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CARDIAC DISORDERS AND PATHOPHYSIOLOGY OF SARCOMERIC PROTEINS

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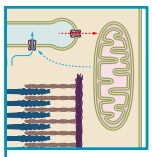

van der Velden J, Stienen GJM. Cardiac Disorders and Pathophysiology of Sarcomeric Proteins. *Physiol Rev* 99: 381–426, 2019. Published October 31, 2018; doi:10.1152/physrev.00040.2017.—The sarcomeric proteins represent the structural building blocks of heart muscle, which are essential for contraction and relaxation. During recent years, it has become evident that posttranslational modifications of sarcomeric proteins, in particular phosphorylation, tune cardiac pump function at rest and during exercise. This delicate, orchestrated interaction is also influenced by mutations, predominantly in sarcomeric proteins, which cause hypertrophic or dilated cardiomyopathy. In this review, we follow a bottom-up approach starting from a description of the basic components of cardiac muscle at the molecular level up to the various forms of cardiac disorders at the organ level. An overview is given of sarcomere changes in acquired and inherited forms of cardiac disease and the underlying disease mechanisms with particular reference to human tissue. A distinction will be made between the primary defect and maladaptive/adaptive secondary changes. Techniques used to unravel functional consequences of disease-induced protein changes are described, and an overview of current and future treatments targeted at sarcomeric proteins is given. The current evidence presented suggests that sarcomeres not only form the basis of cardiac muscle function but also represent a therapeutic target to combat cardiac disease.

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I. INTRODUCTION

Cardiac disorders, representing the main causes of death in Western societies, have a number of different causes. They can originate from structural defects inside cardiac muscle cells; for instance, in Ca^{2+}-handling or contractile properties, they lead to hyper- or hypocontractility of the myocardium (valve defects), volume or pressure overload of the heart [myocardial infarction (MI)], causing loss of myocardial tissue and replacement fibrosis, and systemic triggers such as hypertension, diabetes, and renal failure.

In this review, we will focus on the pathophysiological mechanisms involved in cardiac disorders at the level of the sarcomeric proteins, with special emphasis on human tissue and contractile protein phosphorylation. We will start with a description of sarcomeric proteins at the cellular level and of sarcomeric function and how this relates to cardiac performance at the organ level. Next, an overview will be given of the changes in sarcomeric structure and function in acquired and inherited heart diseases, with special emphasis on primary causes and secondary adaptive or maladaptive changes. The terminology describing cardiac disorders has different origins and therefore is quite diverse: pressure and volume overload (VO) (etiology), concentric and eccentric hypertrophy (anatomy), systolic and diastolic dysfunction (clinical presentation), and heart failure (HF) with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF) (clinical presentation). In the text, we aim to clarify the underlying pathomechanisms. Next, we discuss the techniques to study contractile function at the subcellular/cellular level and describe current and future treatment strategies targeted to sarcomeric proteins.

To provide a lead, we have formulated several questions debated during the last decade, which are summed up below. In the final section of this review, concluding remarks, with answers to these leading questions, will be presented to summarize our view on cardiac disorders and the pathophysiology of sarcomeric proteins.

General basic questions:

1. Does the direction of shift in Ca^{2+} sensitivity of force development dichotomously determine whether cardiac hypertrophy or dilatation occurs? If so, what is the mechanistic basis?

2. Is the Frank–Starling mechanism length-dependent activation (LDA) depressed in HF? If so, what is the mechanistic basis or prevailing/main cause?
3. What is the phosphorylation status of the sarcomeric proteins in a healthy heart? How does it change in specific cardiac diseases?

Translational aspects:

4. What are the guiding principles in the therapy of cardiac disorders caused by sarcomeric dysfunction? Protein isoform shifts or posttranslational modifications? Cardiac energetics, partitioning of energetic costs of basal cardiac metabolism, Ca^{2+} handling and contractile activity, or substrate choice? Reclassification of sarcomere-related cardiomyopathies based on novel insights in disease?

Overall, we aim to provide a bottom-up approach starting with a description of the basic components of cardiac muscle at the molecular level up to cardiac disorders at the organ level. We will try to give credits to all scientists who provided major contributions to understanding the role of sarcomeres in health and disease by preferably citing original papers. We would like to apologize in advance because not in all cases will be within our reach or it will be impossible to cite all because of space limitations.

II. SARCOMERIC FUNCTION IN A HEALTHY HEART: FROM THE CELL LEVEL TO CARDIAC PERFORMANCE IN VIVO

A. Cardiac Muscle Cells

The myocardium largely consists of cardiac muscle cells (cardiomyocytes) (see **FIGURE 1**), which are electrically coupled via gap junctions, or connexins. It thus forms a func-

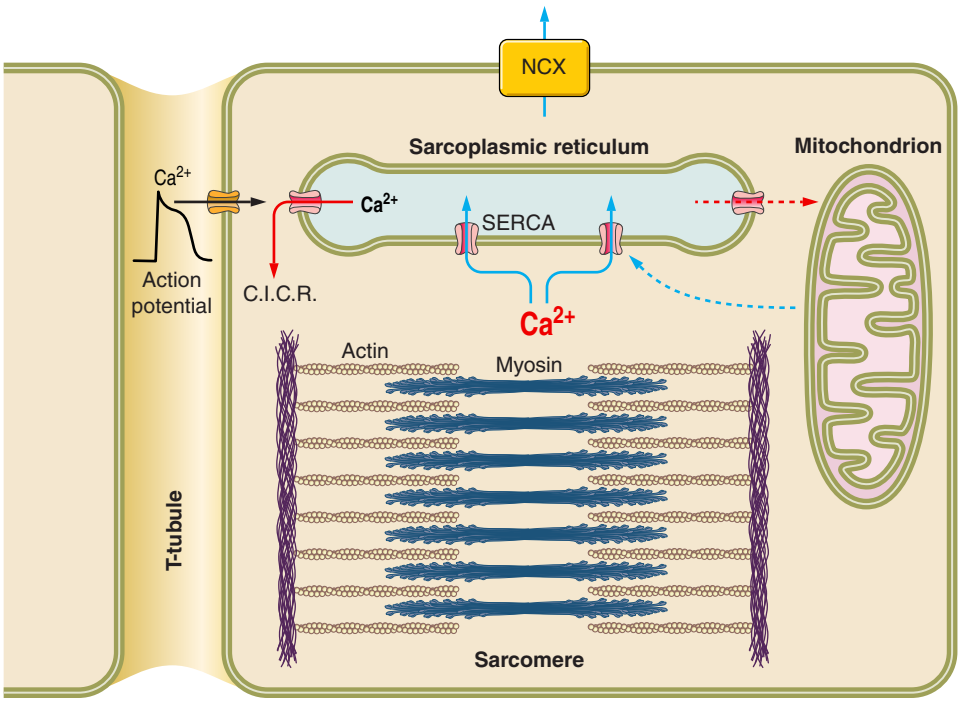


FIGURE 1. Schematic diagram of a cardiac muscle cell and the events during excitation–contraction (EC) coupling. Human cardiac muscle cells (cardiomyocytes) in the wall of the ventricles have a rod shape of ~20 μm in diameter and 100 μm in length. They are surrounded by a surface membrane with tubular invaginations (T-tubules) arranged in a network structure. The T-tubular network ensures that an action potential traveling along the surface membrane is propagated into the interior of the cardiomyocyte. During the plateau phase of the action potential (duration ~100 ms), calcium ions (Ca^{2+}) enter into the cell via the L-type Ca^{2+} channels. This causes a rapid rise in Ca^{2+} concentration in the narrow cleft between the T-tubular membrane and the sarcoplasmic reticulum (SR). This local rise in Ca^{2+} concentration triggers the release of Ca^{2+} stored inside the SR via the ryanodine receptor, a mechanism called Ca^{2+}-induced Ca^{2+} release (C.I.C.R.; solid red line). Ca^{2+} activates regulatory proteins on the thin (actin) filament, which allows binding of myosin heads extending from the thick (myosin) filament to the active sites on the thin filament, resulting in force generation and filament sliding (sarcomere shortening) associated with ATP hydrolysis. Recent evidence suggests that a small fraction of the Ca^{2+} released in the cytosol is taken up by the mitochondria (dotted red line), which is involved in the resynthesis of ATP. During the relaxation phase, the cytosolic Ca^{2+} concentration declines because Ca^{2+} is actively transported back into the SR via the SR-Ca^{2+} pump (SERCA) and out of the cell via the sodium-calcium exchanger (NCX), located in the surface membrane (blue lines). Sarcomeres, mitochondria, and SR occupy ~60%, 35%, and 5% of the cell volume, respectively.

tional electrical syncytium; an action potential originating from the sinus atrial node that propagates along the surface membrane of the cardiomyocytes, and cells of the atrioventricular node and the left and right bundle branches, to the interior of the cells. During a cardiac cycle, the cardiac muscle cells generate force and shorten to eject blood into the systemic and pulmonary circulation and subsequently relax, which enables filling of the heart.

Cardiomyocytes consist of parallel bundles of myofibrils of ~1 μm in diameter surrounded by the sarcoplasmic reticulum (SR). Each myofibril is composed of sarcomeres connected in series at the Z-discs. The sarcomere consists of a regular hexagonal lattice of *1*) thin, actin-containing filaments attached to the Z-disc; *2*) thick, myosin-containing filaments (interconnected in the middle via the M-band), and *3*) titin filaments running from the Z-discs to the M-lines in the middle of the sarcomere. In addition, a large number of accessory proteins are present, forming part of the sarcomeric and extrasarcomeric cytoskeleton, such as myosin-binding protein C (MyBP-C), obscurin, myomesin, nebulette and α-actinin, desmin, and intermediate filaments (microtubules, vimentin) essential for ordered assembly of the actin and myosin filaments into sarcomeres and a number of mechanical, signaling, and transport functions [see (125, 364, 427)].

The sarcomeres represent the contractile building blocks of the cardiomyocytes, which determine muscle contraction and relaxation during the cardiac cycle. To understand how changes in sarcomeric proteins underlie dysfunction of the heart during disease, some background will be provided on the composition of the sarcomere and its role during excitation–contraction coupling in cardiac muscle cells. Therefore, the structural arrangement of sarcomeric proteins and the central events during excitation, contraction, and relaxation of the cardiomyocytes will be described in more detail below.

B. Ca^{2+} Handling

Upon electrical stimulation of cardiac muscle cells, Ca^{2+} enters the cells via the voltage-dependent L-type Ca^{2+} channels and triggers the ryanodine receptor to release Ca^{2+} from the intracellular Ca^{2+} store, the SR **(FIGURE 1)** (32). The latter event is called Ca^{2+}-induced Ca^{2+} release (102). After Ca^{2+}-activated myofilament contraction, cytoplasmic Ca^{2+} levels need to decrease to initiate myofilament relaxation. Four different mechanisms are thought to underlie removal of the Ca^{2+} that is released from the myofilaments after contraction: in human ventricular muscle cells the largest fraction of cytoplasmic Ca^{2+} (~70%) is moved back to the SR via the ATP-dependent SR pump the SR Ca^{2+}-ATPase (SERCA2a). Another relatively large part is removed from the cell via the sodium-calcium exchanger (NCX; ~28%) and the sarcolemmal Ca^{2+}-ATPase (~1%). Ca^{2+} uptake by the mitochondria is generally considered to be rather small (*9*, 47, 485) and serves a regulatory role as it increases ATP production during increased workloads (16, 48). In rodents (rat, mice), reuptake of cytoplasmic Ca^{2+} into the SR is larger (~90%) than in humans because of the larger number of SERCA2a pumps, whereas the contribution of the NCX to Ca^{2+} removal is lower.

C. Cross-Bridge Kinetics

Electrical activation (excitation) of the cardiomyocytes initiates a chain of cellular events which lead to an interaction between the thin (actin) **(FIGURE 2*A*)** and thick (myosin) filaments (cross-bridge formation), causing force generation and/or shortening (32, 140). A model of the myosin molecule is shown in **FIGURE 2*B***. It consists of subfragment 1 (S1) and a long-coiled coil structure, subfragment 2 (S2) with proximal S2, and heavy and light meromyosin (LMM) domains. At the molecular level, a cyclic interaction takes place in which myosin heads (S1) bind to specific sites on the actin filament **(FIGURE 2*C*)**. During this cyclic process, myosin heads attach [form a cross-bridge between the thin (actin) and thick (myosin) filament], generate force, or induce sarcomere shortening and subsequently detach. During this cycle, external work is delivered, fueled by the hydrolysis of ATP (185, 186, 254, 481). In a simplified two-state scheme of the cross-bridge cycle, the attachment process takes place with a rate f_{app}, and the detachment process takes place with a rate g_{app} (51). This implies that the fraction of attached, force-generating cross-bridges is equal to $f_{app}/(f_{app} + g_{app})$.

The rate of ATP hydrolysis is determined by the rate-limiting step in the cycle (presumably the detachment represented by g_{app}). The rates f_{app} and g_{app} vary with the isoform of myosin heavy chain (MHC) expressed; the rates are faster for cross-bridges consisting of the fast (α) MHC than those consisting of the slow (β) MHC. The composition of MHC isoforms is species- and tissue-specific and may change during cardiac disease (described below).

D. Contractile Regulation

1. Thin filament regulation

The thin (actin) filament consists of two strands of globular actin (368) arranged in a helical manner also called filamentous actin (F-actin), two tropomyosin (Tm) strands, and the three troponin (Tn) subunits Tn T (TnT), Tn I (TnI) (393), and Tn C (TnC), which form the Ca^{2+}-regulatory complex of the thin filament **(FIGURE 2*A*)**. Each Tm strand binds to seven actin monomers, whereas Tm strands are bound end to end. This effectively results in a structural repeat of the thin filament of ~38 nm.

At low (resting) [Ca^{2+}], myosin-binding sites on the actin filament are blocked by the Tm–Tn complex. An increase in

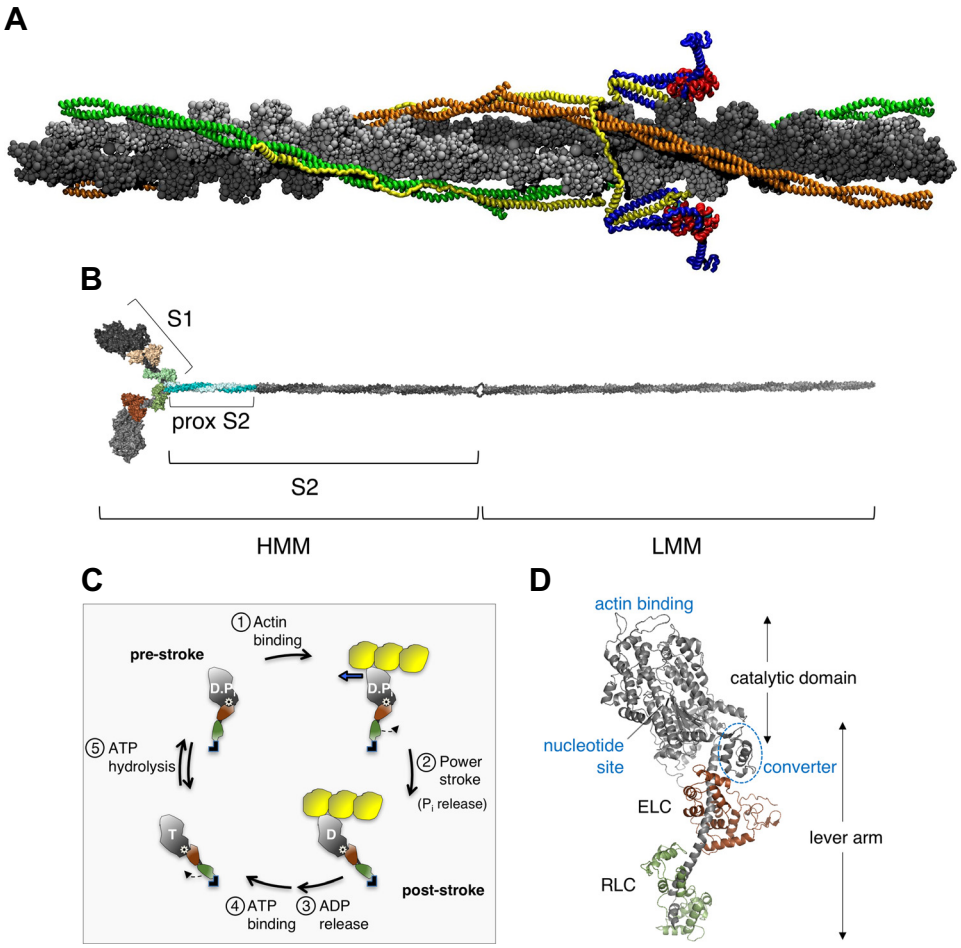


FIGURE 2. *A*: Model of the human thin filament containing human cardiac troponin (cTn), tropomyosin (Tm), and actin: yellow = cTnT; blue = cTnI; red = cTnC; cyan = Ca^{2+} ion; green/orange = overlapping Tm; silver/gray = actin filament. From Manning et al. (263). *B*: Model of the human β-cardiac myosin molecule showing subfragment 1 (S1), proximal subfragment 2 (prox S2), subfragment 2 (S2) and heavy and light meromyosin (HMM and LMM, respectively) domains. The S2 region is a long-coiled coil structure. *C*: Simplified chemomechanical cycle of the interaction of myosin heads with actin. Steps of the chemomechanical cycle are the following: *1*) The prestroke S1 with bound ADP (D) and inorganic phosphate (P_i) binds to actin (yellow); *2*) while bound to actin, the lever arm swings to the right about a fulcrum point (black dot on white star) to the poststroke position, moving the actin filament to the left (bold blue arrow) with respect to the myosin thick filament; *3*) ADP release frees the active site for binding of ATP (T); *4*) ATP binding weakens the interaction of the S1 to actin; and *5*) ATP hydrolysis locks the head into the prestroke state. *D*: Structure of the myosin head (S1) in its prestroke state with the actin binding site, the active (catalytic) domain where ATP hydrolysis takes place, the lever arm and the two light chains: the essential light chain (ELC) or myosin light chain 1 (MLC1), and the regulatory light chain (RLC) or myosin light chain 2 (MLC2). *B*, *C*, and *D* are from Trivedi et al. (424).

cytosolic Ca^{2+} from ~0.1 μM to ~1.6 μM results in binding of Ca^{2+} to cardiac TnC (cTnC). Binding of Ca^{2+} to cTnC changes the conformation of the Tm–Tn complex, which changes the position of Tm and releases myosin-binding sites on the actin filament, causing cross-bridge formation and force development (143, 304, 340) during the active phase of cardiac contraction (systole). A subsequent release of Ca^{2+} from the myofilaments and removal of Ca^{2+} from the cytosol results in relaxation of muscle cells during the diastolic (filling) phase of the heart.

Biochemical and structural studies provided evidence for a three-state model for the regulation of the binding of myosin heads (S1) to the actin filament consisting of a "blocked state" in which binding of S1 is prevented, a "closed state" in which S1 can only bind relatively weakly, and an "open" (ON) state in which S1 can both bind and undergo an isomerization to a more strongly bound rigor-like conformation (236, 273). In the blocked state, Tm sterically blocks myosin-binding sites on actin. In the closed state, Ca^{2+} binds to cTnC and thereby changes conformation of the Tm–Tn complex, resulting in weakly bound cross-bridges. The ON state involves strong binding of tension-generating cross-bridges, which results in force development and myofilament contraction. Cytosolic changes in ATP, ADP, and inorganic phosphate influence binding of myosin heads to actin and thereby modify force generation of the myofilaments. Recently, it was observed that the

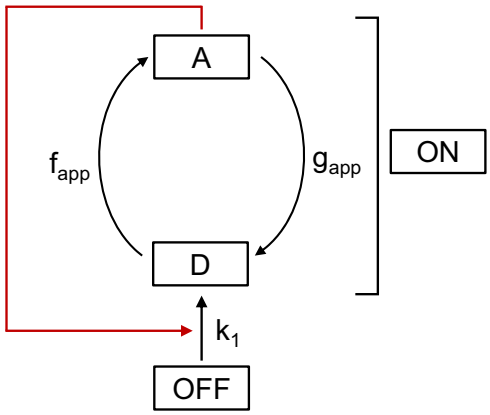


FIGURE 3. Myosin-based regulation of the cross-bridge cycle. Mechanosensing of the force exerted on the myosin filament by the attached cross-bridges provides a positive feedback signal (in red), causing unlocking of myosin heads in the OFF state (via rate k_1). A and D represent the states in which myosin heads are attached and detached, respectively. The axial periodicity of the myosin filament in the OFF state (M3) amounts to 13.34 nm, whereas in the fully ON state this periodicity increases to 13.57 nm. This increase in periodicity (1.7%) is larger than can be accounted for by filament compliance (~0.2%), probably because of the axial movement of myosin heads (187). In the presence of Ca^{2+}, consecutively active ON cross-bridges (~5% of total) enable maximal shortening at low load. At higher loads, these cross-bridges generate sufficient thick-filament stress to unlock myosin heads required for high-load contraction.

activation process consisted of four different components (115), but the biochemical and structural correlates thereof need to be determined.

2. Thick filament regulation

The thick (myosin) filament consists of dimeric myosin molecules to which accessory proteins, such as MyBP-C and titin, are bound. The myosin molecule (~470 kDa) is a dimer composed of two motor domains (S1, the heads) and a coiled coil rod domain (tail) **(FIGURE 2*B*)**. The crystal structure of S1 in the so-called rigor state has been resolved in the early 1990s (356). The myosin rod is composed of light meromyosin (LMM) subfragment and S2, which forms a flexible link from the heads to the filament (126). In the S1 region near S2, two myosin light chains (MLC) are bound, the essential MLC (MLC1) and the regulatory MLC (MLC2). The location of the light chains is illustrated in **FIGURE 2*D***. The α-helical, coiled coil tails form the backbone of the myosin filament. The two myosin heads extend from its surface in three near-helical strands with an overall repeat of ≈43 nm. Each layer of heads (crown) is rotated over 120° relative to the neighboring crown. In the center of the myosin filament, a bare zone is found free of myosin heads as a consequence of the bipolar arrangement of the myosin molecules. At rest, a large fraction of myosin heads **(FIGURE 2*B*)** are folded backward, possibly make contact with the proximal S2 domain, and in this way, adopt the so-called J motif, a conformation in which ATP hydrolysis is largely prevented, also named the OFF state. The small fraction of remaining heads adopts an orientation with the regulatory light chain perpendicular to the filament axis (487, 498). It has been proposed, on the basis of low-angle X-ray diffraction measurements, that force generation in skeletal muscle is also regulated by mechanosensing in the myosin filament (241). Recently, it has been shown that this mechanism may be present in cardiac muscle as well (357). An extension of the two-state cross-bridge scheme discussed above is schematically shown in **FIGURE 3**. This myosin-based feedback mechanism promotes recruitment of cross-bridges during the activation phase as well as parking of myosin heads in the OFF (super relaxed) state, in which ATP hydrolysis is inhibited, during the relaxation phase (178).

Because thin and thick filament regulations most likely act in parallel, it will be difficult to determine their relative contributions in the working heart. Nevertheless, we anticipate that the concept of thick filament regulation will be very important in future research, in particular in relation to the role of mechanosensing and the pathophysiology of mutations in myosin, cardiac MyBP-C (cMyBP-C) and titin, described in detail below (section III).

3. Ca^{2+} sensitivity of tension development

The relation between isometric tension and the free intracellular Ca^{2+} concentration can be described by the modified Hill equation $T(Ca) = T_{max}.Ca^{nH}/(K^{nH} + Ca^{nH})$, in which nH is a measure of the cooperativity in the process of force development (i.e., the steepness of the Hill curve) and K (or EC_{50}) corresponds to the Ca^{2+} concentration required for half-maximal activation. In the classical interpretation, nH would represent the number of Ca^{2+}-binding sites within the regulatory unit (in this case, cTnC) **(FIGURE 4)**. cTnC has four metal-binding EF–hand motifs. It has

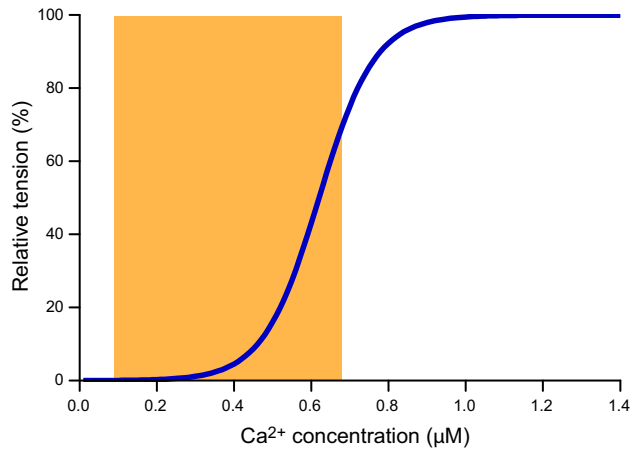


FIGURE 4. Schematic diagram of the Ca^{2+} sensitivity of active isometric tension development in intact cardiomyocytes, according to the modified Hill equation described in the text (section II*D3*). The orange region indicates the physiological range determined by the cytosolic-free Ca^{2+} concentration.

been shown that under the prevailing intracellular conditions (~1 mM of free Mg^{2+}), only Ca^{2+}-binding site II in the cTnC N domain is responsible for regulating cardiac contraction (422).

The Ca^{2+} sensitivity of isometric tension development has been determined in intact preparations by measuring the free Ca^{2+} concentration in conditions in which active tension development was steady (450) and in "skinned" preparations in which the Ca^{2+} concentration can be controlled directly because the surface membrane has been removed or made permeable. Sarcomere control during contraction appeared to be important for accurate measurement of EC_{50} as well as nH (210). Typical values obtained in permeabilized cardiac trabeculae from rats at 15°C and 2.0-μm sarcomere length are EC_{50} of 4 μM and nH of 7.3 (91a). In intact rat trabeculae at room temperature (without sarcomere length control), an EC_{50} of 0.62 μM and an nH of 4.9 were found (121). These nH values are larger than expected on the basis of the available Ca^{2+} sites on cTnC and are thought to represent cooperativity of neighboring Tn complexes and between adjacent myosin heads.

Detailed analysis of the tension-Ca^{2+} relation revealed that the steepness of the relation at low Ca^{2+} concentration ($nH1$) was larger than the steepness of the curve at high Ca^{2+} concentration ($nH2$). These data and measurements of the rate of force redevelopment (k_{tr}), using *N*-ethylmaleimide–modified myosin S1, have been taken as evidence that strongly bound (force-generating) myosin heads at low Ca^{2+} concentration also turn on the actin filament (111, 271). However, it has been argued that the cooperative mechanism underlying the high value of nH is not due to force-generating cross-bridges but that it is an intrinsic property of the thin filaments (408). In addition, the interpretation of the k_{tr} measurements may be reconsidered in light of the myosin-based regulation of contraction (241, 357).

E. Length-Tension Relation

The isometric force per cross-sectional area (equals tension) at saturating Ca^{2+} concentration in striated muscle tissue depends on the degree of overlap between the actin and myosin filaments (141) and thus on sarcomere length **(FIGURE 5)**. This overlap is optimal near the resting sarcomere length (~2 μm) and decreases when sarcomere length is increased above 2.2 μm. This is called the descending limb of the isometric length–tension relation. At sarcomere lengths below resting length the isometric tension declines as well because of double overlap between the actin filaments (<2 μm) and the folding of the myosin filaments between the Z-discs (<1.6 μm), the ascending limb of the isometric length–tension relation. Studies on the changes in sarcomere length during the cardiac cycle indicate active

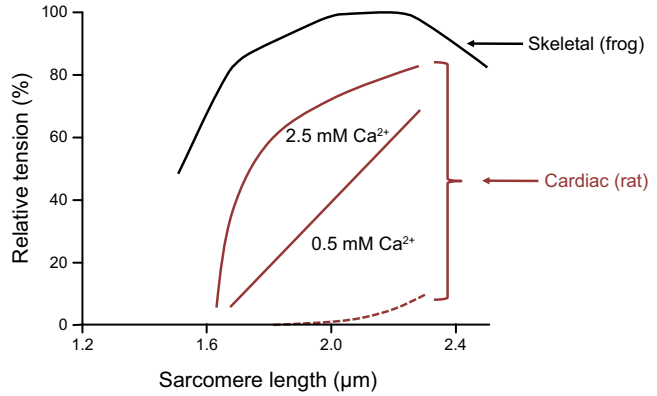


FIGURE 5. Schematic representation of the active sarcomere length–tension relations in frog skeletal muscle fibers [in black; redrawn from Gordon et al. (141)] and in thin rat cardiac trabeculae [in orange, redrawn from ter Keurs et al. (418)], at a high level of activation (2.5 mM of external Ca^{2+}), at a lower level of activation (0.5 mM of external Ca^{2+}), and during quiescence (passive tension, dotted curve). At 2.5 mM Ca^{2+}, active peak tension, in cardiac trabeculae stimulated at 0.2 Hz, approaches the relation in skeletal muscle, whereas at 0.5 mM Ca^{2+}, the relation is rather linear. The relations in cardiac muscle are scaled by assuming that maximum tension set to 100% at 2.2 μm sarcomere length and 2.5 mM Ca^{2+} in (418) corresponds to a submaximal level of activation of 80% of maximum tension in skeletal muscle. Actin filament length in rat papillary muscle is on average ~0.1 μm larger than in frog skeletal muscle (55). This suggests that the predicted descending limb of the maximal force–length relation in cardiac muscle would be shifted to the right by ~0.2 μm.

shortening from ~2 μm to 1.8 μm (393). Hence, cardiac muscle operates on the ascending limb of the isometric length–tension relation in which active tension increases with an increase in sarcomere length.

Studies on the length–tension relation were performed in intact thin cardiac trabeculae from rat in which sarcomere length during contraction could be followed and controlled via Helium–Neon laser diffraction (79, 379, 418). A crucial finding in these studies was that the initial part of the ascending limb of the length–tension relation at submaximal levels of activity was less steep than at near maximal Ca^{2+} levels **(FIGURE 5)**. The latter was attributed to LDA. A subsequent study in permeabilized trabeculae (210) showed that LDA could be attributed to a sarcomere length-dependence of the relation between isometric tension and the free Ca^{2+} concentration (see section II*D3*). According to recent measurements, LDA represents a very fast process, operating at a time scale of fewer than 5 ms (268).

In addition to a very rapid increase in peak tension during electrical stimulation upon a sudden stretch, a slow further increase in tension was observed, named after its discoverer, the Anrep effect (454). It has been shown that this increase resulted from an increase in the amplitude of the Ca^{2+} transient (6), likely because of increased Ca^{2+} entry via the reverse mode of the NCX (342).

F. Force–Velocity Relation

A hyperbolic dependency is observed between the load exerted on an active muscle and the velocity of shortening. The velocity of shortening can be expressed in micrometers per half sarcomere per second to reflect the speed of sliding of the thick and thin filaments relative to each other. At zero load, the velocity of sarcomere shortening reaches a maximal value (V_o) which is species- and myosin isoform–dependent. During a twitch, it has been found that V_o increases until developed force reaches half of its maximum value. Also, at low external Ca^{2+} concentration, which reduces peak force, a reduction in V_o has been observed with a similar force dependency (79). These results indicate that (below 50% of peak force) the level of activity (i.e., the fraction of strongly bound myosin heads) determines V_o. It can be noted that the activity level during the ventricular ejection phase corresponds to >50% of peak force. Studies at different isoproterenol concentration under near physiological $[Ca^{2+}]_o$ indicated that the level of β-adrenergic receptor stimulation did not influence V_o (86). Because a force equilibrium exists between pushing and pulling myosin heads of the actin filament under zero load, the most likely explanation would be that up to a certain level of activity the apparent rate of attachment (f_{app}) increases with the cytosolic Ca^{2+} concentration.

At low sarcomere lengths of 1.5–1.8 μm, V_o increased in sarcomere length. It has been argued that opposing forces below slack length are not sufficient to explain this result. However, the Ca^{2+} sensitivity of the thin actin filament has been shown to increase with sarcomere length, which could lead to an increase in the apparent attachment rate **(FIGURE 4)**. The general picture thus emerges that in the physiological range of sarcomere lengths and activity levels, V_o is a rather constant factor determined by the load-dependent cross-bridge detachment kinetics.

G. Exercise/Stress

To adjust cardiac pump function to increased demand during exercise (fight) or stress (flight), cardiac muscle cells are activated to increase contractility and match the increase in heart rate. Activation of cardiac muscle cells occurs via the β_1-adrenergic receptors (β_1-AR), which leads to phosphorylation of multiple Ca^{2+} handling and myofilament proteins by protein kinase A (PKA) (32, 222). Phosphorylation of the L-type Ca^{2+} channel and ryanodine receptor enhances the level of cytosolic Ca^{2+} released during Ca^{2+}-induced Ca^{2+} release and thereby increases muscle cell contractility **(FIGURE 6*A*)**. To match the increase in heart rate, the velocity of muscle cell relaxation needs to increase, which is regulated via phosphorylation of phospholamban and myofilament proteins (208). Phospholamban inhibits and thereby regulates the activity of SERCA2a. PKA-mediated phosphorylation of phospholamban removes its inhibitory action on SERCA2a and thereby enhances SERCA2a activity. As a consequence, reuptake of Ca^{2+} into the SR increases and thereby enhances muscle relaxation. As reuptake into the SR increases, the affinity of myofilaments (i.e., cTnC) for Ca^{2+} decreases, which is regulated via phosphorylation of TnI **(FIGURE 6*B*)**. PKA-mediated phosphorylation of cardiac TnI (cTnI) decreases Ca^{2+} sensitivity of myofilaments and thereby enables faster Ca^{2+} detachment from the myofilaments. It is thought that, in the cytosol, a balance exists between Ca^{2+} buffering by SERCA2a and cTnC; upon β_1-AR activation, Ca^{2+} binding to SERCA2a increases via phosphorylation of phospholamban, whereas Ca^{2+} binding to cTnC decreases via phosphorylation of cTnI. This has been illustrated by studies using phospholamban knockout mice and mice carrying skeletal TnI, which cannot be phosphorylated by PKA (52). Changes in either system, as occurs during disease (described below), may impair cytosolic Ca^{2+} buffering.

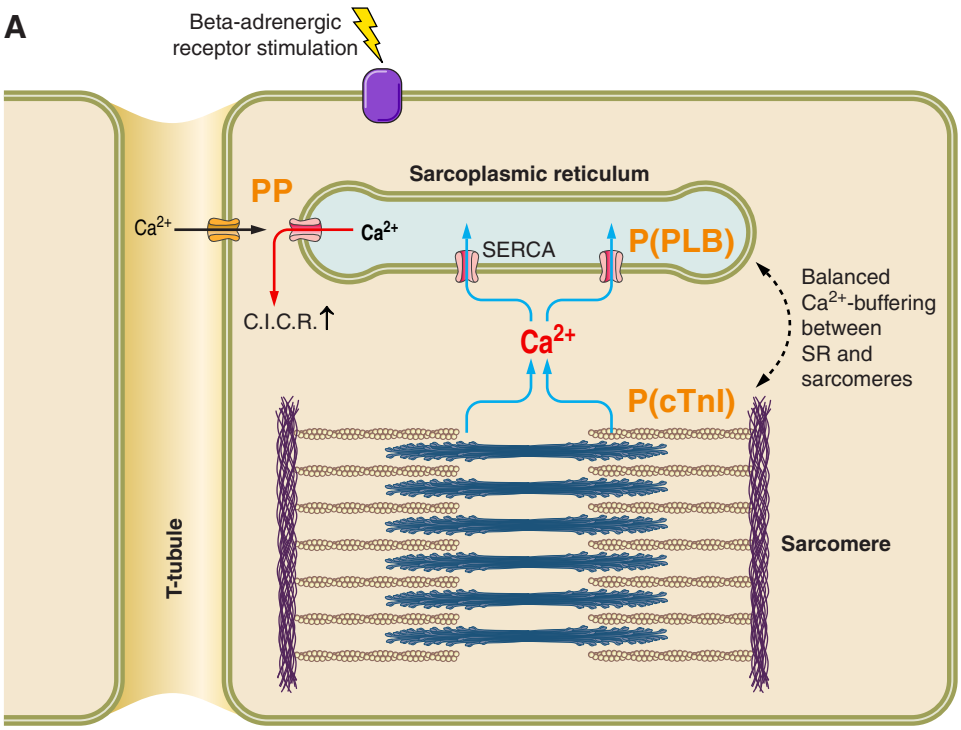
H. Sarcomeric Proteins and Phosphorylation

1. Thick filament proteins (myosin, MyBP-C)

In the adult heart, two isoforms of the MHC are found: the slow (β) MHC and the fast (α) MHC, distinguished on their intrinsic slow and fast rates of actin-activated ATPase activity, respectively. Human ventricular muscle is predominantly composed of the slow (β) MHC, whereas in healthy atria, the fast (α) MHC predominates. In addition, different MLC isoforms are expressed in ventricles (VLC1 and VLC2) and atria (ALC1 and ALC2) (293, 314).

MLC1 phosphorylation has been reported in stress conditions (10). MLC2 can be phosphorylated by the cardiac isoform of MLC kinase at Ser15 (60, 65). In human myocardium, the MLC2 pattern on 2-dimensional gels consisted of four spots, suggestive of the presence of two different isoforms with a similar phosphorylation pattern (438). Results in a more recent study (380) suggest that the doublet observed may be caused by deamination. MLC2 phosphorylation increases Ca^{2+} sensitivity (321, 409) and accelerates cross-bridge kinetics (73, 461). In rodents, differences in MLC2 phosphorylation are observed between subepicardial and subendocardial layers, and have been assigned a central function in cardiac performance, but this issue is still considered controversial (59, 83, 91). A recent study in beating mouse hearts indicated that constitutive MLC2 phosphorylation, which is dependent on a balance between MLC kinase and protein phosphatase, is needed to maintain cardiac performance (63).

cMyBP-C is a 130-kDa-thick filament protein located on seven to nine stripes 43 nm apart in the C-zone of each half A-band (326). The molecule is ~4 nm in diameter and ~40 nm in length and composed of a chain of eleven globular immunoglobulin and fibronectin domains (C0–C10) and a



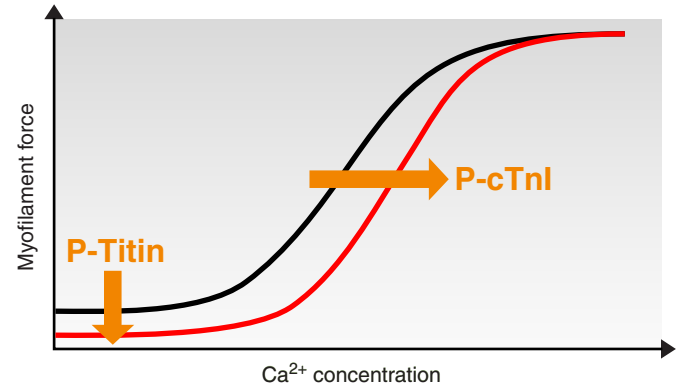


FIGURE 6. *A*: Stimulation of β_1-AR activates protein kinase A-mediated phosphorylation of L-type Ca^{2+} channels and ryanodine receptors, which increases Ca^{2+}-induced Ca^{2+} release (C.I.C.R.) phosphorylation of phospholamban (PLB) and cardiac troponin I (cTnI) increases Ca^{2+} uptake via SR-Ca^{2+} pump (SERCA) and Ca^{2+} release from the sarcomeres, respectively. *B*: The augmented release of Ca^{2+} from the thin filaments is mediated via the PKA-mediated reduction in myofilament Ca^{2+} sensitivity via phosphorylation of cTnI. In addition, PKA-mediated phosphorylation of titin reduces passive stiffness of sarcomeres.

phosphorylatable, intrinsically disordered, extensible M-domain between C1 and C2. The cardiac-specific, C0 domain at the N terminus is connected to C1 via a proline- and alanine-rich linker (19, 112, 126, 253, 325).

C0, C1, C2, and the M-domain are able to bind to F-actin (19, 384) but also to myosin S2 (144). The C-terminal domains C7–C10 bind to the C-zone of the myosin thick filament (252, 253). Recently, it has been shown that C1 binding to F-actin causes a shift in Tm and promotes the ON state of the thin filament (156). Both C0 and C1 fragments were found to compete with myosin S1 for binding to F-actin and inhibit actomyosin interactions when present at high ratios relative to F-actin. Another study (198) indicated that the N-terminal region of cMyBP-C not only stabilizes the ON state of thin filaments but also the OFF state of thick filaments (in which the myosin heads are folded back against the backbone of the thick filament).

Functionally, cMyBP-C is considered to act as a brake on cross-bridges because it modulates cross-bridge kinetics [e.g (300, 367, 396, 397)]. cMyBP-C knockout mice models showed enhanced cross-bridge kinetics [i.e., a faster shortening velocity and k_{tr} of skinned cardiomyocytes (219, 394, 396)], faster stiffening of the heart during systole (307), and a marked abbreviation of the systolic ejection (332, 333). Supposedly, phosphorylation of cMyBP-C relieves this brake because it results in a release of its binding to the S2 region of myosin and to actin and extends the cross-bridges further from the myosin backbone (384, 468). Seventeen phosphorylation sites on cMyBP-C were identified in vivo, with the majority localized in the N-terminal domains C0–C2. The M-domain of cMyBP-C harbors several phosphor-

ylation sites (Ser275, Ser284, Ser304, and Ser311) that have been identified as substrates for PKA, protein kinase C (PKC), or Ca^{2+}-dependent calmodulin kinase II (CaMKII) (74, 126, 193). The three most abundant phosphorylated sites in human tissue, Ser284, Ser286, and Thr290, are located in the regulatory M-domain of cMyBP-C (216). Tandem mass spectrometry analysis identified a novel phosphorylation site on Ser133 in the proline- and alanine-rich linker sequence between the C0 and C1 domains of cMyBP-C. In silico kinase prediction revealed GSK3β as the most likely kinase to phosphorylate Ser133 (227).

A transgenic mouse model in which the phosphorylation sites of cMyBP-C were replaced by alanine demonstrated decreased contraction and relaxation of the heart and highlighted the necessity of cMyBP-C phosphorylation for proper cardiac function (370). The involvement of cMyBP-C and its phosphorylation in the stretch activation response has been clearly demonstrated (396, 398, 440). This stretch activation might play an important role in the development of systolic pressure (394, 396). Moreover, evidence suggests that the impact of cMyBP-C phosphorylation on contractile function is site specific (459).

It has been argued on the basis of recent evidence that cMyBP-C phosphorylation increases the cooperative recruitment of cross-bridges and that the coordinated phosphorylations of cMyBP-C and Ca^{2+}-handling proteins tune the kinetics of cross-bridge detachment to the kinetics of Ca^{2+} decay (300). A recent study (345) showed that cMyBP-C phosphorylation could have its greatest effect on tuning cMyBP-C's Ca^{2+} sensitization of thin filaments at the low Ca^{2+} levels between contractions, whereas Ca^{2+} levels at the peak of contraction would allow cMyBP-C to remain a potent contractile modulator, regardless of its phosphorylation state.

2. Thin filament proteins (G-actin, Tm, and Tn)

The thin filament consists of a double stranded helix of G-actin. G-actin in adult human, pig and bovine myocardium (both ventricular and atrial) consists of ~80% α-cardiac actin and ~20% α-skeletal actin, which are encoded by *ACTA1* and *ACTC* genes, respectively, and only differ in 4/375 amino acid residues (448). In mice enhanced expression of α-skeletal actin is associated with increased contractility (166). In diseased human heart samples the percentage of α-skeletal actin was increased to 50%–70% (74), suggesting that this upregulation might be beneficial.

At the functional level it can also be noted that one myosin head directly interacts with two adjacent actin monomers (24). This binding of myosin to actin is a multi-step process: the initial weak binding is mainly ionic, and the subsequent strong binding is mainly hydrophobic (24, 175).

Tm is composed of 284 amino acids and is ~40 nm in length. It is composed of two coiled-coil polypeptides with 7 periodic repeats corresponding with the periodicity of the G-actins. In adult human heart several Tm isoforms are present: 90%–94% α-Tm, 3%–5% β-Tm, and 3%–5% κ-Tm. κ-Tm is a recently discovered Tm isoform which is generated from alternative splicing of the *TPM1* gene (353). A replacement of α-Tm with β-Tm in skinned cardiac muscle fibers resulted in an increased myofilament Ca^{2+} sensitivity but decreased *nH* (250).

Both α-Tm and β-Tm have a potential phosphorylation site at Ser-283, a region critical for Tm-TnT [cardiac TnT (cTnT)] interaction, and the head-to-tail overlap between adjacent Tm's on the actin-filament (159, 257). In adult cardiomyocytes 10%–30% of α-Tm is phosphorylated (160, 492), but β-Tm is not phosphorylated (160). In vitro studies showed that α-Tm phosphorylation had no effect on its binding to actin (161), but promoted the head-to-tail interaction between two neighboring Tm's (355). Pseudophosphorylation of α-Tm has been shown to prolong the slow phase of relaxation in myofibrils supporting a role for Tm in the regulation of muscle relaxation (317).

Cardiac Tn consists of a Ca^{2+}-binding subunit (cTnC), an inhibitory subunit (cTnI), and a Tm-binding subunit (cTnT). Its structure **(FIGURE 7)** and role in the regulation of cardiac muscle contraction has been the subject of many reviews [e.g (212, 391)]. In this study, we highlight a few points and summarize the effects of site-specific phosphorylation. cTnC contains four metal binding sites but only one of them is able to bind Ca^{2+} at the free Mg^{2+} concentration inside a cardiac cell (~1 mM). The affinity of Ca^{2+} binding to cTnC can increase 10-fold depending on its structural arrangement (opening of the cTnC cleft) governed by an intricate interplay of Ca^{2+}, cTnI, and Tm (292). Ca^{2+} binding to the cTnC N-lobe promotes interaction between the cTnC N-lobe and the switch peptide of cTnI. This binding alters the interaction between the Tn complex and Tm and exposes binding sites for myosin on the thin filament (179).

cTnI contains a number of phosphorylation sites targeted by different kinases and phosphatases (212, 391). Early studies concentrated on the Ser23/24 sites on cTnI (human sequence) because they are substrates for PKA, which is activated upon β_1-AR stimulation **(FIGURE 6)** (290, 320, 323, 349, 390, 405). A number of additional phosphorylation sites have been identified, including Ser42, Ser44, Ser76 (or Thr77), Thr143, Ser150, and Ser198 both in rodents and in humans (56, 216, 495, 496). Pseudophosphorylation of the Ser23/24 sites on cTnI results in an interaction between the NH_2 terminus and the inhibitory peptide of cTnI and could explain the increased rate and decreased duration of the slow component of force relaxation observed in myofibrils (69); although PKA treatment of human

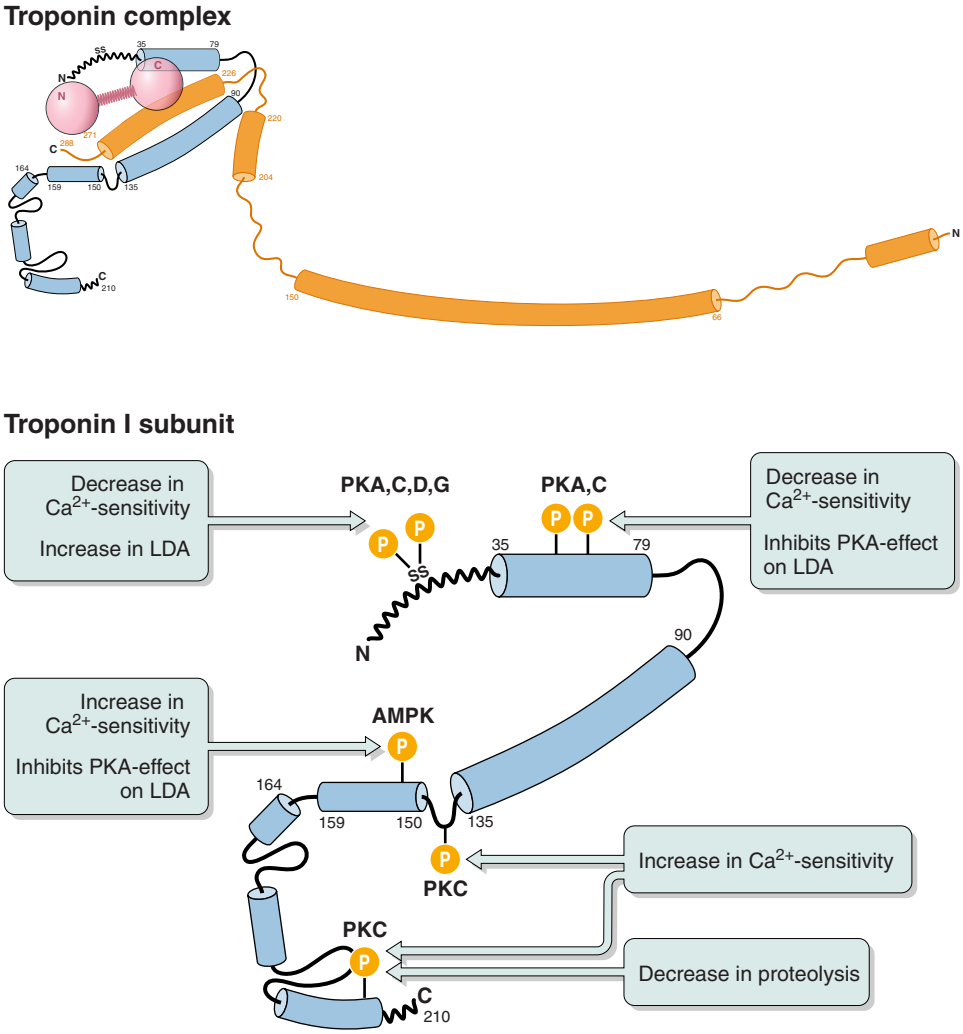


FIGURE 7. Interaction of troponin I (blue) relative to troponin C (14) and troponin T (orange). The structure is based on the crystal structure of troponin (416). The enlarged scheme of troponin I shows the main phosphorylation sites, which are phosphorylated by different kinases and alter Ca^{2+} sensitivity of myofilament, length-dependent activation (LDA), or proteolysis.

cardiac myofibrils decreased myofilament Ca^{2+} sensitivity but did not alter cross-bridge kinetics independent of the tension level (i.e., the relationship between cross-bridge kinetics and tension was unaltered by PKA) (456). The latter study indicates that PKA-mediated cTnI phosphorylation accelerates cardiac muscle relaxation via myofilament Ca^{2+} desensitization irrespective of changes in cross-bridge cycling.

To investigate the effects of phosphorylation on tension production and its Ca^{2+} sensitivity in human myocardium, studies were performed in which endogenous Tn was exchanged with reconstituted recombinant human Tn in membrane-permeabilized human cardiomyocytes isolated from cardiac tissue obtained during heart transplant surgery. To study the overall effects of different kinases on cTnI in concert, the unphosphorylated recombinant Tn complex was treated with PKA, PKCα, or PKCε to phosphorylate the accessible sites in vitro before exchange (215, 217, 218). To study site-specific effects of cTnI phosphorylation, pseudophosphorylated cardiac Tn complexes were generated with mutation of the serine or threonine sites on cTnI to aspartic acid (D) (215, 472, 475, 476). In line with previous studies in rodents (179, 362), these experiments provided evidence that Ser23/24 needs to be bisphosphorylated to cause a reduction in myofilament Ca^{2+} sensitivity (476). Surprisingly, this reduction was already maximum at ~55% pseudo-bisphosphorylated cTnI. This indicates that pseudophosphorylation (most likely random) of half of the cardiac Tn complexes along the thin filament is sufficient to induce a structural change in the Tn complexes along the thin filament (476).

Pseudophosphorylation of Ser42/44 was found to decrease, whereas Thr143 was found to increase Ca^{2+} sensitivity of myofilaments (476). Studies in rodents indicated that pseudophosphorylation of Ser150 causes an increase in Ca^{2+} sensitivity (318). The endogenous level of Ser199 phosphorylation in human myocardium appeared to be low (496). However, this does not preclude functional significance because a relatively low level of pseudophosphorylation (5.9%) already causes a significant increase in Ca^{2+} sensitivity and reduces calpain I–mediated proteolysis of cTnI (474). Ser199 phosphorylation may protect the heart during ischemia–reperfusion via reducing cTnI degradation (section II*E1*).

Human cTnT also contains a fair number of PKC phosphorylation sites including: Ser179, Ser198, Thr203, and Thr284 (195, 322, 404). Two-dimensional SDS gel electrophoresis of donor and end-stage failing human tissue revealed two major spots originating from cTnT, representing dephosphorylated (27%) and monophosphorylated cTnT (63%) (437). In rat myocardium cTnT was found to be largely monophosphorylated (374). Thr206 in mice, which corresponds with Thr203 in human cTnT, appears to be the functionally relevant site for reduction in Ca^{2+} sensitivity, cooperativity, Mg-ATPase activity, and maximum tension generation (406). Ser179 has been identified as a substrate of PKCα (218). Pseudophosphorylation at additional sites increased the Ca^{2+} sensitivity (376).

3. Z-disc and M-line

The Z-disc in cardiac muscle is ~100 nm thick and consists of six layers of α-actinin (251). Its structure and its components are reviewed in detail elsewhere (125, 214, 426). The M-band is also ~100 nm thick and consists mainly of myosin, myomesin, obscurin, and titin. For a review, see Reference 382. Myosin filaments are cross-linked in the M-band by myomesin dimers, which are linked with obscurin, which in turn binds the C-terminal domain of titin (125). The M-band shows up to five lines, and its structure appears to correlate roughly with heart rate at rest (337).

4. Titin

Titin, a giant protein with a molecular weight of ~3 MDa, is also called the third filament within the sarcomere (225, 237, 426). In human adult myocardium, two titin isoforms are present, called N2B and N2BA. The latter is different from N2B because it has an insertion and therefore has a larger molecular weight and is less stiff. Stiffness of titin also depends on site-specific phosphorylation (167). During exercise, PKA-mediated titin phosphorylation reduces sarcomere stiffness (**FIGURE 6B**). Multiple sites that are phosphorylated by different kinases [e.g., PKA, protein kinase G (PKG), and CaMKII] have been reported and are reviewed in Reference 151. Apart from its function in regulating cardiac stiffness, titin represents a central hub for cellular signaling (224).

5. Overview of sarcomeric targets of kinases and phosphatases

Multiple in vitro studies with isolated sarcomeric proteins have identified specific phosphorylation sites, which are target of one or more kinases. The physiologic relevance of site-specific phosphorylation and dephosphorylation of sarcomeric proteins is an ongoing topic of research and is the subject of recent reviews on the sarcomeric targets of both kinases and phosphatases (245, 389). Phosphatases known to target myofilament proteins include MLC phosphatase, protein phosphatase I, and protein phosphatase 2A [recently reviewed in (245)]. An overview of the sarcomeric proteins, phosphorylation sites, and kinases reported in the literature is provided in **TABLE 1**.

The combined activity of multiple proteins, kinases, and phosphatases will define myofilament Ca^{2+} sensitivity, and the related Ca^{2+} buffering of Tn, and thereby actively modulate Ca^{2+} homeostasis and contractile function in cardiac muscle cells. In **FIGURE 7**, we highlight effects on myofilament Ca^{2+} sensitivity of different site-specific cTnI phosphorylations, some of which may be activated during acute cardiac stress or chronic disease. Phosphorylation of cTnI is of particular interest as different sites interact (i.e., phosphorylation at one site alters phosphorylation, and associated functional effects, at another site of cTnI). Examples of the "cross-talk" between phosphorylation at Ser23/24 and Ser42/44 and Ser23/24 and Ser150 are illustrated in **FIGURE 7**. Pseudophosphorylation of Ser42/44, which decreases myofilament Ca^{2+} sensitivity, blunted the length-dependent increase in myofilament Ca^{2+} sensitivity, which is mediated by PKA phosphorylation of Ser23/24 (476). Ser42/44 is a target of PKC, which is upregulated in several disease conditions. Likewise, Ser150 phosphorylation most likely occurs during disease (ischemic conditions) as it has been proposed to be a target of AMP-activated protein kinase (AMPK). AMPK is an energy-sensing kinase (327, 373), which is activated during stress-induced ATP depletion to stimulate metabolic pathways aimed to restore the AMP/ATP balance. Although AMPK-mediated phosphorylation of Ser150 is associated with an increased myofilament Ca^{2+} sensitivity (318, 327), Ser150 phosphorylation blunted PKA-mediated myofilament Ca^{2+} desensitization and the length-dependent increase in myofilament Ca^{2+} sensitivity. Ser150 phosphorylation (319) was shown to counteract the reduction in myofilament Ca^{2+} sensitivity mediated by a low pH as occurs during ischemia (434). Although the Ser23/24 phosphorylation-mediated Ca^{2+} desensitization was blunted under these conditions, the accelerated Tn Ca^{2+} release upon Ser23/24 phosphorylation was maintained (319). The latter indicates that Ser150 phosphorylation may increase contractility under ischemic conditions, whereas it does not interfere with Ca^{2+} release from Tn needed for proper relaxation.

6. Species differences

Whereas phosphorylation sites are highly conserved, the major differences between small rodents and humans are isoform composition of sarcomeric proteins, in particular MHC and titin. Although the slow (low ATPase activity) β-MHC dominates in the ventricles of humans, mice and rats predominantly express the fast (high ATPase activity) α-MHC. The MHC isoform composition matches species-related resting heart rate (153). The I-band region of titin, referred to as molecular spring, is central in regulating passive stiffness of cardiomyocytes. Cardiomyocyte stiffness correlates with the

Table 1. *Overview of site-specific phosphorylation (human sequence) and involved kinases*

	PKA	PKC	PKD	PKG	AMPK	MLCK	CaMKII	GSK3β
cTnI	**Ser23/24** (290)	**Ser23/24** (320) **Ser42/44** (323) **Thr144** (323) **Ser199** (218)	**Ser23/24** (157)	**Ser23/24** (234)	**Ser150** (327, 373)			
cTnT		**Ser198 Thr203 Thr284** (195) **Ser179** (218)						
Tm			**Ser283** (several kinases) (257)					
MLC2						**Ser15** (60)		
MyBP-C	**Ser275 Ser284 Ser304** (126)	**Ser275**	**Ser304** (18)				**Ser304**	**Ser133** (227)
	Ser311 (193)	**Ser304** (369)					**Ser284** (369)	
Titin			Multiple sites/multiple kinases [reviewed in (151)]					

MLCK, MLC kinase.

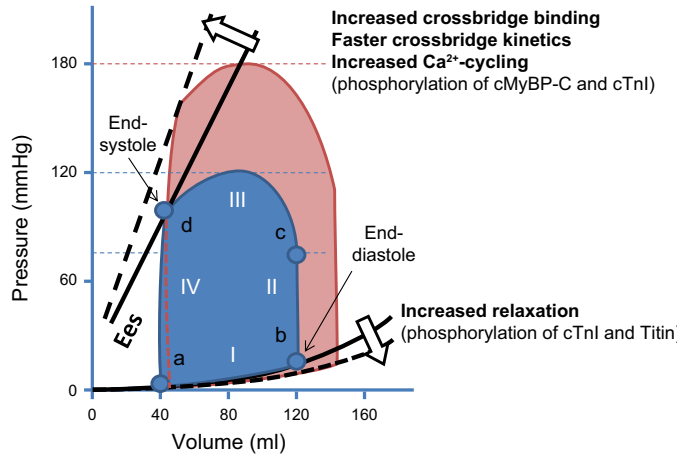


FIGURE 8. Four different events in the LV contraction cycle at rest (depicted in blue): a, opening of the mitral valve; b, closure of mitral valve; c, opening of the aortic valve; d, closure of the aortic valve. Four different phases, I–IV: I. diastolic filling ~600 ms, II. isovolumetric contraction ~50 ms, III. ejection phase ~300 ms, and IV. isovolumetric relaxation ~50 ms for a normal heart rate at rest 60 bpm (i.e., 1,000 ms in total per beat). Stroke volume (SV) (100) of 80 ml corresponds to cardiac output of 4.8 liter/min. Diastolic filling (phase I) and aortic valve closure (d) are determined by the end-diastolic and end-systolic pressure volume relations, respectively (solid lines). Ejection fraction (EF): SV/end-diastolic volume = (120–40 ml)/120 ml or 0.67 (or 67%). The area within the P-V loop indicates the amount of external work delivered per heartbeat (~80 × 100 ml.mmHg/s or ~1 Watt). The blue line depicts ventricular contractility (end-systolic elastance [Ees]). End-systolic pressure increases (red line) at increased loading of the ventricle (i.e., Frank–Starling mechanism). The diastolic pressure-volume relation is illustrated by the black line and reflects the increase in pressure during the filling phase, which, in healthy individuals, is minimal because of a highly compliant (relaxed) ventricle. Upon sympathetic activation of the heart, contractility and compliance of the ventricle increases, in part via PKA-mediated phosphorylation of sarcomere proteins, thereby improving systolic and diastolic performance of the heart.

length of titin's spring segment with a higher stiffness when the I-band region is shorter (229, 330). Fetal cardiac titin has a very long spring segment and is very compliant, whereas the adult cardiac N2B isoform has a short spring and is therefore rather stiff. The length of the spring segment in the adult N2BA isoform is intermediate between the fetal and adult N2B titin isoforms. Studies in different species revealed predominantly stiff (smaller) N2B isoforms in small rodents, whereas a higher level of compliant (long) N2BA isoform is present in pigs and humans (58, 435).

I. Pressure–Volume Relation

Cardiac pump function can be described by the relation between [left ventricular (LV)] pressure and volume as illustrated in **FIGURE 8**. The cardiac cycle consists of four phases (**FIGURE 8** illustrates the left part of the heart). First, blood flows passively from the atrium to the ventricle during the filling phase (phase I) (45, 46). During this phase, the volume of the ventricle increases, whereas pressure remains

low as the ventricle is relaxed and compliant. After the atrial kick (atrial contraction), which increases the ventricular volume by 10%, the mitral valve closes, and the ventricle contracts isovolumetrically, resulting in an increased LV pressure (isovolumetric contraction phase; phase II). Once LV pressure has increased to a level that is higher than the pressure in the aorta, the aortic valve opens, and the LV empties its content into the systemic circulation during the ejection phase (phase III). At the end of the ejection phase, LV pressure drops below aortic pressure, and the aortic valve closes, after which the LV relaxes isovolumetrically and pressure decreases (isovolumetric relaxation phase, phase IV). When LV pressure decreases below atrial pressure, the mitral valve opens, and blood enters the LV, initiating the next cardiac cycle. A standard measure of ventricular contractility is the end-systolic elastance **(FIGURE 8)**, which is defined by the slope and intercept of the end-systolic pressure–volume (P-V) relationship (202, 371). The diastolic curve **(FIGURE 8)** depicts the pressure increase during the filling phase. In a healthy individual, the increase in LV pressure during filling is small.

The sarcomeres play a central role in regulation of cardiac pump function in response to increased demand. Cardiac output increases upon increased loading of the ventricle (increased end-diastolic volume). The increased systolic pressure upon increased ventricle filling (Frank–Starling mechanism; see below) is explained by an increase in force generation by the myofilaments. During increased sympathetic drive (e.g., exercise), contractility increases, reflected by increased steepness of the end-systolic elastance, and ventricular compliance increases, reflected by a downward shift of the diastolic P-V relationship (**FIGURE 8**: dotted lines during increased sympathetic activation) (45). At the cardiomyocyte level, increased contractility is explained by increased cytosolic Ca^{2+} levels or increased Ca^{2+} sensitivity, which both increase the number of force-generating cross-bridges **(FIGURE 6)**. Activation of β_1-AR enhances PKA-mediated phosphorylation of sarcomere proteins cTnI, cMyBP-C, and titin. Phosphorylation of cMyBP-C will increase the rate of cross-bridge attachment to match to exercise-related increase in heart rate. PKA phosphorylation of cTnI decreases Ca^{2+} sensitivity of myofilament, which enables faster detachment of Ca^{2+} from the myofilaments and subsequent reuptake by the SR. In addition, Ca^{2+}-desensitized myofilaments will result in a more relaxed cardiomyocyte. In addition to the reduced myofilament Ca^{2+} sensitivity, PKA-mediated phosphorylation of titin will lower passive stiffness of cardiomyocytes, making the heart more compliant. Increases in titin-based compliance have been associated with increased ventricular compliance in vivo in mice expressing different titin isoforms (280).

J. Frank–Starling Mechanism

Otto Frank (113) and Ernest Starling and colleagues (339) may be considered as the founding fathers of the relation between end-diastolic volume and cardiac performance (systolic pressure; stroke volume), now known as the Frank–Starling law of the heart. It consists of two components: a relatively slow one (the Anrep effect, see section II*E*), related to alterations in Ca^{2+} handling (6), and a fast component operating on a beat to beat basis. The basis of this fast component resides inside the sarcomere. It reflects two independent myocardial properties: the sarcomere length dependence of the maximal force-generating capacityand the midpoint of the force–Ca^{2+} relation (EC_{50}), collectively referred to as LDA.

The cardiomyocyte in vivo operates at the ascending limb of the tension–sarcomere length relationship (between ~1.8 and 2.0 μm) (418) **(FIGURE 4)**. Increased diastolic filling results in an increase in sarcomere length and thus an increase in the tension-generating capacity as more myosin heads within the cardiac myocytes are able to bind to the thin filament. The second aspect of LDA, the decrease in EC_{50} (i.e., increase in myofilament Ca^{2+} sensitivity) with sarcomere length, is much less understood.

Given the isovolumical behavior of muscle cells (269), an increase in length directly results in a decrease in muscle width. An increase in sarcomere length thus implies that the distance between the actin and myosin filament decreases. Because the myofilaments are negatively charged, such change in distance between them may influence the affinity of binding of positively charged Ca^{2+} ions. Evidence in favor of this hypothesis was obtained through experiments in skinned (permeabilized) muscle cells [e.g (136, 267, 401, 463)]. Osmotic compression of skinned muscle cells by inert high molecular weight polymers, such as PVP or dextrans, that did not penetrate the myofilament lattice indeed resulted in a decrease in EC_{50} similar to that observed when the cells were stretched. However, X-ray diffraction studies at different sarcomere lengths (213) revealed that there is not a unique relation between myofilament lattice spacing and EC_{50} in osmotically compressed permeabilized cardiac trabeculae. In addition, evidence has been provided that modification of a single residue on cTnI (threonine 144; Thr>Pro) modulated the effect of sarcomere length on EC_{50} (413). Moreover, PKA-mediated phosphorylation at Ser23/24 of cTnI enhances LDA (476), which is blunted by phosphorylation at other residues on cTnI **(FIGURE 7)**. Apparently, the model of reduced muscle width (lattice spacing) is too simplistic, and other mechanisms [e.g., radial forces (477)] are involved in or contribute to LDA of myofilaments.

In addition to Tn, a role in LDA has been assigned to the giant protein titin. In a mouse model, increased levels of compliant titins were associated with a blunted LDA evident from a reduced length-dependent increase in myofilament Ca^{2+} sensitivity (280). A similar blunted LDA was observed in a patient sample expressing highly compliant

titin isoform (30). However, in the latter case, the myofilament response to length was corrected after treatment with exogenous PKA, indicating that the blunted LDA was due to hypophosphorylation of PKA targets within the sarcomere. Recent evidence suggests that β-arrestin, a multifunctional scaffold that interacts with many proteins and protein kinases, may modulate LDA because the length-dependent increase in myofilament Ca^{2+} sensitivity was absent in mice lacking β-arrestin1 or β-arrestin2 (2). Overall, recent studies thus suggest that LDA of cardiomyocytes involves a complex of sarcomeric and nonsarcomeric proteins, which may be modified upon stretch.

III. PATHOPHYSIOLOGY OF SARCOMERIC PROTEINS IN ACQUIRED HEART DISEASE

A. Introduction

Acquired heart diseases, by definition, develop after birth. The most common forms are the following: coronary artery disease (343) because of atherosclerosis leading to MI; valvular heart disease (stenosis or regurgitation); hypertension leading to damage of arteries and venes and to HF, MI, stroke, and kidney failure; and myocarditis and pericarditis and rheumatic heart disease. In general, they are associated with morphological changes in the heart with hypertrophic or dilated features. MI resulting from coronary artery disease, for instance, leads to scar tissue and fibrosis while the remaining myocardium hypertrophies. Valvular heart disease may lead to cardiac hypertrophy (in case of stenosis) or cardiac dilatation (in case of regurgitation). In this chapter, both causes and consequences of acquired heart diseases will be discussed. In addition, the main changes at the level of the sarcomeric proteins will be summarized that have been reported in different cardiac disease models.

B. Concentric and Eccentric Remodeling of the Failing Heart

The nomenclature used for the classification of HF can be based on the underlying cause (e.g., pressure or VO), clinical presentation (e.g., HFrEF or HFpEF), and thus is quite diverse. At the morphological level, a distinction can be made between concentric (hypertrophic) and eccentric (dilated) remodeling of the heart. Typical P-V relations under these conditions are shown in **FIGURE 9**.

Tension development of the individual cardiomyocytes is transmitted via longitudinal (intercalated discs) and lateral [extracellular matrix (ECM)] connectivity and leads to macroscopic average tension in the myocardial wall (σ) and pressure inside the cavity. Approximating the LV by a homogeneous, isotropic sphere with internal radius R and wall thickness h, transmural pressure (P) can be derived from the law of Laplace (288):

$$P = \sigma\left(\frac{2h}{R} + \frac{h^2}{R^2}\right)$$

This equation indicates that pressure increases with an increase in wall thickness and with a decrease in the internal diameter if cardiomyocyte properties [i.e., wall tension (σ)] are constant. This qualitatively explains the positive impact of cardiac hypertrophy on pressure development with a reduction in stroke volume in the P-V loops shown in **FIGURE 9*B***. Similarly, cardiac dilatation, an increase in the internal diameter, results in a decrease in pressure development **(FIGURE 9*C*)**.

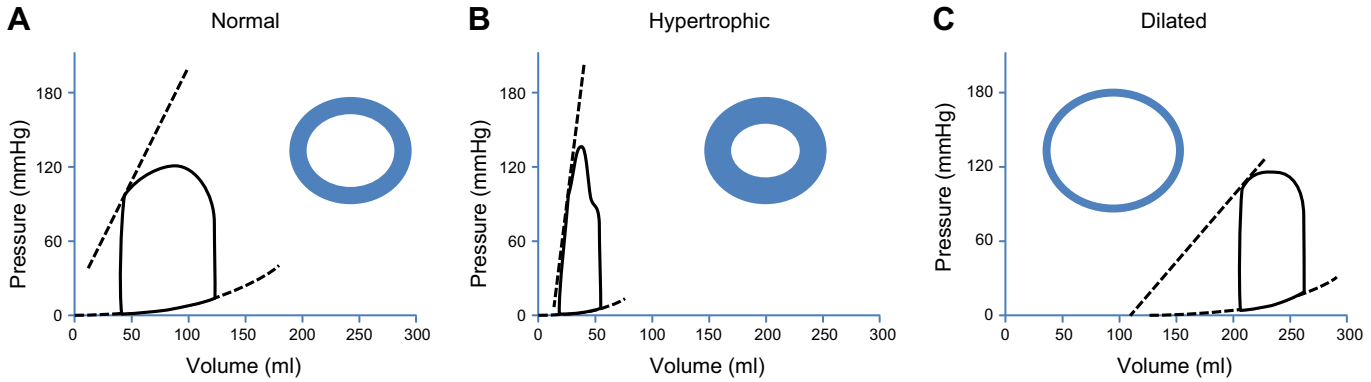


FIGURE 9. Left ventricular pressure–volume loops in hearts exhibiting concentric (hypertrophic) remodeling and eccentric (dilated) remodeling. *A*: Pressure-volume relation in a normal (healthy) individual. *B*: Concentric hypertrophy is characterized by increased contractility (as a result of the increase in ventricular mass) reflected by the steeper end-systolic pressure–volume relation (upper interrupted line in *B*). In addition, the entire pressure-volume relation is displaced to the left because of the increase in wall stiffness that limits diastolic filling. *C*: A dilated heart is characterized by reduced contractility and reduced chamber stiffness illustrated by a rightward shift of the pressure–volume relation. In both types of cardiac remodeling, the area within the P-V loops is reduced in comparison to normal.

In addition to remodeling of the cardiomyocytes, characterized by cell thickening in concentric and cell elongation in eccentric remodeling, changes occur in the fibrous proteins of the ECM, such as collagens, elastins, fibronectins, and laminins. Fibrous collagen is rather stiff, has a high tensile strength, and in the healthy myocardium, it is present in relatively low amounts (2%–4%) (53). The fibrillar collagens found in the myocardium include types I, III, and V. Type I collagen is the predominant type (normally >50%), followed by type III (between 10% and 45%) and type V (<5%) (53). The stiffness of the collagen network is also determined by the degree of cross-linking. Cross-linking is a process whereby collagen fibers are covalently linked, resulting in increased material stiffness and greater resistance to degradation. The most extensively characterized and predominant type of cross-linking is considered to be associated with advanced glycation end products (324). Cardiac fibroblasts are the major cell type in the heart responsible for synthesis of the ECM (22). The conversion of fibroblasts to collagen-secreting myofibroblasts is enhanced by transforming growth factor beta (301). ECM degradation is regulated via matrix metalloproteinases 1, 8, and 13, tissue inhibitors of metalloproteinase, and mast cells. Mast cells are known to store and release a variety of biologically active mediators including TNF-α and proteases such as tryptase and chymase, which can induce matrix metalloproteinase activation (90, 191). In general, changes in the ECM (e.g., increased fibrosis) are thought to contribute to reduced contractility and impaired relaxation of the myocardium and appear to be a general feature of advanced cardiac disease.

C. Volume and Pressure Overload: Causes and Signaling Pathways

VO of both LV and right ventricle (RV) occurs for instance with mitral or tricuspidal valve regurgitation. Because of retrograde blood flow, ventricular filling has to increase to maintain stroke volume. In animal models, this can be achieved by inducing damage of an atrioventricular valve or by a shunt (fistula) between the abdominal aorta and the inferior vena cava (123). A less invasive, but less frequently used, method is based on the addition of minoxidil to the drinking water (106, 431).

A pure VO, without an increase in systolic pressure, increases diastolic load and results in adverse eccentric LV hypertrophy and remodeling manifested by wall thinning, cardiomyocyte elongation, and a decrease in LV mass to volume (135). The mechanical stimulus of a pure stretch/VO has multiple myocardial events including inflammation and matrix loss, cardiomyocyte and mitochondrial oxidative stress, and increased adrenergic drive (135).

VO-induced diastolic dysfunction is characterized by increased end-diastolic pressure (EDP), increased diastolic wall stress, and a decreased slope of the EDP volume relation (135). The progression of VO in the aortocaval fistula model occurs in three distinct phases: acute stress (0–2 wk), compensatory remodeling (2–10 wk), and decompensated HF (>10 wk) (184). Acute stress is characterized by net degradation of ECM and collagen isoform switching, resulting in a more compliant ventricle. The compensatory remodeling phase is characterized by wall thickening and an increase in ECM content that normalize wall stress imposed by the increased preload (148, 191, 244). Decompensated HF occurs when the stress imposed by VO exceeds the ability of the ventricle to normalize or compensate (301).

Pressure overload occurs for instance with aortic valve stenosis (AS), systemic, or pulmonary hypertension. It usually affects one side of the heart, but RV hypertrophy can also impair performance of the LV (230). Frequently used animal models are transaortic constriction (TAC), spontaneous hypertensive animals, exposure to chronic hypoxia, or monocrotaline-induced damage (38, 338, 399).

In general, concentric hypertrophy is a result of systolic pressure overload, whereas eccentric hypertrophy is a consequence of VO (116).

Calcineurin, which affects gene expression through dephosphorylation of the transcription factor NFAT, is an important promoter of pathological cardiac hypertrophy (291). Increased expression and activity of calcineurin are found in pressure overload (240). A comparative study with pressure overload (TAC) and a shunt model of VO in mice indicated that pressure overload resulted in maladaptive fibrotic hypertrophy with CaMKII–dependent altered Ca^{2+} cycling and apoptosis (423). In contrast, VO resulted in more benign hypertrophy without fibrosis, and was associated with Akt activation, but not with CaMKII or ERK1/2 activation. Akt activation is generally believed to promote a more adaptive hypertrophic phenotype (15, 385). In the shunt-induced VO, Ca^{2+} cycling was normal, and none of the pressure overload–related signals were activated (423).

The roles of MAPKs in the heart have been extensively reviewed (365) and discussed (329). Activation of the ERK1/2 pathway is observed in pressure overload but not in the development of physiological hypertrophy. Activation of JNK and p38, which is also observed in the setting of pressure overload, seems to antagonize pathological hypertrophy (31). In vitro data have implicated ERK5 in serial sarcomere assembly, suggesting a role in eccentric hypertrophy (316).

D. Systolic and Diastolic Dysfunction: HFrEF and HFpEF

In **FIGURE 10**, a schematic diagram is shown of the changes in the LV P-V relation associated with systolic and diastolic

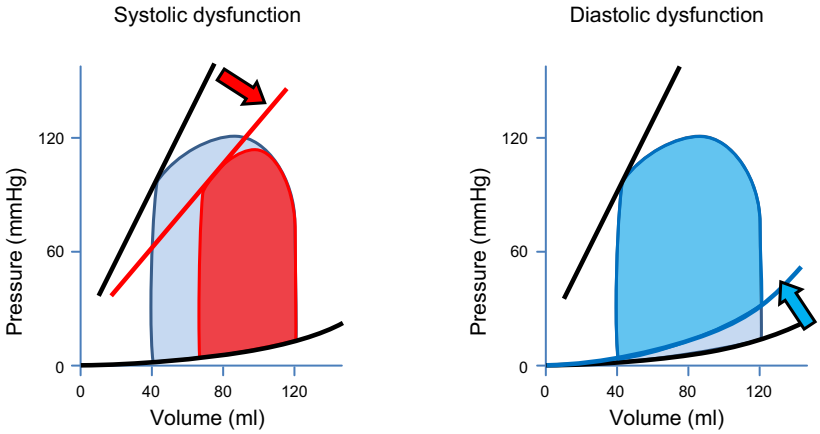


FIGURE 10. Left ventricular pressure–volume loops in systolic dysfunction and diastolic dysfunction. In systolic dysfunction (left panel), contractility is depressed and the end-systolic pressure–volume line is displaced down compared with control; there is diminished capacity to eject blood into a high-pressure aorta. In diastolic dysfunction (right panel), chamber stiffness is increased and the diastolic pressure–volume relation is displaced up compared with controls; there is diminished capacity to fill at low diastolic pressures.

dysfunction. It can be seen that in pure systolic dysfunction, the EF is reduced, and the EDP is normal. In pure diastolic dysfunction, the EF is normal, and the EDP is elevated. Approximately half of all patients with HF have preserved EF, but show diastolic dysfunction, whereas HFrEF patients in general show both systolic and diastolic dysfunction. As the main risk factors of HFpEF involve obesity, diabetes, and hypertension, the prevalence of HFpEF will continue to grow in the aging population (45).

A study on human biopsies from HFpEF patients identified increased cardiomyocyte passive stiffness relative to controls as cause of diastolic dysfunction in addition to a larger collagen volume fraction (44). HFpEF was historically considered to be caused exclusively by LV diastolic dysfunction, but research during the last 15 yr has identified several other contributory factors, including limitations in LV systolic reserve, systemic and pulmonary vascular function, nitric oxide (NO) bioavailability, chronotropic reserve, right heart function, autonomic tone, left atrial function, and peripheral impairments (45). A novel paradigm for HFpEF has been proposed in which coronary microvascular endothelial dysfunction and inflammation play an important role. Microvascular endothelial inflammation was presumed to trigger an unfavorable molecular cascade leading to diastolic LV dysfunction because of stiff and hypertrophied cardiomyocytes and interstitial collagen deposition because of a loss of NO bioavailability, which resulted in cardiomyocyte deprivation of cGMP and lower activity of PKG. Three primary mechanisms may impair cGMP signaling in HFpEF myocardium: *1*) enhanced degradation through upregulation of cardiac phosphodiesterases (PDEs), especially PDE5 and PDE9; *2*) decreased NO stimulation and/or responsiveness of soluble guanylate cyclase; and *3*) reduced synthesis via impaired natriuretic peptide activation of transmembrane particulate guanylate cyclase receptors (145).

The main cause of HFrEF is MI. MI is often caused by coronary artery occlusion or damage. It leads to cell necrosis, inflammation, and formation of fibrotic scar tissue in the affected region. During the initial phase, the myocardium is stunned (41) and may turn into a phase of hibernation (37, 165); both conditions are reversible if reperfusion of the affected region occurs in time. Reperfusion may result in Ca^{2+} overload of the cells and induce injury (12, 335) and PKCα activation by the activation of Ca^{2+}-dependent protease activity (e.g., calpain-1) (200). Preload of the depressed myocardium also activates Ca^{2+}-dependent proteases (107, 334). Vital tissue in the remote myocardium has to hypertrophy to compensate for the loss of myocardium. Clinical outcome is determined by size of the infarcted region. If the infarcted region is small, the incidence of life-threatening arrhythmias is small, and hypertrophy of the remaining tissue can be compensatory, but larger infarctions have poor prognosis and lead to decompensated HFrEF.

E. Sarcomere Changes in Acquired Forms of HF

1. Acute and chronic sarcomere changes in ischemic heart disease

In animal models, research on the changes in sarcomeric proteins during cardiac remodeling has been focused on the early phases, whereas studies in humans involve more advanced disease stages because of tissue availability.

Acute changes upon MI have been studied mainly in animal models of ischemia–reperfusion, in which hearts were exposed to brief periods of ischemia (30–45 min), followed by a brief period of reperfusion (30–45 min) **(FIGURE 11*A*)**. This type of intervention results in so-called stunned myocardium which shows reduced contractility (228). Studies in rat and pigs showed a reduction in myofilament Ca^{2+} sensitivity in the stunned cardiac myocytes (119, 122, 174), and several studies showed a reduction in maximal force generation (119, 122). The reduction in myofilament Ca^{2+} sensitivity was assigned to the reperfusion phase, indicating that reperfusion injury of the myofilaments underlies dys-

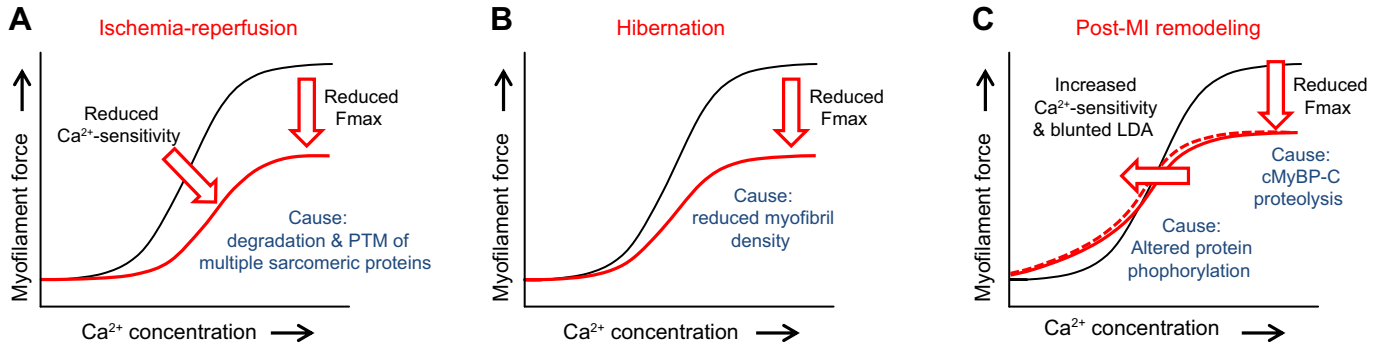


FIGURE 11. Changes in myofilament function upon acute and chronic ischemia. All stages are characterized by reduced maximal force generation (Fmax), the sum of active and passive isometric force, although with a different underlying cause. *A*: Acute ischemia–reperfusion is characterized by degradation and posttranslational modifications (PTM) of multiple proteins, although cTnI proteolysis appears to be the main cause of sarcomere dysfunction. *B*: Hibernating myocardium is characterized by reduced myofibril density that causes reduced cardiomyocyte Fmax. *C*: Post-MI–remodeled cardiomyocytes show increased myofilament Ca^{2+} sensitivity and a blunted response to stretch (blunted LDA), whereas myofilament Ca^{2+} sensitivity is reduced during acute ischemia–reperfusion and contributes to contractile dysfunction. Post-MI–remodeled hearts show defects in the beta-adrenergic signaling pathway and altered phosphorylation of cTnI and MLC-2.

function of stunned myocardium (287). Reduced myofilament function was initially attributed to calpain I–mediated proteolysis of cTnI, resulting in a truncated cTnI form (120). Proteomic studies of stunned rat myocardium revealed protease-mediated damage of cTnI and covalent complex formation (272). However, Tn exchange experiments in single human and rat cardiomyocytes revealed that truncated cTnI enhances myofilament Ca^{2+} sensitivity (313, 412). These experimental data were confirmed by structural analysis of the Tn-Tm position on thin filament actin, which revealed that truncated cTnI stabilized the Ca^{2+}-activated state of Tm on actin consistent with an increased force development (117). Truncated cTnI caused increased cross-bridge kinetics and reduced maximal force in rat cardiomyocytes (412). cTnI proteolysis may thus explain part of the dysfunction observed in the stunned myocardium. In subsequent years, proteomic analyses of stunned myocardium and calpain I–incubated cardiac tissue revealed a multitude of protein changes: degradation of multiple structural and sarcomeric proteins (5, 72, 335, 443, 470, 471, 478) and altered posttranslational modifications of sarcomeric proteins (33, 68, 491). Based on these studies, it can be concluded that reperfusion injury reduces contractility of myofilaments via a myriad of sarcomeric protein changes and contributes to systolic dysfunction of the stunned heart. Interestingly, a recent study revealed that hyperphosphorylation at Ser199 of cTnI (**TABLE 1**; **FIGURE 7**) reduced cTnI proteolysis and protected the heart against ischemia–reperfusion injury (239), which indicates that Tn degradation is centrally involved in the contractile dysfunction of stunned myocardium.

During acute ischemia, contractile performance of myofilaments will be significantly diminished because of the ischemia-mediated reduction in intracellular pH and increase in inorganic phosphate (7, 98, 434), which will contribute to systolic dysfunction during ischemia. Chronic hypoperfusion of the heart because of a severe coronary stenosis (but without infarction) results in hibernated myocardium which is characterized by reduced contractility (**FIGURE 11*B***). The cardiac contractile deficit is reversible after revascularization. Studies in pig models of myocardial hibernation showed myofibril loss (36, 37, 66), which resulted in reduced maximal force generation of single cardiomyocytes (36). The extent of functional recovery of hibernating myocardium is related to the degree of ultrastructural cellular changes (67).

After an MI, the surviving cardiomyocytes hypertrophy in an attempt to maintain cardiac output. Apart from cellular remodeling and development of fibrosis, the intrinsic properties of the hypertrophied cardiomyocytes change (**FIGURE 11*C***). Early changes in MI-induced, remodeled myocardium have been studied in rodents and pigs. A reduction has been found in the maximal force-generating capacity of cardiomyocytes in mouse, rat, and pig MI (80, 88, 436). A study in trabeculae from post-MI rats indicated that the reduction in maximal force generation occurs at a relatively late stage (28 wk) after MI as no change in maximal force was observed 12 wk post-MI (80). Moreover, the reduction in maximal force appears to be most severe in the endocardial layer of the heart (435). Another striking observation in post-MI models is the loss of transmural differences in myofilament properties, which appears to be crucial for cardiac pump function (83). The largest changes in myofilament properties were observed in the endocardial layer, whereas the epicardial layer was not affected, or changes were minor (59, 435). Reduced phosphorylation of MLC-2 appears to be a consistent finding in post-MI–remodeled hearts (59, 94). In contrast, reports on cTnI phosphorylation are diverse, with studies reporting no difference (59) and reduced (150) and increased cTnI phosphorylation (238). The diverse changes in cTnI phosphorylation may be explained by disease stage (457). Most studies reported an increased

myofilament Ca^{2+} sensitivity (14, 88, 150, 436, 451) and a blunted LDA activation evident from a reduced length-dependent increase in Ca^{2+} sensitivity (59, 80) in post-MI–remodeled hearts compared with controls. As indicated above, degradation of cTnI was shown to increase myofilament Ca^{2+} sensitivity using Tn exchange experiments in single cardiomyocytes. Although a low percentage of truncated cTnI has been reported in post-MI–remodeled pig myocardium (436), calpain I–mediated cTnI degradation appears to be transient (i.e., during ischemia and reperfusion) and does not explain the high myofilament Ca^{2+} sensitivity observed in post-MI–remodeled hearts. The high myofilament Ca^{2+} sensitivity may rather be explained by reduced PKA phosphorylation of cTnI as reduced β_1-AR signaling has been found at different levels (i.e., reduced levels of β_1-AR, reduced PKA activity, reduced in vivo cardiac response to noradrenaline, and blunted cTnI phosphorylation upon in vivo stimulation of β_1-AR with dobutamine) (42, 88, 150, 436). In addition, treatment of myofilaments with exogenous PKA normalized myofilament Ca^{2+} sensitivity of post-MI cardiomyocytes to control values (436). The changes reported in post-MI myofilament properties (reduced maximal force, increased myofilament Ca^{2+} sensitivity, and blunted LDA) will contribute to systolic and diastolic dysfunction of the heart, and the high myofilament Ca^{2+} sensitivity has been suggested to be a substrate for ventricular arrhythmias (451).

2. Sarcomere changes caused by pressure overload

The most commonly used model to study effects of pressure overload of the LV in animals is TAC of the aorta, which results in a severely hypertrophied heart with reduced systolic and diastolic function. Studies on myofilament function in TAC-induced cardiac disease models did not show consistent changes: studies reported a reduction (rat) (26), an increase (mice and rat) (105, 428), and no change (mice) (201) in maximal force and Ca^{2+} sensitivity of myofilaments. TAC mice showed a significant increase in myofilament passive stiffness (428). High passive stiffness of myofilaments was also found in patients with AS (104), which causes concentric hypertrophy of the heart. Moreover, these human studies revealed that stiffness of the sarcomeres was worse in AS patients with diabetes as comorbidity. High myofilament stiffness was also observed in pressure-overloaded RV. The RV of the heart is exposed to pressure overload in patients with pulmonary arterial hypertension (PAH). PAH patients die of severe RV failure with impaired diastolic function. Studies in human explanted heart tissue from pulmonary hypertension patients revealed an increase in myofilament passive force (351, 352). Experiments in rats with mild and severe pulmonary aortic banding (PAB) revealed that the increase in RV stiffness in rats with mild PAB is due to an increase in myofilament stiffness, whereas in severe PAB, both myofilament stiffness and fibrosis impair diastolic function (350). Several studies showed reduced PKA-mediated phosphorylation of titin and cTnI (350–352). Blunted cTnI phosphorylation was associated with impaired frequency-dependent myofilament desensitization in PAB rabbits with RV hypertrophy (449). However, in contrast to studies in MI models, pressure overload–induced myofilament changes were not corrected by exogenous PKA treatment (351, 428), indicating that signaling pathways other than the beta-adrenergic pathway induced posttranslational protein modifications that cause myofilament dysfunction. The high passive stiffness of myofilaments is most likely related to titin modifications (see below) and contributes to diastolic dysfunction of the pressure-overloaded heart.

3. Myofilament Ca^{2+} sensitivity at early and advanced stages of human cardiac disease

Increased myofilament Ca^{2+} sensitivity has been demonstrated in a dog model with dilated cardiomyopathy (DCM) (483). This observation was subsequently confirmed in human end-stage DCM samples, which were obtained during heart transplantation or LV assist device implantation (482). High myofilament Ca^{2+} sensitivity was corrected to control values upon treatment with PKA, indicating impaired β_1-AR–mediated phosphorylation of sarcomere proteins. Myofilament studies in less advanced HF patients were performed in cardiomyocytes isolated from small cardiac biopsies taken during valve replacement surgery (433). Single cardiomyocytes were isolated from these biopsies, permeabilized, and exposed to various calcium concentrations to assess myofilament Ca^{2+} sensitivity. Patients had volume or pressure overload and were classified based on New York Heart Association (NYHA) classes into mild (class I) to severe (stage IV) HF. Interestingly, the patient with the most severe stage of HF (class IV) showed a conspicuous increase in myofilament Ca^{2+} sensitivity compared with all other less severely affected (class I–III) patients. The increased myofilament Ca^{2+} sensitivity was indicative for reduced PKA-mediated phosphorylation of cTnI due to downregulation and desensitization of β_1-AR or may be caused by a sarcomere gene mutation (described below in section III). Later studies in large sets of end-stage failing human DCM hearts, with idiopathic DCM (IDCM) and ischemic heart disease (ISHD), confirmed increased myofilament Ca^{2+} sensitivity compared with nonfailing donor hearts (439). The high myofilament Ca^{2+} sensitivity was normalized to control values after treatment with exogenous PKA and correlated with low cTnI phosphorylation. Myofilament changes were more severe in IDCM compared with ISHD, characterized by a higher myofilaments Ca^{2+} sensitivity and lower cTnI phosphorylation in IDCM compared with ISHD hearts (150). Studies in myocardial biopsies from HFpEF patients, who were classified as NYHA classes II and III, compared with a control group showed no difference in Ca^{2+} sensitivity or cTnI phosphorylation between groups (44, 430). Comparison of HFpEF and HFrEF patients also did not show a Ca^{2+} sensitivity difference between groups (152, 444). Overall, these hu-

man cardiomyocyte studies indicate that the increase in myofilament Ca^{2+} sensitivity occurs at a relatively late stage of disease. Tn exchange studies in human cardiomyocytes showed that maximal myofilament Ca^{2+} desensitization is reached with 55% of bisphosphorylated cTnI (472). Thus, the level of bisphosphorylated cTnI has to decrease considerably to enhance Ca^{2+} sensitivity of myofilaments in cardiac disease. This is supported by a study in post-MI pigs, in which PKA-mediated cTnI phosphorylation upon dobutamine stimulation was blunted, whereas the initial difference in myofilament Ca^{2+} sensitivity was corrected to control values (42), indicating that the functional window of cTnI PKA-mediated bisphosphorylation ranges between 0% and 55% of cTnI phosphorylation.

Apart from reduced PKA-mediated phosphorylation of cTnI, multiple other posttranslational protein modifications may alter myofilament Ca^{2+} sensitivity (391, 475). Such posttranslational protein modifications have been identified in rodent and pig models, which showed increased PKC-mediated Tn phosphorylation (25, 26, 457), altered cMyBP-C phosphorylation (42), and oxidative stress–induced modifications of sarcomere proteins [reviewed in (395)]. A study in human cardiac biopsies indicated that reduced PKA-mediated phosphorylation is not the only cause of altered myofilament Ca^{2+} sensitivity. Comparison of human biopsies with HFrEF and HFpEF revealed a larger PKA-mediated reduction in Ca^{2+} sensitivity in HFrEF compared with HFpEF. As a consequence, myofilament Ca^{2+} sensitivity was significantly higher in HFpEF than HFrEF cardiomyocytes after PKA treatment (444). A similar observation has been made in TAC mice, in which myofilament Ca^{2+} sensitivity remained significantly higher compared with controls after treatment with PKA (428). The latter studies suggest that pressure overload of the LV increases myofilament Ca^{2+} sensitivity via a PKA-independent mechanism. The complex interaction of sarcomere protein posttranslational modifications during early and advanced cardiac disease in humans warrants further investigation as it may represent a target for therapy. Future studies using pluripotent stem cell–derived human cardiomyocytes (described below: sections III and IV) may shed light on the early pathophysiologic changes caused by MI and pressure overload in humans.

4. High myofilament passive stiffness as cause of diastolic dysfunction

Diastolic dysfunction is a hallmark of many forms of acquired cardiac disease. Rather counterintuitive, studies in end-stage failing IDCM and ISHD human hearts revealed more compliant muscle preparations compared with controls, which was explained by a shift in titin isoform composition from the stiff N2B to the more compliant N2BA isoform (150, 258, 308, 315). The shift to compliant titin isoform could thus not explain diastolic dysfunction observed in HF. It is thought that the switch to the compliant N2BA titin isoform represents a beneficial adaptation to counter fibrosis.

Studies in HFpEF and HFrEF cardiac biopsies from NYHA class II and III patients identified high myofilament stiffness as cause of impaired relaxation (44, 444). Passive force measurements in single cardiomyocytes revealed a significantly higher passive force in HFpEF compared with controls, and values in HFrEF cardiomyocytes were intermediate. Exogenous PKA treatment significantly decreased passive force in both patient groups, indicating that hypophosphorylation of titin caused the high stiffness. As indicated above (section II*H4*), in addition to isoform switching, cardiomyocyte stiffness is regulated by phosphorylation of titin. Sites within the N2B element of titin are phosphorylated by PKA and PKG and reduce passive force (223, 488), whereas sites in the PEVK domain are phosphorylated by PKC and CaMKII and increase passive force (8, 168, 180). Based on the observation of reduced PKG activity and correction of passive stiffness with PKG in HFpEF cardiomyocytes (43, 445), a paradigm was proposed in which a systemic inflammatory state was linked with impaired endothelial function and a subsequent reduction in NO-mediated PKG activation in cardiac muscle cells, which would explain hypophosphorylation of titin and high passive force (341). The systemic inflammatory state is related to the multiple comorbidities (overweight/obesity, diabetes mellitus, and hypertension), which are thought to cause HFpEF.

In an attempt to link comorbidities with cardiomyocyte stiffness, comparative studies were performed in HFpEF with and without diabetes. Diabetic HF patients showed worse diastolic dysfunction than patients without diabetes mellitus (104, 446). A higher cardiomyocyte passive stiffness was observed in diabetic compared with nondiabetic patients, which was associated with hypophosphorylation of titin (104). A study in hypertensive patients with and without HFpEF showed increased collagen-dependent and titin-dependent stiffness in hypertensive HFpEF patients compared with controls and hypertensive patients without HFpEF (497). The high titin-based stiffness was associated with reduced phosphorylation of the N2B element, and increased phosphorylation of the PEVK element. The authors suggest that increased PKCα activity may cause PEVK phosphorylation and decrease N2B phosphorylation via activation of PP1 (50). Overall, these studies show that hypertension and diabetes aggravate diastolic dysfunction, which is, in part, caused by altered titin isoform phosphorylation.

Several HFpEF patients showed high cardiomyocyte stiffness without an increase in fibrosis (44), suggesting that the titin changes are the primary defect causing increased cardiac stiffness. The studies in a PAH rat model described above (350) suggest that at an early stage of cardiac disease, high passive stiffness of the heart is due to altered titin

phosphorylation, whereas collagen-based stiffness contributes at a later disease stage. High cardiac stiffness subsequently may trigger a shift from stiff to compliant titin isoform in an attempt to improve diastolic function. Changes in titin isoform composition and phosphorylation thus are complex and depend on stage of disease and etiology and comorbidities. Therefore, a treatment which targets one specific phosphorylation site may be too narrow. Treatment options targeting high passive stiffness are discussed in section V.

An overview of the maladaptive and adaptive changes in the ventricle in HFrEF and HFpEF, described above, are illustrated in **FIGURE 12**.

5. Disease-related sarcomere changes in the atria

Atrial fibrillation (AF) is the most common clinical tachyarrhythmia associated with significant morbidity and mortality and is expected to affect ~30 million North Americans and Europeans by 2050 (177). AF is a frequent feature in HF patients with diastolic dysfunction (493), indicating that impaired ventricular relaxation poses a stress on the atria, which induces remodeling of atrial cardiomyocytes. HFpEF patients with AF show worse outcome, evidenced by reduced ventricular pump function, neurohumoral activation, exercise intolerance, and increased HF hospitalization (278, 299, 493). The contribution of left atrial contraction (atrial kick) to filling of the LV becomes more important when passive filling is hindered by the stiffened ventricle. Sustained AF causes a decrease in cardiomyocyte myofibril density (13). The AF-induced structural remodeling of human atria was associated with reduced cardiomyocyte maximal force-generating capacity (101). In addition to structural changes, isoform shifts in MHC and titin isoform composition were observed in AF human atria (28, 312). A shift from the fast α-MHC to the slow β-MHC correlated with reduced kinetics of contraction and relaxation and reduced maximal force of myofibrils (28). The AF-related shift from stiff N2B to compliant N2BA titin isoform was associated with reduced passive stiffness of myofibrils. Deterioration of LV function in AF patients may be in part explained by reduced function of the remodeled atrial sarcomeres.

IV. PATHOPHYSIOLOGY OF SARCOMERIC PROTEINS IN INHERITED HEART DISEASE

A. Inherited Cardiac Diseases and Defects in Cardiac Function

Genetic studies revealed that the sarcomere is central in inherited cardiac disease, in particular, in hypertrophic cardiomyopathy (HCM), which has been referred to as "disease of the sarcomere." Strikingly, mutations in genes expressing sarcomeric proteins are associated with different clinical phenotypes ranging from HCM, noncompaction cardiomyopathy to DCM (171, 458, 465). Although multiple attempts have been made to establish cardiomyopathy-specific mutation effects, cardiomyopathy onset and progression is complex, and the mutation-induced changes are diverse dependent on the affected gene and type of mutation. In this section, we will summarize several gene-specific changes in cardiomyocyte function and discuss changes which are secondary to the disease process and appear to be a common factor in cardiomyopathy development.

In 1989, the first HCM-associated mutation was identified in the gene *MYH7*, encoding the thick filament protein MHC (130, 192). Currently, more than 1400 HCM muta-

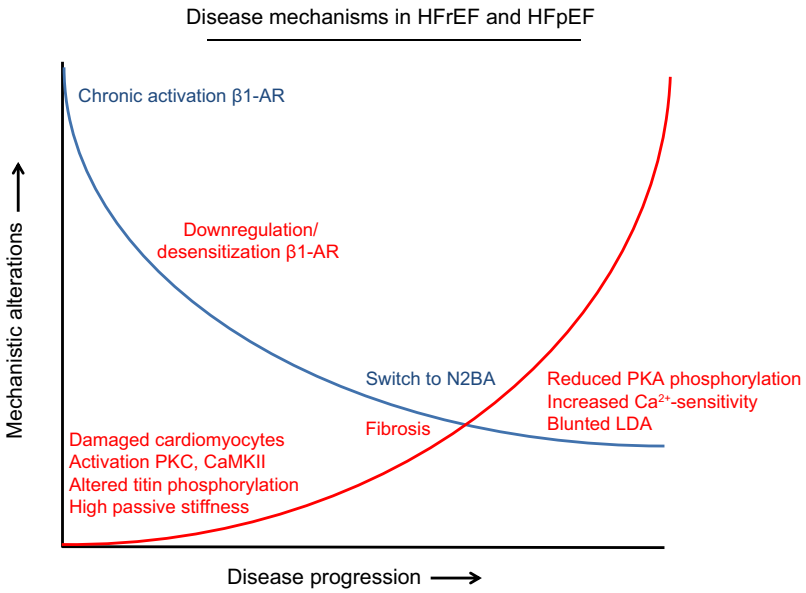


FIGURE 12. Overview of maladaptive (in red) and adaptive (in blue) changes in the ventricle in HFrEF and HFpEF based on studies in human and experimental animal models. The initial detrimental changes include damage of cardiomyocytes (in HFrEF) caused by ischemia and changes in sarcomeric protein phosphorylation due to perturbations in kinases and phosphatases. Altered titin phosphorylation has been reported in both HFrEF and HFpEF and causes sarcomere stiffness. Activation of the β_1-adrenergic receptors (β_1-AR) is initially beneficial and preserves cardiac output. However, chronic β_1-AR stimulation is detrimental as it causes downregulation and desensitization of β_1-AR with a coincident reduction in cTnI phosphorylation, increased myofilament Ca^{2+} sensitivity, and blunted length-dependent activation (LDA).

tions have been identified, of which ~90% reside in the genes encoding thick and thin filament proteins of the sarcomere. The three most frequently affected genes (*MYH7*, *MYBPC3*, and *TNNT2*) encode the sarcomeric proteins MHC, cMyBP-C, and cTnT. HCM is extremely common, with an estimated prevalence of 1:500 to 1:200 in the general population (360, 381). The clinical presentation of the disease is enormously variable, with mutation carriers that remain asymptomatic during their entire life, whereas others develop severe HCM, end-stage HF, or die of acute cardiac arrest (282, 328). As most mutation carriers are heterozygous and carry one normal and one disease (mutant) allele, onset of the disease appears to be determined by the dose of mutant protein. This is illustrated by rare cases of homozygous mutation carriers who show a severe and early (childhood) disease onset (188, 264, 285, 361). In general, disease onset in heterozygous mutation carriers occurs between the age of 20–50 yr. HCM is characterized by asymmetric hypertrophy of the ventricle, most frequently of the septum, which may obstruct blood flow to the aorta (obstructive cardiomyopathy). In addition to hypertrophy, the remodeled heart of HCM patients shows myofibrillar and cellular disarray, fibrosis, and capillary rarefaction (146). Although cardiac remodeling is evident at a later disease stage, imaging studies in asymptomatic mutation carriers revealed diastolic dysfunction and reduced myocardial efficiency even before development of hypertrophy (131, 147, 172, 283, 421, 480). These early changes in cardiac function may be explained by the direct mutation-mediated defects in sarcomere function. Although HCM is characterized by diastolic dysfunction, DCM patients show systolic dysfunction. DCM is not only caused by sarcomere mutations, but also involves mutations in structural proteins of the heart. Overall, studies indicate that DCM is associated with loss of function, which contributes to systolic dysfunction and initiates dilation of the heart. Establishing pathogenicity of gene mutations is a challenge and continuous matter of debate. Studies on the cellular function of sarcomere proteins and effects of sarcomere mutations aid in understanding the complex pathogenesis of cardiomyopathies.

B. Overview of Effects of Sarcomere Gene Mutations

1. Thin filament mutations: increase and decrease in myofilament Ca^{2+} sensitivity in HCM and DCM

The most frequently affected thin filament gene causing HCM is the *TNNT2* gene, which encodes cTnT. DCM has been associated with mutations in genes encoding cTnT, cTnI, cTnC, Tm, and actin (*TNNT2*, *TNNI3*, *TNNC1*, *TPM1*, and *ACTN*). Overall, thin filament mutations represent ~5%–10% of all HCM and DCM mutations. After the identification of sarcomere gene mutations as cause of HCM and DCM (197, 419), studies emerged to define the functional effects of mutations using engineered mouse models and in vitro protein assays. These studies revealed that mutations in thin filament proteins cause HCM and DCM via an opposing functional defect. HCM mutations cause increased myofilament Ca^{2+} sensitivity, whereas DCM mutations have been shown to reduce myofilament Ca^{2+} sensitivity. Studies on the effects of thin filament HCM and DCM mutations on myofilament Ca^{2+} sensitivity are summarized in **TABLES 2–5**. It has been proposed that the opposing effects of HCM and DCM mutations on myofilament Ca^{2+} sensitivity and coincident changes in buffering of the Ca^{2+} transient would underlie distinct disease pathways (363). Studies performed on *TNNC1* mutations in mice that caused either HCM or DCM (82) provided evidence that increased tension induced by the HCM mutation triggers hypertrophic signaling via activation of calcineurin and MEK1–ERK signaling and causes an increase in cardiomyocyte width and cardiac mass characteristic of concentric hypertrophy. Reduced tension induced by the DCM mutation also activated calcineurin signaling but reduced MEK1–ERK signaling (82). Inhibition of MEK1–ERK signaling results in cardiomyocyte elongation (206), which is characteristic for eccentric hypertrophy. Thus, the tension-based activation of diverse hypertrophic signaling is proposed to underlie the hypertrophy and dilated cardiac phenotypes.

In addition to cardiac remodeling, HCM is the most frequent cause of cardiac arrest in the young (181). Several studies showed that the Ca^{2+}-sensitizing effects of HCM mutations may be a substrate for ventricular arrhythmias (21, 211, 336, 378) because of increased Ca^{2+} binding of thin filaments. Studies in mice harboring different Tn mutations showed increased afterdepolarizations in mice with increased myofilament Ca^{2+} sensitivity. These and other studies showed that the extent of myofilament Ca^{2+} sensitization depends on the specific mutation as HCM Tn mutations cause a small to large increase in myofilament Ca^{2+} sensitivity (21, 109, 249). The occurrence of arrhythmias in Ca^{2+}-sensitized cardiac muscle has been linked to focal energy deprivation upon stress (increased pacing) and a coincident reduction in gap junctional coupling (182). Alternative mechanisms which could underlie increased risk of cardiac arrest in genetic heart disease involve disease-related electrophysiological perturbations because of altered expression/function of ion channels and/or structural remodeling of t-tubules and the development of fibrosis (75, 78). The latter indicates that an increase in Ca^{2+} sensitivity is not the only mechanism underlying development of HCM and sudden cardiac arrest. Indeed, several other pathomechanisms have been observed and are described below.

2. Impaired response to PKA-mediated phosphorylation

Phosphorylation of cTnI reduces myofilament Ca^{2+} sensitivity and thereby regulates cardiac muscle relaxation

Table 2. *Effects of HCM- and DCM-associated TnT mutations*

Mutation	Phenotype	Type of Experiment	Sarcomere Effect	Reference
179N	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} of tension and ATPase activity	(298)
		Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension	(410, 489)
		Transgenic mice	Increased pCa_{50} tension and ATPase activity	(286)
			Decreased Fmax and maximum ATPase	
R92Q	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension and ATPase activity	(298, 489)
R92W		Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension	(410)
R92L		Transgenic mice, cardiac muscle	Increased pCa_{50} tension	(61, 109)
		Transgenic mice, cardiac muscle	Increased pCa_{50} tension	(62)
		Transgenic mice, cardiac muscle	Increased pCa_{50} tension	(62)
R94L	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension	(249)
R94H		Recombinant proteins in guinea pig fibers	Increased pCa_{50} tension, blunted LDA	(284)
F110I	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased maximum ATPase activity	(489)
		Human recombinant proteins in porcine cardiac muscle	Increased Fmax, pCa_{50} tension unaltered	(311)
			Increased pCa_{50} tension	(410)
R131W	DCM	Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
R134G	DCM	Human recombinant proteins in porcine cardiac muscle	pCa_{50} tension unaltered	(164)
R141W	DCM	Human recombinant proteins in rabbit cardiac muscle	Decreased pCa_{50} tension	(249)
		Human recombinant proteins in porcine cardiac muscle	pCa_{50} tension unaltered	(452)
		Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
		Human recombinant proteins in guinea pig trabeculae	Decreased pCa_{50} tension	(289)
		Human recombinant Tn in mouse cardiac fibers	Decreased pCa_{50} tension	(138)
		Knock-in mice	Decreased pCa_{50} tension, impaired response to β-AR	(354)
R151C	DCM	Human recombinant proteins in porcine cardiac muscle	Decreased pCa_{50} tension	(164)
R159Q	DCM	Human recombinant proteins in porcine cardiac muscle	Decreased pCa_{50} tension	(164)
ΔE160	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} ATPase activity and tension	(155, 296)
		Transgenic mice, cardiac muscle	Increased pCa_{50} tension, increased tension cost	(62)
E163K	HCM	Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension	(410)
E163R		Transgenic mice, cardiac muscle	Increased pCa_{50} tension, increased tension cost, increased resting ATPase	(109)
R205W	DCM	Human recombinant proteins in porcine cardiac muscle	Decreased pCa_{50} tension	(164)
		Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
R205L		Human recombinant proteins in guinea pig trabeculae	Decreased pCa_{50} tension	(289)

Continued

Table 2.—*Continued*

Mutation	Phenotype	Type of Experiment	Sarcomere Effect	Reference
ΔK210	DCM	Human recombinant proteins in rabbit cardiac muscle	Decreased pCa_{50} tension and ATPase activity	(296)
		Human recombinant proteins in porcine cardiac muscle	Decreased Fmax and pCa_{50} tension	(452)
		Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
		Knock-in DCM mouse model	Decreased pCa_{50} tension, reduced LDA	(93, 189)
E244D	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased maximum ATPase activity	(489)
			Increased pCa_{50} tension, increased Fmax	(311)
R270N	DCM	Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
K273E	HCM to DCM	Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension	(452)
R278C	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} ATPase activity	(489)
		Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension, decreased Fmax	(297)
			Increased pCa_{50} tension	(410)

pCa_{50}, myofilament Ca^{2+} sensitivity; Fmax, maximal force; β-AR, beta-adrenergic receptor. In gray: DCM.

(FIGURE 6). Based on structural studies of the Tn complex, it has been proposed that phosphorylation of the PKA site at the NH_2 terminus of cTnI induces an interaction between the NH_2 terminus and inhibitory peptide of cTnI **(FIGURE 13)**. This interaction is proposed to destabilize the Ca^{2+}-induced interaction between the cTnI switch peptide and the cTnT N-lobe and thereby enhances relaxation (69). Mutations in both the NH_2-terminus and inhibitory peptide of cTnI reduced responsiveness to PKA. The HCM mutation R21C in cTnI is closely located to the PKA phosphorylation sites (Ser23/24) and impaired both in vitro and in vivo phosphorylation by PKA. The mutation R145G, which is located in the inhibitory peptide of cTnI, did not affect PKA-mediated cTnI phosphorylation, but did result in a blunted functional response to PKA. Based on molecular dynamics stimulations, it is proposed that these mutations inhibit formation of the interaction between the NH_2 terminus of cTnI and the inhibitory peptide (71, 242). Impaired response to PKA has also been reported in in vitro motility assays on isolated Tn from HCM and DCM patients (23, 279). Reduced myofilament response to PKA would result in impaired in vivo responsiveness to β-AR stimulation. Mice expressing the Ca^{2+}-sensitizing *TNNT2* mutation I79N showed worsening of systolic and diastolic function upon β-AR stimulation with isoproterenol compared with wild-type mice and mice harboring the *TNNT2* mutation R278C, which does not alter myofilament Ca^{2+} sensitivity (386). A similar response was observed with the Ca^{2+}-sensitizer EMD57033, which indicated that the mutation-induced Ca^{2+} sensitization impairs the response to β-AR stimulation (386). A blunted response to β-AR stimulation has been found in several transgenic mice carrying both thin and thick filament proteins (310, 354, 462). Reduced cTnI phosphorylation has been observed in cardiac samples from HCM patients with thin and thick filament mutations (221, 442) and are associated with an increase in myofilament Ca^{2+} sensitivity as observed in end-stage failing human hearts (section II). These studies indicate that β-adrenergic responsiveness in HCM is impaired by the mutation and by secondary disease remodeling.

3. Perturbed LDA

As described in section I, two main mechanisms exist which regulate myofilament Ca^{2+} sensitivity in response to increases in stress and during exercise: PKA-mediated myofilament Ca^{2+} desensitization and LDA of myofilaments. Measurements in human cardiac samples harboring HCM- and DCM-associated mutations revealed a blunted LDA characterized by a reduced length-dependent increase in myofilament Ca^{2+} sensitivity compared with controls (40, 383). The blunted LDA was restored upon exchange of the endogenous mutant by normal recombinant human Tn in HCM and DCM samples harboring *TNNT2* and *TNNI3* mutations (40, 383). Reduced LDA has been observed in cardiac muscle preparations from several transgenic mouse models as well **(TABLE 2)** and indicates that a blunted LDA may underlie reduced cardiac response during exercise in these animals (129).

4. Hypercontractility

As HCM is characterized by a preserved or even enhanced systolic function and DCM hearts show impaired systolic function, HCM and DCM mutations have been associated with hypercontractility and hypocontractility, respectively.

Table 3. *Effects of HCM-associated TnI mutations*

Mutation	Phenotype	Type of Experiment	Sarcomere Effect	Reference
R21C	HCM	Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension, reduced response to PKA	(139)
		Knock-in mice	Reduced in vitro and in vivo cTnI phosphorylation	(462)
		Rat recombinant proteins in rat cardiac muscle	Reduced in vitro cTnI phosphorylation	(71)
			Reduced myofilament response to PKA	
P83S	HCM	Rat recombinant proteins in rat cardiac muscle	Reduced myofilament response to PKA	(70)
R145G	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension and ATPase, reduced maximum ATPase	(414, 415)
		Human recombinant proteins in porcine cardiac muscle	Increased pCa_{50} tension	(232)
		Transgenic mice	Increased pCa_{50} tension and ATPase activity	(469)
		Rat recombinant proteins in rat cardiac muscle	Reduced Fmax, unaltered maximum ATPase	(71)
		In vitro protein assays	Reduced myofilament response to PKA	(89)
			Increased pCa_{50}, reduced response to PKA	
R145Q	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension and ATPase activity	(414)
R162W	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension and ATPase activity	(414)
ΔK183	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension and ATPase activity	(414)
G203S	HCM	Human recombinant proteins in rabbit cardiac muscle	pCa_{50} tension and ATPase activity unaltered	(414)
K206Q	HCM	Human recombinant proteins in rabbit cardiac muscle	Increased pCa_{50} tension and ATPase activity	(414)

pCa_{50}, myofilament Ca^{2+} sensitivity; Fmax, maximal force.

Although this concept appears to work for thin filament mutations, which either increase or decrease myofilament Ca^{2+} sensitivity, evidence for mutations in the thick filament proteins MHC and cMyBP-C is less consistent. This merges from the observation that *MYH7* mutations show different effects on a α-MHC compared with a human-like β-MHC background in mice (247, 248). In vitro motility assays using human β-cardiac myosin S1 also reported diverse effects for HCM-causing *MYH7* mutations. Early-onset HCM was attributed to mutations causing significantly increased intrinsic force of myosin heads, actin gliding velocity, and ATPase activity compared with wild-type myosin (3), which is consistent with hypercontractility. However, studies on adult-onset HCM mutations showed diverse changes in these three parameters (intrinsic force, velocity, and ATPase activity); some changes would con-

Table 4. *Effects of HCM- and DCM-associated TnC mutations*

TNNC1	Phenotype	Type of Experiment	Sarcomere Effect	Reference
A8V	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} tension	(231)
		Knock-in mice	Increased pCa_{50} tension	(266)
L29Q	HCM	Human recombinant proteins in porcine myofibrils	pCa_{50} tension unaltered	(97)
		In vitro protein assays	Reduced response to PKA	(377)
A31S	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} tension	(336)
C84Y	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} tension	(231)
E134D	HCM	Human recombinant proteins in porcine myofibrils	pCa_{50} tension unaltered	(231)
D145E	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} tension	(231)
G159D	DCM	Human recombinant proteins in porcine myofibrils	Decreased pCa_{50} tension and ATPase, decreased maximum ATPase	(97)
		Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)

pCa_{50}, myofilament Ca^{2+} sensitivity; Fmax, maximal force. In gray: DCM.

Table 5. *Effects of HCM- and DCM-associated Tm mutations*

Mutation	Phenotype	Type of Experiment	Sarcomere Effect	Reference
E40K	DCM	Human recombinant proteins in porcine myofibrils	Decreased pCa_{50} ATPase activity	(64)
		Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
E54K	DCM	Human recombinant proteins in porcine myofibrils	Decreased pCa_{50} ATPase activity	(64)
		Human recombinant proteins in in vitro motility assay	Decreased pCa_{50}	(289)
E62Q	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} ATPase activity	(64)
E180G	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} ATPase activity	(64)
L185R	HCM	Human recombinant proteins in porcine myofibrils	Increased pCa_{50} ATPase activity	(64)
D230N	DCM	Transgenic mice, in vitro motility assay	Decreased pCa_{50}	(255)

pCa_{50}, myofilament Ca^{2+} sensitivity. In gray: DCM.

tribute to hypercontractility, whereas others would cause hypocontractility (204, 305). Based on these observations, a model has been proposed (306) that may explain the seemingly opposite findings obtained with *MYH7* mutations, in part analogous to the scheme shown in **FIGURE 3**. Thick filament HCM mutations may perturb function via increasing the number of myosin heads in the sarcomere that are functionally available to interact with actin. A study in cMyBP-C knockout mice revealed that myosin heads in the super relaxed state are significantly less abundant in the absence of cMyBP-C (275). Similar findings were obtained in a study (274) in human HCM cardiac tissue with *MYBPC3* mutations, which have reduced expression of full length cMyBP-C (i.e., haploinsufficiency) (265, 441).

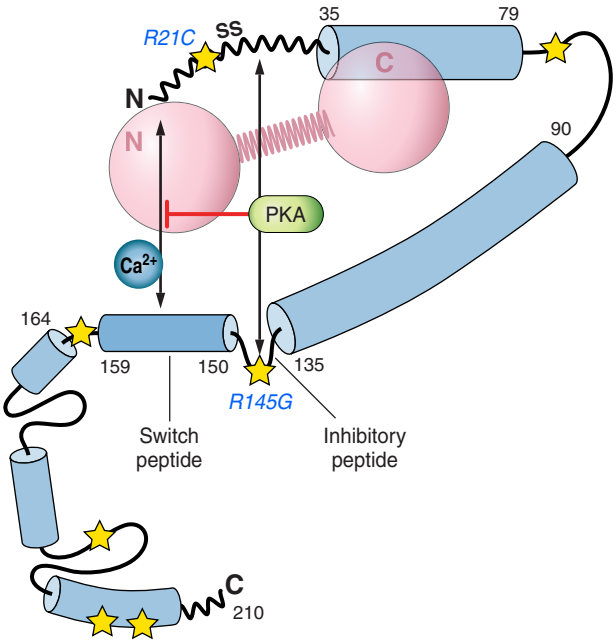


FIGURE 13. Schematic of the troponin I (cTnI) and troponin C (cTnC) subunits illustrating the interaction between cTnC and the switch peptide of cTnI when calcium binds to the N-lobe of cTnC. Bisphosphorylation of Ser23/24 (ss) by PKA stimulates the interaction between the NH_2 terminus and inhibitory peptide of cTnI and thereby destabilizes the interaction between the cTnC N-lobe and cTnI switch peptide. Stars indicate the positions of HCM-associated mutations in cTnI described in **TABLE 4**. The mutations R21C and R145G impair the response to PKA.

5. Increased crossbridge kinetics and ATP utilization

Defects in in vivo cardiac energetics have been observed in HCM mouse models (158) and HCM patients (77). Based on changes in the energetic status of the heart, it was proposed that HCM mutations would cause energy depletion. HCM mutations were proposed to increase ATP turnover during the crossbridge cycle and cause inefficient cardiac performance (11). Indirect proof for increased ATP utilization was given by studying myofibril kinetics in human HCM myocardium with the R403Q *MYH7* mutation (29). Direct measurements in cardiac muscle strips showed increased tension cost (ATP utilization/force development) in HCM mouse models (62, 109) and human HCM samples (479). Tension cost measurements in a large group of human HCM samples showed increased ATP utilization for force development in HCM harboring *MYBPC3* and *MYH7* mutations (480). Imaging studies revealed reduced myocardial efficiency in asymptomatic HCM mutation carriers compared with controls (147, 421, 480), indicating that the energy deficiency is already present at an early stage of the disease before development of hypertrophy.

6. Titin mutations

Next-generation sequencing revealed titin as being the most frequently affected protein causing DCM, in particular truncating titin (TTN) variants (163) [recently reviewed by (411)]. A second disease hit appears to trigger cardiac disease, as titin variants have been found in peripartum cardiomyopathy and chemotherapy-induced DCM (243, 447). Functional studies on mutation effects were performed in patient-specific induced pluripotent stem cell (iPSC)–derived cardiomyocytes (169). These studies revealed that some TTN mutations are pathogenic, whereas others are genetic modifiers of disease. TTN mutations caused significant reductions in contractility and a reduced response to

β-AR stimulation, which is in line with reduced PKA-protein phosphorylation observed in human DCM and peripartum cardiomyopathy myocardium (39).

In addition, mutations in the splicing factor RNA binding factor 20 (RBM20), which regulates titin isoform composition (149), are observed in human DCM (49, 359). Studies in human cardiac tissue from end-stage DCM patients and patient-derived, iPSC-derived cardiomyocytes revealed that the RBM20 mutations cause a shift to compliant titin isoforms, which is associated with reduced passive stiffness (30, 403). Both TTN and RBM20 mutations lead to disorganized sarcomere organization in iPSC-derived cardiomyocytes (169, 403, 486), highlighting the central role of titin in sarcomere architecture.

7. Primary mutation–related and secondary disease–related changes in HCM

Primary mutation–induced and secondary disease–related changes in HCM are depicted in **FIGURE 14**. The sequence of changes is based on studies in human cardiac tissue and experimental HCM models (described above). The HCM mutation–induced sarcomere changes trigger adverse remodeling and causes electrophysiologic changes, evidenced by a prolonged action potential, related to CaMKII-mediated changes in late sodium (Na^+) and Ca^{2+} currents (76). Cardiomyocytes isolated from HCM patients and HCM mouse models show increased occurrence of cellular arrhythmias and higher diastolic [Ca^{2+}], which is explained by disease-related phosphorylation changes of sarcomere and Ca^{2+}-handling proteins. These secondary disease-related changes are expected to advance disease progression.

V. TECHNIQUES TO STUDY FUNCTIONING OF SARCOMERIC PROTEINS AT CELLULAR/SUBCELLULAR LEVEL

A. Intact Tissue

The study of the effects of sarcomeric proteins at the cellular level benefited enormously from the development of Langendorff perfused mammalian heart named after Oscar Langendorff (233). Subsequently, mechanical and heat measurements were performed on smaller preparations such as thin papillary muscles, muscle strips, and trabeculae superfused with oxygenated Tyrode solution [for instance (1, 54, 134)]. As outlined in section I, this type of studies provided insight in the force development, power output, and energy utilization of cardiac muscle tissue. X-ray diffraction studies provided insight in the structural alterations at the subcellular level [e.g., (213, 270, 357)]. A major step forward was made by using laser diffraction techniques that allowed scientists to record and to control sarcomere length during contraction of thin preparations (79). Recently, the striation follower designed by A. F. Huxley and originally used in intact skeletal muscle fibers from frogs was employed to record sarcomere length changes and the isotonic velocity transients in intact cardiac trabeculae with submillisecond time resolution (57). The use of intact muscle strips was first performed in human tissue obtained during open heart surgery (302). These techniques were also used in a number of studies in tissue from patients with HF [e.g., (303)].

Experiments are also performed on isolated cardiomyocytes obtained by enzymatical digestion [e.g., (344)]. These cells can be loaded with various fluorescently labeled indi-

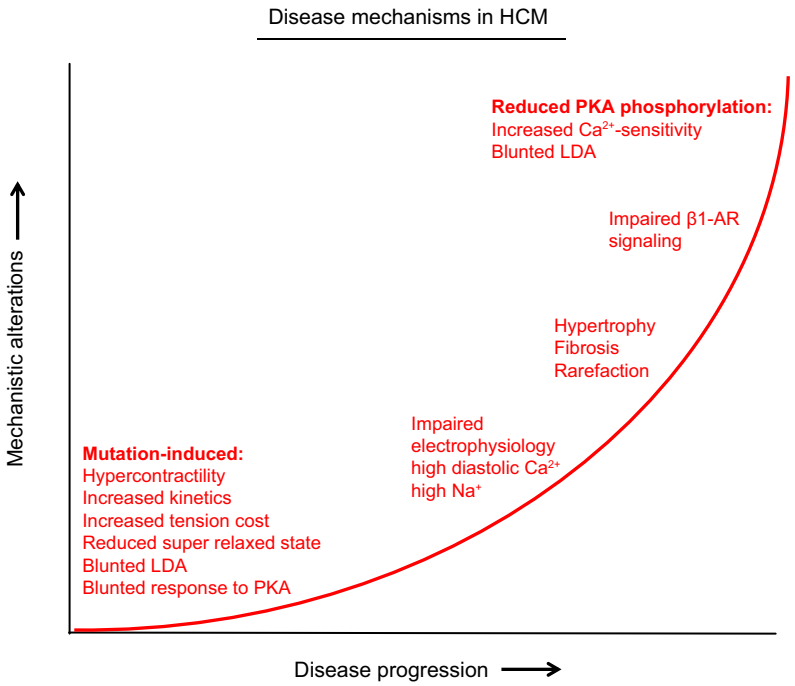


FIGURE 14. Overview of primary mutation–related sarcomere changes and secondary adaptive/maladaptive changes in hypertrophic cardiomyopathy (HCM). Mutations in thin and thick filament proteins exert diverse effects on sarcomere function, which in general, increase force generation and ATP utilization. Overall, HCM mutation cause inefficient sarcomere performance, which is thought to trigger adverse remodeling (hypertrophic signaling, capillary rarefaction, and fibrosis). Studies in HCM mice and human cardiac samples revealed high diastolic calcium (Ca^{2+}) and high sodium (Na^+), which further advances disease progression. Advanced HCM is characterized by a secondary disease–related increase in myofilament Ca^{2+} sensitivity and blunted LDA caused by reduced cTnI phosphorylation.

cators that allow the registration of the changes in, for instance, calcium, sodium, magnesium concentration, reactive oxygen species, and pH in different cellular compartments (331, 425). Force measurements in isolated cardiomyocytes remained challenging for a long period of time because cells need to be attached to a force transducer. The first measurements were made with the use of carbon fibers to which the cardiomyocytes adhered (124). Recently, a biological compatible glue has become available (347) that allows attachment of the cardiomyocytes to sensitive force transducer and a piezoelectric motor and the registration of work loops (162). Exciting new developments in intact muscle cells are the use of genetically targeted protein expression (196), providing insight in subcellular signal transduction and super resolution microscopy (455), allowing imaging of cellular structures at ~60-nm resolution.

B. Permeabilized Tissue, Myofibrils, and the In Vitro Motility Assay

Considerable insight in the factors controlling force development and energy utilization has been obtained by using thin papillary muscles, muscle strips, trabeculae, and enzymatically or mechanically isolated cardiomyocytes made permeable by means of detergents or prolonged storage in glycerol. These studies provided insight in Ca^{2+} handling [e.g., (103)] and in the determinants of cross-bridge cycling and the associated ATP hydrolysis (20, 98, 203, 207, 209). Moreover, the impact of phosphorylation and dephosphorylation of sarcomeric proteins could be studied by the endogenous application of various kinases and phosphatases and by protein exchange in permeabilized cardiomyocytes (439, 472). In many of these studies, well-characterized human tissue was obtained from the Sydney Heart Bank from explanted hearts and from healthy donor hearts (92).

Measurements in isolated myofibrils (190), which are ~1 μm in diameter, have the advantage that diffusion distances are minimal and factors controlling force development can be studied at high time resolution. Similar techniques are used to study sarcomere properties in human cardiac tissue (28, 479) and effects of sarcomere mutations associated with cardiomyopathy (96, 226, 343).

In vitro motility assays enable the study of actomyosin interaction at the molecular level (110, 176, 367). Important insight is to be expected because site-directed mutagenesis makes it possible to study, for instance, the impact of disease-causing mutations of sarcomeric proteins on the function of the molecular motor (204).

C. Stem Cells and Engineered Heart Tissue

The research on stem cells and engineered heart tissue has grown tremendously over the last decade and is the subject of many excellent reviews. In this review, we highlight recent studies that may have direct implications studying or improving cardiac function [e.g., (392, 407, 467, 484, 494)]. Both cell systems enable one to study effects of sarcomere mutations causing cardiomyopathy (34, 84, 402, 473).

One major hurdle has been that the cells obtained were far from an adult phenotype (429, 490). Therefore, methods have been developed to mature human iPSC-derived cardiomyocytes sufficiently to harvest isolated myofibrils for mechanics measurements (343) and to culture engineered heart tissue with human origin for studies on cardiac remodeling and repair (420). The success of these efforts is illustrated by a recent study (366). **FIGURE 15** provides an overview of different techniques which are used to study sarcomere properties.

VI. CURRENT AND FUTURE TREATMENTS TO TARGET SARCOMERIC PROTEINS

A. Targeting Diastolic and Systolic Dysfunction

1. Current pharmacological treatments

HF is responsible for ~50% of deaths in the Western population. Current pharmacological treatment of HF is mainly concentrated on the improvement of systolic dysfunction by angiotensin-converting enzyme inhibitors, angiotensin receptor 1 (AT1) inhibitors and β-AR blockers and on the relief of symptoms of HF by diuretics. Over the last decade, it has become clear that ~50% of patients with HF have diastolic dysfunction with an HFpEF. Unfortunately, no specific treatment of HFpEF exists, and therefore, also in this case, treatment medication for HFrEF are often applied.

2. Targeting hypophosphorylation of titin

Novel treatments for HFpEF have been based on studies showing hypophosphorylation of titin. Low titin phosphorylation and high passive stiffness of sarcomeres has been related to low PKG activity (section II). PKG activity may be increased via inhibition of PDEs, which would increase cyclic guanosine monophosphate (cGMP). Treatment with sildenafil, a PDE5 inhibitor, showed beneficial effects in a dog model with impaired diastolic function (35). However, clinical trials using sildenafil in HFpEF patients were neutral (173, 358). A recent study revealed that PDE9 inhibition may be more potent than PDE5 inhibition (235). Studies (127, 154) have shown that β_3-AR stimulation may counterbalance the detrimental effects caused by reduced disease-related β_1-AR signaling in the heart. β_3-AR activation increases the activity of PKG via cGMP, which has been shown to phosphorylate PKA phosphorylation sites

on sarcomeric proteins and reduce myofilament Ca^{2+} sensitivity (234). As such, β_3-AR activation provides a novel route to decrease Ca^{2+} sensitivity and enhance relaxation of the sarcomeres. Activation of β_3-AR is mediated via a pathway that is distinct from β_1-AR. Moreover, β_3-AR is resistant to desensitization and remains operative despite chronic adrenergic drive. Therefore, this pathway provides an attractive novel target for therapeutic interventions to counterbalance detrimental secondary disease-related effects of β_1-AR downregulation.

3. Targeting titin isoform composition

As described in section II, a shift to compliant titin isoform occurs in response to increased collagen formation in the heart. This adaptive response of the heart is explored as therapy to correct diastolic dysfunction in experimental models. As indicated above, the composition of titin isoforms is regulated by the splicing factor RBM20. The role of RBM20 in regulating cardiac stiffness was uncovered by a rat model which expressed giant titin isoforms caused by deletion of RBM20 in the heart (149). Reducing RBM20 activity increases compliant titin isoform expression and may thereby correct impaired relaxation. Improved diastolic function was observed upon experimental reduction of RBM20 activity in mouse models with diastolic dysfunction. The shift toward more compliant titin was associated with decreased diastolic stiffness, reduced EDP, improved exercise capacity, and a partial normalization of gene expression related to the hypertrophic response and fatty acid metabolism (170, 280, 281). Adaptive cardiac growth was related to four and a half LIM domain proteins that trans-

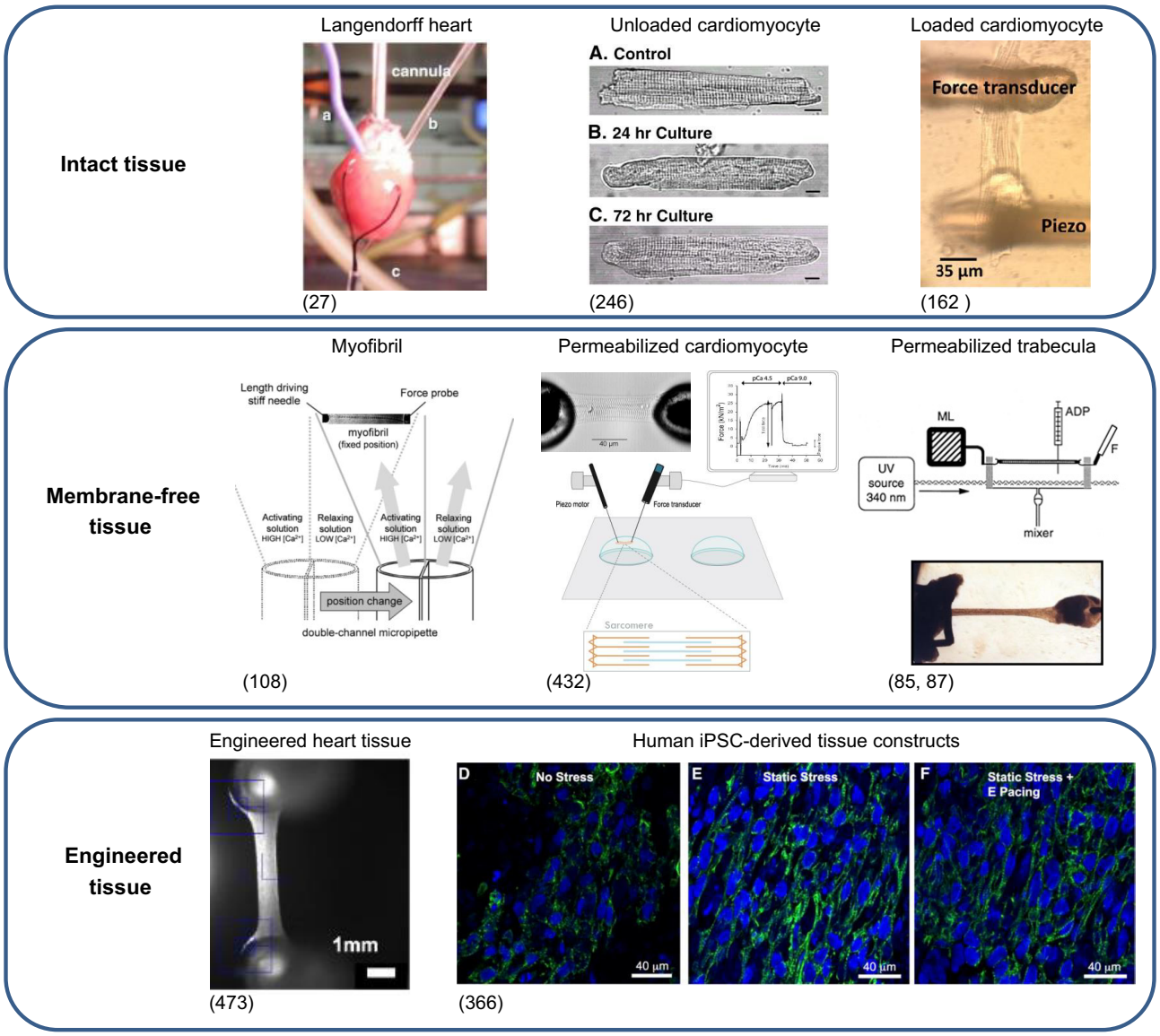


FIGURE 15. Techniques to study the properties of sarcomeres in intact heart and cardiomyocytes, membrane-free cardiac preparations, and engineered cardiac tissue. The figure is composed of figures presented in Refs. 27, 85, 87, 108, 162, 246, 366, 432, 473.

late mechanical stress into hypertrophy signals. Improvements in diastolic performance come at the expense of mechanisms regulating systolic function as reduced RBM20 also resulted in an attenuated Frank–Starling mechanism, reduced maximal and submaximal force generation, and slowed cross-bridge kinetics (348). Thus, there appears to be a tight window of treatment by titin isoform switching; when the titin isoform switch is too large, it may impair systolic performance and cause HF. The latter is illustrated by patients with RBM20 mutations who express giant titin isoforms and develop DCM with severe systolic dysfunction (30, 359). Although a shift to more compliant titin isoform may rescue concentrically remodeled hearts with diastolic dysfunction, a recent study showed that a shift to stiff titin isoform (decrease in N2BA/N2B ratio) limited eccentric remodeling (183). These studies indicate that modulating titin isoform composition and passive stiffness represents a therapy to prevent both concentric and eccentric remodeling of the heart.

4. Small molecule myosin activators and inhibitors

Decreased cardiac contractility is considered a central feature of systolic HF. Existing drugs increase cardiac contractility indirectly through signaling cascades but are limited by their mechanism-related adverse effects. To avoid these limitations, omecamtiv mecarbil (OM; CK-1827452), a small-molecule direct activator of cardiac myosin, has been developed (295). It has been shown (259) to bind to the myosin catalytic domain and to operate by an allosteric mechanism to increase the transition rate of myosin into the strongly actin-bound, force-generating state. OM accelerated the transition rate from the weakly bound to the strongly bound force-producing state. The rate of the subsequent step, ADP release, that governs the length of time in the strongly bound state did not change, nor did the rate of ATP binding and myosin release from the actin filament. A recent study showed that the effect of OM on isometric force development strongly depends on [Ca^{2+}], with activation at relatively low, physiologic [Ca^{2+}] (199). In contrast, inhibition of isometric force and cross-bridge kinetics were observed at maximal [Ca^{2+}] (199, 261). In addition, OM blunted the length-mediated increase in myofilament Ca^{2+} sensitivity in guinea pig myofibers (137). In animal models, OM increases cardiac function by increasing the duration of ejection without changing the rates of contraction (259). This illustrates that cardiac myosin activation may provide a new therapeutic approach for systolic HF, although the myosin-activating properties highly depend on OM concentration and [Ca^{2+}] in the cardiac muscle cells (199, 262).

As described in section III, HCM is characterized by preserved or even increased contractility (hypercontractility). Recently, a small molecule, MYK-461, has been identified that reduces contractility by decreasing the ATPase activity of the cardiac MHC (142) [see also the accompanying editorial (464)]. These authors demonstrated that early, chronic administration of MYK-461 suppresses the development of ventricular hypertrophy, cardiomyocyte disarray, and myocardial fibrosis and attenuates hypertrophic and profibrotic gene expression in mice harboring heterozygous human *MYH7* mutations. These data indicate that hyperdynamic contraction is central in HCM pathobiology and that inhibitors of sarcomere contraction may be a valuable therapeutic approach for HCM. A recent study (400) showed that MYK-461 also exerted beneficial effects in an acute setting.

Small molecules targeting the sarcomeric protein MHC may thus represent a therapy to correct both hypercontractility and hypocontractility. Therapies targeting sarcomere mutation–related changes in myofilament Ca^{2+} sensitivity and tension cost have been recently reviewed (417).

5. Heat shock proteins

The protein quality control system includes the intracellular surveillance by a large and diverse family of proteins termed chaperones, of which the largest group consists of heat shock proteins (HSPs). HSPs assist in the correct folding of nascent or incomplete proteins, preventing them from forming insoluble aggregates (460). HSPB1 (Hsp27), HSPB5 (or αB-crystallin), HSPB6 (Hsp20), HSPB7 (cvHsp), and HspB8 (Hsp22) are considered the first line of defense against proteotoxicity by stabilizing the contractile apparatus and assisting in removal of damaged sarcomeric proteins (205). Some forms of cardiomyopathy are, in fact, caused by mutations expressing chaperones (453). Inversely, this would imply that one can also rescue derailed protein homeostasis to alleviate or delay cardiomyopathy onset by boosting protein quality control, as shown for HSPB5 mutation–induced desmin cardiomyopathy in mice (372). The binding of αB-crystallin to myofilaments upon ischemia (17) may represent a protection to prevent myofibril degradation. Treatment of human cardiomyocytes with αB-crystallin protected human cardiomyocytes from titin aggregation (220) and lowered passive stiffness (114).

6. Cell therapy

As indicated above, cell therapy may be used to repair the injured heart. Interestingly, transplantation of neonatal rat cardiomyocytes in the infarct region of rat hearts 1 wk post-MI–enhanced myofilament Ca^{2+} sensitivity in the remote uninjured part of the LV (294). As the remote post-MI myocardium hypertrophies, the enhanced force-generating capacity of myofilaments in this region may represent a major contributor to improved LV function after cell transplantation. A recent study in guinea pigs demonstrated that three-dimensional human heart muscle constructs can repair the injured heart (466). Cardiosphere-derived cells secrete exosomes which reduce scarring, attenuate adverse

remodeling, and improve function in acute and chronic porcine MI (118). Administration of cardiosphere-derived cells has been shown to halt adverse remodeling in a phase 1 trial (260)

B. Gene Therapy

An exciting novel concept is the repair of sarcomere gene mutations using genome-editing techniques. Silencing of mutant RNA using RNA interference in a HCM mouse model carrying the R403Q MHC mutation prevented hypertrophy and fibrosis (194). Beneficial effects have been shown of exon skipping, transsplicing, and gene replacement in a mouse model of HCM and human iPSC-derived cardiomyocytes carrying a *MYBPC3* mutation (128, 277, 346). In addition, systemic administration of *MYBPC3* via adeno-associated virus increased mRNA and protein levels of cMyBP-C and suppressed the accumulation of mutant mRNA in HCM mice with a *MYBPC3* mutation (276). Direct correction of a heterozygous mutation in *MYBPC3* was established in human embryos using CRISPR/Cas9 technology (256).

C. Effects of Exercise

Exercise is an effective and safe way to improve peak oxygen consumption and quality of life in both HFrEF and HFpEF patients (99, 100, 132, 133, 387). However, optimal exercise modalities, efficacies, and the mechanisms involved are a matter of debate. Beneficial exercise effects appear to be partly mediated via a positive effect on the heart in HFrEF (132, 133). Results in HFpEF are not consistent: a beneficial effect has been shown on diastolic dysfunction (99), whereas other studies did not show a clear exercise-mediated cardiac effect (387, 388). Beneficial effects of exercise were observed in MI mouse models (VO), whereas exercise in TAC mouse (pressure overload) aggravated cardiac disease (95, 428). Experimental MI models showed that exercise-induced changes in myofilament Ca^{2+} sensitivity may underlie improved cardiac function (88, 375). Overall, evidence suggests that exercise improves cardiac function in MI-related HF models, which is partly related to myofilament changes (4, 88, 375).

Exercise in a HCM mouse model showed different effects on myofilament properties in male and female mice (309). A study in human HCM patients showed that the energetic status (PCr/ATP ratio) worsened during acute exercise (81). A recent study showed a small increase in exercise capacity in HCM patients who underwent a 16-wk exercise protocol (368).

VII. CONCLUDING REMARKS

Insight in the role of sarcomeric proteins in cardiac disorders has grown over the last decade at a remarkable pace. It turned out that the interaction between actin and myosin functions was a well-tuned, but very robust, contractile motor. A major step forward has been made in this period by the insight in the functions of cMyBP-C and titin within this process, the importance of posttranslational modifications of sarcomeric proteins, and the impact of mutations in genes expressing sarcomeric proteins. At the functional level, it has become apparent that not only systolic dysfunction but also diastolic dysfunction are important in HF. In fact, in part, as a consequence of population aging and overweightedness, diastolic dysfunction will become relatively more important and novel, more specific treatment options are urgently needed.

Animal models are key in providing mechanistic insight in the progression of cardiac diseases, but the translation to humans remains a challenge. Evidence mainly from animal studies suggested that compensatory processes, such as cardiac hypertrophy, were accompanied by a shift toward fetal protein expression, as expected because of cell growth. Studies in human tissue did not provide evidence for a MHC isoform shift in this direction and by and large, stressed the importance of protein phosphorylation in the regulation of contractile function. An exciting era is in front of us in which engineered heart tissue of human origin will be more generally available and will provide an alternative for animal models and enable more direct drug testing. Novel imaging techniques will enable insight in local Ca^{2+} handling and cell signaling in compartments/subcompartments of cardiac muscle cells, and gene editing may become applicable to treat inherited cardiomyopathies.

To address the new perspectives of the advances described above, we would like to first answer the general basic questions put forward in sect. I:

1. Does the direction of shift in Ca^{2+} sensitivity of force development dichotomously determine whether cardiac hypertrophy or dilatation occurs? If so, what is the mechanistic basis?
   The available evidence (described in sections II and III) suggests that, in answering this question, a distinction needs to be made between acquired and inherited cardiac diseases. Cardiac hypertrophy, the addition of sarcomeres in series or in parallel as a result of pressure or VO, respectively, arises via different cellular signaling pathways in which calcineurin and MEK1–ERK signaling are involved. The role of changes in Ca^{2+} sensitivity of force in these cases is still unclear but most likely secondary. In inherited cardiomyopathies, the evidence presented in **TABLES 2–5** indicates a direct link between the effect of thin filament gene mutations on Ca^{2+} sensitivity of force and the prevailing phenotype: an association between HCM and DCM with an increase or decrease in Ca^{2+} sensitivity of force, respectively. The situation in thick filament gene mutations (or in genes expressing accessory pro-

teins [e.g., cMyBP-C]) is less clear, as is the case for titin gene mutations. Mechanistically, it remains to be established whether a reduction of the force-generating capacity (maximal force), altered myosin-based regulation or stress-sensing mechanisms (for instance, via titin) are causal factors.

2. Is the Frank–Starling mechanism (LDA) depressed in HF? If so, what is the mechanistic basis or prevailing/main cause?
   The Frank–Starling mechanism (described in section I) is considered of crucial importance in the balance between the systemic and pulmonary circulation and in the increase in cardiac output during physical exercise. The current available evidence suggests that it is indeed depressed in the various forms of HF and thus may explain, at least in part, the exercise intolerance in patients with HF. Studies on the role of sarcomere protein phosphorylation showed that multiple protein modifications may alter LDA. Tn-exchange studies in single cardiomyocytes provided proof that PKA-mediated phosphorylation is a regulator of LDA (i.e., an increase in PKA-mediated cTnI phosphorylation enhances the length-dependent increase in myofilament Ca^{2+} sensitivity). Although sarcomere mutations may directly impair LDA, the secondary disease–related reduction in β_1-AR signaling will contribute to a depressed Frank–Starling mechanism in failing hearts.
3. What is the phosphorylation status of the sarcomeric proteins in a healthy heart? How does it change in (specific) cardiac diseases?
   Actually, this issue has not been addressed in detail in this review. In general, findings in human tissue indicate that sarcomeric proteins are dephosphorylated in HF, but it is unclear whether this is the case only in end-stage failing hearts or (less likely, at least in general) intrinsic to the disease processes and also present during the early stages of HF. Medication (β-AR blockers) precludes systematic research in this area in humans. Studies in experimental animal models indicate reduced β_1-AR signaling at a relatively early stage of disease. Although minor changes in protein phosphorylation may have a significant effect on sarcomere function, a decrease in protein phosphorylation status may have to be extremely large to have a functional impact. PKA-bisphosphorylation of cTnI has to decrease $<55\%$ to become functionally relevant.
   Next, we would like to address the translational aspects of these basic findings:
4. What are the guiding principles in the therapy of cardiac disorders caused by sarcomeric dysfunction? Protein isoform shifts or posttranslational modifications? Cardiac energetics: partitioning of energetic costs of basal cardiac metabolism, Ca^{2+} handling and contractile activity, or substrate choice? Reclassification of sarcomere-related cardiomyopathies based on novel insights in disease?
   Because MHC isoform shifts are minor in the failing human ventricle, therapy targeted to adaptive and maladaptive posttranslational modifications would be expected to be the most promising. In addition, modulation of the phosphorylation status and isoform composition of titin appears to be attractive mechanisms to direct remodeling of the heart and prevent diastolic dysfunction. Based on the sequence of changes during the progression of acquired and inherited cardiac disease (illustrated in **FIGURES 12 AND 14**), choice and timing of interventions appears to be crucial and dependent on the stage of disease. As the initial defect in inherited cardiomyopathies is the gene mutation, site-directed interventions will be required. Based on imaging studies in asymptomatic mutation carriers, inefficient cardiac pump function and a perturbed energetic status of the heart appears to be an early target to prevent HCM. Increased knowledge on the different pathomechanisms induced by sarcomere mutations obtained during the past decade has provided a broad basis for the development of therapies that target high myofilament Ca^{2+} sensitivity, impaired energetics, and perturbed excitation-contraction coupling. Future studies will reveal which therapy is most suited to prevent or reverse disease at an early or late stage of cardiac disease. The main ATP-consuming processes during contraction are active Ca^{2+} reuptake into the SR via SERCA2a and cross-bridge cycling. Therefore, even small shifts in the Ca^{2+} sensitivity of force development, cytosolic Ca^{2+} buffing, or substrates for ATP resynthesis can be beneficial when the demands are high, even for the well-tuned cardiac contractile machinery. Based on the knowledge of disease mechanisms collected during past years, the classification of genetic cardiomyopathies may be reconsidered. Classification of HCM is currently based on the clinical observation of cardiac hypertrophy, whereas in asymptomatic mutation carriers, who are phenotype-negative (i.e., hypertrophy-negative), the mutation-induced disease mechanisms may have been initiated. Thus, there is an urgent need to develop novel diagnostics to identify cardiac dysfunction in seemingly healthy, asymptomatic sarcomere mutation carriers, as in recent years it has become evident that disease initiation and progression start well before the development of hypertrophy.

Finally, to conclude this review in one sentence: sarcomeres not only form the basis of cardiac muscle function but also represent a therapeutic target to combat cardiac disease.

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