes in several of these defined parameters can occur simultaneously having either additive or compensatory effects. For example, it has been demonstrated that F_{ave} is similar in V1 (αMyHC) and V3 (βMyHC) despite longer t_{on} and t_{cycle} in V3 because F_{uni} and the duty ratio are similar between the two isoforms [81, 105, 106]. Therefore, changes in any of these parameters will be important in determining changes in the cross-bridge dynamics that influence the dynamics of cardiac function.

Modulation of the parameters that define cross-bridge dynamics can be both calcium-dependent and calcium-independent. MgATP binding and the resulting detachment of myosin from actin (t_{on}) determines the maximal velocity of the cross-bridge cycling. The regulation of this process is calcium-independent and depends on the ability of the myocyte to maintain high levels of MgATP and low levels of MgADP, P_i, and H^+ [60]. In a state of energy deprivation, as observed in end-stage heart failure, the altered velocity of cross-bridge cycling becomes a significant contributor to the overall contractile dysfunction [33]. Maximal force production (F_{avg}) is determined by calcium-regulated mechanisms that include the number of rigor cross-bridges formed and the time these cross-bridges spend in the rigor state (duty ratio). Any observed change in contractile function associated with the sarcomere ultimately alters cross-bridge dynamics (kinetic properties of the cross-bridge cycle). These properties include changes to the velocity of shortening, the duty cycle, and/or the unitary force generated by the strongly bound cross-bridges, intrinsically related to the state of both the thick and thin filament. In the context of heart failure, evidence has supported alterations in the state of both the thick and thin filament as a major contributor to the associated contractile dysfunction. Such alterations can arise via extrinsic factors, such as changes in kinase activity and, thus, the post-translational state of the myofilament, and from intrinsic factors, such as changes in myofilament protein expression. In the following sections we will discuss how the changes in sarcomeric protein expression and post-translational state observed in heart failure alter cross-bridge dynamics and contribute to cardiac dysfunction.

Function of the Sarcomere in the Failing Heart

Over half a century ago, the first study was published which demonstrated a reduction in myofibrillar ATPase activity in the failing heart, suggesting a role for the myofilament in the development of heart failure [1]. We now know that many changes occur within the sarcomere to cause reduced cardiac function. There are three primary mechanisms that account for alterations in sarcomeric function observed during heart failure: changes in gene expression, protein proteolysis, and post-translational modification of the myofilament proteins. Of these, the role of protein proteolysis in cardiac dysfunction associated with heart failure appears to be minimal and may only occur as an initial response to ischemia-reperfusion injury [18, 64, 94]. Our focus in this section is on the alterations in both gene expression and the post-translational state of the sarcomeric proteins that have been shown to contribute to the reduced cardiac function in heart failure. While studies of these changes have provided sufficient evidence to suggest the sarcomere is a significant contributor to contractile dysfunction, one must also take into consideration the effects of such alterations in the context of an altered intramyocellular environment. Here we focus exclusively on how changes in sarcomeric protein composition and post-translational state directly relate to depressed cardiac function.

Changes in Composition of Sarcomeric Proteins and Their Biophysical Consequences

Thick Filament and Associated Proteins

Myosin Heavy Chains

The human myocardium expresses two isoforms of MHC: α-MHC and β-MHC. These two isoforms display 93 % amino acid identity [67], but show different biophysical properties. The isoform expression of MHC is different in atria and ventricles and changes in hypertrophy, heart failure, and other diseases. Non-failing human atria express about 90–100 % of α-MHC, but this amount decreases to 50–55 % in heart failure [91, 96, 113], whereas human non-failing ventricles express small (0–15 %) amounts of α-MHC [8, 68]. In hypertrophy and heart failure, the amount of α-MHC in the ventricle decreases to 0–4 % [70, 91, 96]. Although this observed shift in MHC isoform expression is relatively small, the two isoforms differ in ATPase activity, actin filament sliding velocity, and power output. These differences may still have a significant effect on the function of the sarcomere. In humans this is a complex task, not only because access to human samples is limited but also because in hypertrophy and heart failure there are dynamic changes in the level of myofilament proteins making it difficult to separate their individual effects. Thus, animal models of hypertrophy and heart failure are often employed to both circumvent these limitations and provide a longitudinal system for study.

It is well documented that α-MHC exhibits a several-fold higher actin-activated ATPase activity [55] and higher actin filament sliding velocity [35]. Moreover, the force generated by different cardiac preparations is dependent on the levels of expression of MHC isoforms. Herron et al. [39] reported that the force generated by myocytes expressing α-MHC is about 3 times higher than the force generated by myocytes expressing β-MHC. The rates of force development and unloaded shortening velocity are also shown to be increased in preparations expressing α-MHC [25]. Moreover, cross-bridge cycling kinetics is depressed linearly with decreased expression of α-MHC [93]. The power output, measured in skinned rat myocytes and isolated working heart preparations, is also linearly related to MHC content and decreases as expression of β-MHC increases [49]. A similar reverse relationship was observed between unloaded shortening velocity and β-MHC expression [49]. Very interesting data were published by the same group that compared loaded velocities, power output and peak normalized power in myocytes expressing either 0 or 12 % α-MHC. Peak normalized power output was 52 % greater in myocytes expressing 12 % vs. 0 % α-MHC [40]. These data suggest that even small shifts in α-MHC expression, as has been observed in human heart failure, may have a significant effect on overall heart function.

Myosin Light Chains

In addition to the shifts in MHC isoforms in hypertrophic and failing human hearts, there are reports regarding the shift in both the isoforms and the level of expression of essential and regulatory light chains. In the human heart, three isoforms of regulatory light chains (atrial, ALC-2, ventricular-a, VLC-2a, and ventricular-b, VLC2-b) and two isoforms of essential light chains (ventricular, VLC-1, and atrial, ALC-1) have been identified (for further review, see [38, 72, 75]). In the normal human heart the ratio of MLC1/MLC2 is 1:1 [57], but while it may change in pathological conditions the reported data are not consistent. Morano et al. [74] reported no change in MLC1/MLC2 ratio in failing hearts, but recently, Li et al. [54] have presented data indicating that the expression of MLC-2 is down-regulated in heart failure and the degree of the down-regulation is associated with the class of heart failure (New York Heart Association stages II–IV). Also, Margossian et al. [57] found that in idiopathic dilated cardiomyopathy the ratio of MLC1/MLC2 varied from 1:0.1 to 1:0.69. This decreased level of MLC2 was linked to the presence of an active protease and associated with a decrease in V_m of actin-activated myosin ATPase but no changes in the rates of ATP binding to myosin were detected.

In normal human heart the expression of ALC-1 is restricted to the atria, although it can be reexpressed in the ventricles in the context of different disorders. Patients with dilated cardiomyopathy expressing variable amounts (2.4–10.3 %) of ALC-1 showed increased myofilament calcium sensitivity that correlated with the amount of ALC-1 expression [75]. However, van der Velden showed no significant correlation between ALC-1 expression and calcium sensitivity in human end-stage donor samples [115]. One possible explanation for the difference between the two studies is the etiology of failure in the donor samples. In the study by van der Velden, the correlation was conducted using primarily samples taken from ischemic heart disease patients, whereas samples from patients with dilated cardiomyopathy were used in the former study. In addition it has been reported that increased expression of ALC-1 in skinned fibers prepared from human hearts with congenital heart diseases resulted in increased detachment rate and the rate of force development [76]. The increased cross-bridge kinetics was partially due to weaker interactions between the N-terminal domain of ALC-1 and actin [73]. The functional role of these changes has yet to be established, although studies using different TG mouse models strongly support the importance of the myosin light chain isoforms on cardiac function [13, 84].

Myosin-Binding Protein-C

As mentioned previously (see section "Basic Function of the Sarcomere"), MyBP-C is a critical component of the sarcomere and an important regulator of cardiac function. Three isoforms are expressed in muscle, but only one isoform, MYBPC3, is found in cardiac myocytes (for review, see [47]). Currently, no known changes in isoform expression of MyBP-C have been identified.

Titin

Titin is the largest protein in mammals and is often referred to as the third filament of striated muscle. It is responsible for the determination of passive tension and is a major sensing and signaling molecule. Two major isoforms, both which show distinct biophysical properties, are expressed in the human heart and their relative amounts change in heart failure. For a complete discussion of titin, see Chap. 10.

Thin Filament Proteins

Troponin Complex

Troponin is a trimeric complex that comprises TnT (Tn-tropomyosin), TnI (Tn-inhibitory), and TnC (Tn-calcium). TnT plays both a modulatory and structural role within the thin filament and binds to both Tm and TnI-TnC. Cardiac TnT is encoded by a single gene encoding multiple isoforms: one adult isoform (TnT3) and three fetal isoforms (TnT1, TnT2, and TnT4). While TnT3 is normally the only isoform expressed in the adult human heart, tissue samples from failing hearts have revealed the re-induction of the fetal isoforms [2, 69]. Moreover, Barton et al. reported expression of the slow skeletal muscle TnT gene in end-stage heart failure [4]. The functional significance of the expression of different TnT isoforms in human heart failure is not clear. It has been shown that human cTnT isoforms affect the calcium sensitivity of force development and the ability to inhibit the actomyosin ATPase activity, but no differences in maximal actin-Tm-activated myosin ATPase activity were observed [27]. Myofilaments reconstituted with human TnT1 and TnT2 isoforms that showed increased calcium sensitivity when compared with the TnT3 and TnT4 isoforms [27].

TnI and TnC are expressed as cardiac isoforms in the human heart. While changes in the ability to activate or inhibit actomyosin activity have been observed in the presence of ssTnI [28], ssTnI is only expressed during development [58] and there are no reports of re-expression of ssTnI in heart failure. The same is true for cTnC, in which expression levels appear to remain unaltered during heart failure.

Actin

The human heart expresses two actin isoforms: skeletal muscle α-actin and cardiac α-actin that only differ by two amino acid substitutions and a transposition [6, 97, 117]. The healthy human heart contains about 20 % of skeletal muscle α-actin [117] and in pathological conditions the same [6, 97, 117] or higher levels of skeletal muscle α-actin have been reported [108]. Recently, Copeland et al. reported that end-stage failing hearts express 53 % skeletal muscle α-actin compared to 21 % in normal heart [19]. However, they did not find any differences in the motility assay between those two actin isoforms. These functional data are not surprising, since the only difference between the two actin isoforms is two amino acid substitutions and one transposition of amino acids [118].

Tropomyosin

Tropomyosins are a family of actin-binding proteins encoded by four different genes. The adult human heart expresses predominantly α-Tm [86], but also β- and κ-Tm [24, 87]. Interestingly, in heart failure and dilated cardiomyopathy the expression level of κ-Tm increased by twofold [87], while β-Tm levels were reduced (personal communication from Dr. David Wieczorek). κ-Tm is structurally less stable and binds more weakly to actin as compared to α-Tm. When expressed in Tg mice, it results in decreased systolic and diastolic function with decreased myofilament sensitivity to calcium [87] suggesting that its altered expression in human failing hearts could contribute to the cardiac dysfunction.

Post-translational Modifications of Sarcomeric Proteins in Heart Failure

Sarcomeric Protein Phosphorylation

In the healthy heart, protein–protein interactions within the sarcomere are finely tuned by extrinsic signal transduction and subsequent post-translational modifications. Under pathological conditions, however, where the extrinsic signal is continually present, such post-translational modifications are no longer able to sufficiently tune contractile performance and these modifications become deleterious. The activities of several protein kinases and phosphatases have been shown to be altered in heart failure, resulting in changes in the post-translational state of many sarcomeric proteins. More recent studies have also provided evidence for a functional role of oxidative stress in modifying the post-translational state of sarcomeric proteins, contributing to contractile dysfunction.

Elevations in the level of catecholamines during the development of early heart failure result in increased β-adrenergic signaling, however, over time, chronic exposure leads to β-adrenergic receptor desensitization and reduced expression [10–12, 34]. As a result of depressed β-adrenergic signaling, generation of cAMP is greatly reduced in end-stage heart failure and PKA activity is low. This has significant effects on the sarcomere, as several of the sarcomeric proteins are known to be phosphorylated by PKA leading to altered cardiac dynamics. Interestingly, it has been shown that the etiology of the disease may also determine the degree to which PKA signaling is reduced [115]. Besides changes in PKA activity observed during heart failure, PKC activity is also altered. The initiation of hypertrophic remodeling causes activation of PKC within the myocardium, most notably activation of PKCβ and PKCα isoforms [9].

It appears that the reduction in PKA activity also plays a role, at least in part, in altering protein phosphatase activity in heart failure. One of the downstream targets of PKA is Inhibitor 1 (I-1), whose activity is dependent on phosphorylation by PKA. The main function of I-1 is to prevent protein phosphatase 1 (PP1) activity. Therefore, as a consequence of reduced PKA activity and, thus, reduced I-1 phosphorylation, PP1 activity is enhanced in the failing heart [77]. Studies in skinned myocytes from human failure samples suggest that the reduced PP1 expression in heart failure contributes directly to alterations in the myofilament response to calcium through changes in the phosphorylation state of MLC-2 and cTnI [114]. Finally, it appears that the activity of PP2A, while important in balancing the phosphorylation state of sarcomeric proteins in the healthy heart, does not change during heart failure [77].

Phosphorylation of Thin Filament Proteins: cTnI and cTnT

The putative PKA sites within the cTnI protein sequence (Ser-23 and Ser-24) are located within the N-terminal extension of cTnI that is unique to the cardiac isoform. This region is important for the interaction of cTnI with the regulatory domain of cTnC, found in its N-lobe, and for modulation of TnC's calcium-binding affinity. In the unphosphorylated state, the N-terminal extension remains highly flexible and binds to TnC, enhancing calcium-binding affinity. Consequently, the responsiveness of the myofilament to calcium is increased. Indeed, myofilament calcium sensitivity is increased in human failure samples and is returned to control levels upon PKA treatment [115, 121]. This effect was shown to be linked to reduced cTnI phosphorylation [115].

TnI can also be phosphorylated by PKCs and in some cases the sites of phosphorylation are isozyme-dependent. The majority of PKC isoforms expressed in the heart (PKCα, βI and βII, δ, ε, and ζ) target cTnI at Ser-43 and Ser-45 and alter ATPase rate with little to no effect on calcium sensitivity. However, PKCδ was shown to preferentially phosphorylate the PKA sites on cTnI and alter calcium sensitivity [80] in addition to reducing ATPase rate. Serine 43 and 45 of cTnI are located in the region of cTnI that participates in intermolecular binding to the C-lobe of cTnC. Phosphorylated cTnI at Ser-43/-45 promotes the blocked state of thin filament activation reducing actomyosin affinity and as a result, the calcium-stimulated ATPase rate is reduced [80]. This post-translational modification is likely to play a role in maladaptive remodeling during the transition from early to late stages of heart failure, as evidenced by the up-regulation of several PKC isoforms (ε, α, β) in end-stage heart failure [9, 48, 78, 100].

While phosphorylation of cTnI at Ser-43 and Ser-45 by PKCs appears to have the greatest consequence on contractile function of the failing myocardium, it has been shown that phosphorylation at threonine 144 may also play a significant role. Using the in vitro motility assay, Burkart et al. were able to demonstrate that the effects of PKC phosphorylation of cTnI on actin–myosin interactions exhibited site specificity, inasmuch as reconstituted preparations with cTnI pseudo-phosphorylated at Ser-43, Ser-45, and Thr-144 displayed both a decrease in maximal sliding speed and calcium sensitivity, while preparations with cTnI pseudo-phosphorylated only at Thr-144 displayed a reduction in calcium sensitivity without changes in sliding speed [14]. This implies that phosphorylation of Thr-144 is necessary for regulation of myofilament calcium sensitivity. Indeed, it is possible that the introduction of a negative charge within the inhibitory peptide region of cTnI, where Thr-144 is located, would be likely to alter the ability of cTnC to bind calcium and augment the calcium responsiveness of activation. Also of interest is the fact that the cardiac isoform of TnI is the only isoform that contains a phosphorylatable residue at position 144 (Pro-144 in slow skeletal and fast skeletal TnI). This may provide an alternative means for cTnI to regulate contractility in cardiac muscle, where the ability to recruit additional motor units for regulation is lacking.

The adult cardiac isoform of TnT (TnT3) contains four identified sites of phosphorylation by PKC, Thr-197, Ser-201, Thr-206, and Thr-287, all located in the C-terminal region of the protein. This region of TnT aids in the control of actin–myosin interactions in a calcium-dependent manner via interactions with cTnI and TnC. Specifically, changes in the C-terminal region can be propagated across the TnT structure and alter N-terminal interaction of TnT with Tm, thereby playing a direct role in modulating the calcium-dependent actomyosin ATPase activity [85]. It has been suggested that in end-stage congestive heart failure PKCα induces hyperphosphorylation of TnT, with little change in the early stages of failure [5]. Moreover, one study using detergent skinned mouse papillary muscles showed that PKCα-dependent phosphorylation of cTnT significantly depressed actomyosin ATPase rate, calcium sensitivity, and cooperativity of the myofilament and maximal tension development. They also demonstrated that Thr-206 appears to be the critical site for TnT regulation of reduced maximal tension, as mimicking phosphorylation (by glutamic acid substitution) at this site alone reduced isometric tension and calcium sensitivity while pseudo-phosphorylation of the other sites had no effect on these parameters [107]. Despite these findings, earlier studies suggest that the phosphorylation of cTnT alone cannot account for the full reduction in actomyosin ATPase rate [79] but rather, that a reduced affinity of TnT for actin-Tm potentiates the calcium-regulated myosin binding.

Phosphorylation of Thick Filament Proteins: MLC-2, MyBP-C, and Titin

MLC-2 exists in the human myocardium in two isoforms, LC-2 and LC-2*, distinguished by their increasing acidity and, therefore, difference in their isoelectric points. Both forms are targeted for phosphorylation by PKC, PKA, and myosin light chain kinase (MLCK) at Ser-15, while rodent MLC-2 is also phosphorylated at Ser-19. These two residues are located at the N-terminal end of MLC-2, a portion of the light chain that interacts with the C-terminal end of myosin's lever arm. Introduction of negative charges in this interacting region were shown to promote disorder of the helical array of myosin heads within the lattice structure, shifting them closer to the thin filament [53]. More recently, human MLC-2 was shown to exist in three predominate forms—the unphosphorylated species, a singly phosphorylated species, and a phosphorylated/deamidated species [99]. The deamidation occurs at the Asn adjacent to Ser-15, resulting in a switch from Asn to Asp and introduction of a negative charge at this residue (either position 14 or 16). The authors were unable to detect any phosphorylation at Ser-19 in the human samples.

Functionally, phosphorylation of MLC-2 serves to aid in basal cardiac contraction and ejection via alterations in myosin cross-bridge kinetics [95, 98]. Scruggs et al. demonstrated that ablation of basal MLC-2 phosphorylation reduced tension cost (ATPase activity/force produced), a measurement of the rate of cross-bridge detachment, without altering calcium sensitivity [98]. The lack of a change in myofilament response to calcium observed was consistent with the initial study characterizing this mouse model [95]. However, Morano et al. demonstrated increases in myofilament calcium-dependent tension development with MLC-2 phosphorylation, due to an increase in the rate of weak-to-strong cross-bridge transition [71]. Despite the discrepancy in calcium-sensitivity changes, these studies all concluded that MLC-2 phosphorylation is important for cardiac contractility. In human heart failure, it has been shown that the state of MLC-2 phosphorylation, but not MLC-2*, is reduced [114]. Although this reduction would be suspected to decrease calcium-dependent force production in the failing heart, the authors found the opposite to be true. They attribute the observed increase in calcium sensitivity to the greater functional effect of cTnI dephosphorylation on the myofilament properties. Despite this apparent masking of the functional role of MLC-2 phosphorylation in cardiac failure, the same authors showed that further dephosphorylation of MLC-2 by PP1 in failure has a much greater effect on the response of the myofilament to calcium than it does in the healthy heart. This was proposed to be a possible "last resort" mechanism that the myocardium employs to improve diastolic function in end-stage failure [114].

The level of MyBP-C phosphorylation has also been shown to be reduced in heart failure, with unphosphorylated MyBP-C serving as the predominate species and only a small portion existing in the mono-phosphorylated form [20, 45]. This reduction in the phosphorylation state of MyBP-C is likely due to a reduction in PKA activity, as PKA can phosphorylate MyBP-C at Ser-273, Ser-282, Ser-302, and Ser-307. However, MyBP-C is also a substrate of PKC phosphorylation and, therefore, the idea of increases in MyBP-C phosphorylation throughout cardiac remodeling cannot be excluded. In fact, in a mouse model of dilated cardiomyopathy caused by cardiac-specific PKCε overexpression, it was shown that MyBP-C phosphorylation at Ser-302 was significantly increased and likely contributed to cardiac dysfunction [122]. All of the phosphorylatable residues on cardiac MyBP-C are located within the M domain of the protein. This region interacts with both titin and myosin, restricting myosin movement [43]. More recently, Weith et al. demonstrated that MyBP-C also interacts with actin through the M domain and acts as a viscous load against filament sliding [120]. Upon phosphorylation by PKA, MyBP-C's ability to interact with actin was weakened resulting in greater myosin motility observed using the in vitro motility assay. It has also been shown that phosphorylation of MyBP-C causes an extension of the myosin head away from the myosin backbone [119], increasing the ATPase rate [63] and enhancing both contraction and relaxation. Interestingly, the post-translational state of MyBP-C also appears to affect protein stability. Following ischemia-reperfusion, MyBP-C was shown to be dephosphorylated, promoting its degradation [23, 94]. What effect this has on the myocardium during failure is not fully understood, but one would expect it to likely contribute to the observed reduction in the actomyosin ATPase rate.

We mention, only in brief, alterations to the post-translational state of titin demonstrated in human failure. The reader is referred to Chap. 10 for a more in-depth discussion. Dephosphorylation of titin at Ser-469 within the N2B region of titin's spring element has been shown to occur during failure, resulting in increased passive stiffness [7, 51]. The increased passive stiffness can be returned to healthy, control levels following PKA treatment [59, 116], suggesting that the reduced β-adrenergic signaling associated with failure is likely the cause for the depression in titin phosphorylation. Moreover, it seems that elevations in passive stiffness are not a result of weakly bound cross-bridge cycling as BDM treatment, which inhibits actomyosin interaction, did not reduce the passive stiffness observed in failing cardiomyocytes [7].

Sarcomeric Protein Oxidation

While it is well-known that oxidative stress occurs in heart failure [42, 88, 101], more recently increases in reactive oxygen species (ROS) have been linked to modifications of the sarcomere. Modifications include oxidation, *S*-glutathionylation, and carbonylation. Of the sarcomeric proteins, actin, Tm, and titin all have been shown to be modified by ROS during heart failure. Oxidation of actin was associated with reduced contractility via a reduction in the maximal force production and a depression in the force-frequency relationship [22]. Cys-374 is likely the modified residue, as oxidation at this site is associated with reduced ATPase activity and sliding velocity [22]. Actin was also shown to be carbonylated in human heart failure patients, as was Tm. These two modifications, aling with Tm disulfide cross-bridge formation strongly correlated with the associated cardiac contractile impairment [15]. Oxidation of Tm, presumably at Cys-190, has also been shown to occur in mice following MI [3]. Cys-190 is located within the TnT-interacting region of Tm and oxidation at this site is likely to decrease contractility through altered Tn–Tm interaction, as well as affecting Tm flexibility during the development of heart failure [16, 17, 41]. The same study by Avner et al. that demonstrated Tm oxidation also showed increased *S*-glutathionylation of a high molecular weight protein that they suggest is likely titin [3]. Interestingly, in vitro studies have pointed to three residues in titin that contain disulfide bridges under oxidative conditions. These modifications were localized to the N2B region of titin and resulted in increases in passive stiffness due to a reduction in the extensibility of titin [32].

Several other oxidative modifications have been observed within the sarcomere but their role in cardiac pathology remains unexplored. Myosin has been shown to be *S*-glutathionylated at Cys-400 and Cys-695 both within the myosin head region, and Cys-947 resulting in reduced ATPase activity [83]. Acutely, following an MI, myosin heavy chain was shown to be oxidized at Cys-707 and Cys-697. This was associated with reduced sliding velocity, suggestive of reduced unloaded shortening velocity of the cross-bridge. In addition, following the acute phase post-MI, MyBP-C carbonylation was shown to be increased [89]. Whether these acute, early changes in sarcomere protein oxidation remain present later on in heart failure progression are yet unknown.

Intrinsic Modulation of Sarcomere Function by Genetically Linked Mutations

To this point we have discussed the maladaptive changes to the sarcomere that occur during heart failure, in the presence of chronic extrinsic stressors. However, our understanding of the mechanisms underlying cardiac dysfunction associated with heart failure have broadened in recent years to include maladaptive changes to the sarcomere and cross-bridge dynamics caused by genetically linked mutations, or the so-called intrinsic stressors. Such genetically linked mutations are most frequently associated with mutations in sarcomeric proteins and lead to either hypertrophic or dilated cardiomyopathies (HCM or DCM, respectively). In part due to the complexity of the cardiac contractile apparatus, the precise mechanisms whereby mutations in sarcomeric proteins cause human cardiomyopathies are likely to be vast (for recent reviews, see [26, 36, 109, 110]). In the context of the preceding discussion, it is interesting to note that some of the proposed alterations in sarcomeric structure caused by thin filament mutations are predicted to change either the accessibility of the post-translational substrate site and/or the physiologic response to phosphorylation. Ongoing longitudinal studies of genotyped cohorts will continue to provide crucial data regarding the role of sarcomeric mutations in the progressive remodeling that occurs in many of the genetic cardiomyopathies [92].

Summary

Collectively, the modifications in both protein expression and post-translational state modulate the contractile state of the heart via direct effects on the biophysical properties of the cardiac sarcomere. The overall complexity of both the sarcomeric complex and the resultant patterns of cardiac ventricular remodeling observed in heart failure create a seemingly endless array of potential downstream pathogenic mechanisms. Coupling a deeper understanding of the primary biophysical causes of changes in contractile function to a more complete understanding of the resultant pathogenic ventricular remodeling that occurs over time will allow for both significant advances in disease management and new points of therapeutic intervention.

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