DISEASE MODIFIERS IN HYPERTROPHIC CARDIOMYOPATHY: INTERACTION BETWEEN CALCIUM, TITIN AND BETA-ADRENERGIC RECEPTOR STIMULATION

Aref Najafi
The work presented in this thesis was performed at the department of physiology of the VU University medical center, Amsterdam Center for Cardiovascular Sciences, in Amsterdam, the Netherlands.

VRIJE UNIVERSITEIT

Disease modifiers in hypertrophic cardiomyopathy: interaction between calcium, titin and beta-adrenergic receptor stimulation

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan de Vrije Universiteit Amsterdam, op gezag van de rector magnificus prof.dr. V. Subramaniam, in het openbaar te verdedigen ten overstaan van de promotiecommissie van de Faculteit der Geneeskunde op donderdag 8 november 2018 om 15.45 uur in de aula van de universiteit, De Boelelaan 1105

The work presented in this thesis was performed at the department of physiology of the VU University medical center, Amsterdam Center for Cardiovascular Sciences, in Amsterdam, the Netherlands.

Cover design: Vasco Sequeira and Joska Sesink, persoonlijkproefschrift.nl
Layout and design by Joska Sesink, persoonlijkproefschrift.nl.
Printing: Ridderprint BV | www.ridderprint.nl

ISBN: 978-94-6375-053-0

Copyright: Copyright © Aref Najafi 2018
No part of this work may be reproduced or transmitted in any form or by any means without written permission of the author.
The research described in this thesis was supported by a grant of the Dutch Heart Foundation (CVON ARENA, CVON 2011-11) and we acknowledge support from the Netherlands organization for scientific research (NWO; VIDI grant 91711344), the 7th Framework Program of the European Union (“BIG-HEART”, grant agreement 241577), and from ICIN-Netherlands Heart Institute and Ionoptix.

Financial support by the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.
# CONTENT

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>General introduction and thesis outline</td>
<td>9</td>
</tr>
<tr>
<td><strong>Part 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Altered myofilament function induced by post-translation modifications</td>
<td>27</td>
</tr>
<tr>
<td>3</td>
<td>( \beta )-adrenergic receptor signalling and its functional consequences in the diseased heart. <em>Eur J Clin Invest.</em> 2016</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Selective phosphorylation of PKA targets after ( \beta )-adrenergic receptor stimulation impairs myofilament function in Mybpc3-targeted HCM mouse model. <em>Cardiovascular research.</em> 2016</td>
<td>55</td>
</tr>
<tr>
<td>5</td>
<td>Sexual dimorphic response to exercise in hypertrophic cardiomyopathy-associated MYBPC3-targeted knock-in mice. <em>Pflugers Arch.</em> 2015</td>
<td>93</td>
</tr>
<tr>
<td><strong>Part 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Mimicking the cardiac cycle in intact cardiomyocytes using diastolic and systolic force clamps; measuring power output. <em>Cardiovascular research.</em> 2016</td>
<td>121</td>
</tr>
<tr>
<td>6</td>
<td>Pre-activation of cardiomyocytes determines contractile force; role of titin and calcium. <em>In preparation.</em></td>
<td>123</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td>Summary, general discussion and future perspectives</td>
<td>151</td>
</tr>
<tr>
<td><strong>Chapter 8</strong></td>
<td>Nederlandstalige samenvatting</td>
<td>169</td>
</tr>
<tr>
<td><strong>Appendix</strong></td>
<td>Curriculum vitae</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>List of publications</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Acknowledgments/Dankwoord</td>
<td>193</td>
</tr>
</tbody>
</table>
General introduction and thesis outline
1. CARDIAC FUNCTION

1.1 Cardiovascular system
The heart is a muscle that contracts continuously throughout our life at a rate of ~60 beats per minute during resting conditions. Myocardial contractions (i.e. heart beats) are triggered by electrical impulses generated by the sinoatrial node (SA node; i.e. the pacemaker cells) of the heart. During each contraction, the heart pumps blood throughout a network of blood vessels thereby providing the body with oxygen and nutrients. The delivery of oxygen (and removal of CO₂) to all cells in the body is regulated via two circulations of the cardiovascular system: the pulmonary and systemic circulation. Deoxygenated blood that returns to the heart from the organs and other body tissues arrives in the right atrium and is subsequently, via right ventricular contraction, pumped to the lungs for reuptake of oxygen (and removal of CO₂). The oxygenated blood returns to the left atrium (LA) and subsequently to the left ventricle (LV) and contraction of the LV leads to blood flow to the systemic circulation thereby providing the body with oxygen and nutrients. This sequence of events is repeated roughly every second throughout the life of a human being (around 2.5 billion times during a normal life span). Because the demand of blood from the body varies (for instance exercise versus rest), the heart is able to match the amount of blood supply to the body relative to demand. How the heart is able to match output to demand is the subject of the next sections.

1.2 Cardiac cycle
A cardiac cycle is characterized by a period of contraction (systole) and relaxation (diastole), which are central to ejecting and filling of the heart. During systole, the heart contracts and pumps blood to the lungs and the systemic circulation, while during the diastolic phase, the heart relaxes and fills with blood (figure 1A). The driving mechanical force during the cardiac cycle is the change in blood pressure that moves the blood in and out of the ventricles (the same holds true for the atria, but as the ventricles are the most important for determining cardiac output, the cardiac cycle of the LV is described). The cardiac cycle is described by changes in pressure and volume in the ventricle. The cardiac cycle of the LV is divided in four phases: A) filling of the ventricle, B) isovolumic contraction, C) ejection of blood into the aorta and D) isovolumic relaxation. These phases are depicted in a pressure volume (PV) diagram in Figure 1B. During diastole -the ventricle is at its lowest volume and pressure- point 1 of the PV diagram of the LV. Due to the low pressure in the ventricle blood flows from the LA to the LV (the valve between the atrium and ventricle, the mitral valve, is open), leading to a small increase in pressure and a large increase in LV volume (phase A). This ventricular filling phase includes the atrial contraction (i.e. atrial kick, which contributes to the last part of LV filling) and ends just after the ventricle starts contracting (point 2). This point represents the end-diastolic pressure (EDP) and end-diastolic volume (EDV) of the LV. Once the LV pressure exceeds the pressure in the LA, the mitral valve closes to prevent blood from flowing back into the atrium, and the ventricle starts to contract isovolumetrically (point 2 and phase B respectively in figure 1B). During the isovolumic contraction, an increase in LV pressure occurs, while the ventricular volume remains the same, because both the mitral valve and aortic valve are closed. Once the LV pressure exceeds aortic pressure, the aortic valve opens (point 3 on figure 1B) and the ejection of blood into the aorta starts (phase C on figure 1B). During this ejection phase the LV volume decreases as LV pressure increases to a peak value (peak systolic pressure), and then decreases as the ventricle begins to relax. When the aortic valve closes (at point 4), the ventricle relaxes isovolumetrically, meaning that LV pressure drops, while the LV volume remains unchanged. From the point where the LV starts to relax (point 4), we can determine the end-systolic volume (ESV). When the LV pressure falls below LA pressure, the mitral valve opens (point 1) and the ventricle is filled again. A considerable amount of information of cardiac performance can be determined from the pressure vs. volume relation of the heart. The PV relation changes upon myocardial filling (i.e. preload) and the load which against the heart pumps blood through the circulation (i.e. afterload). Many parameters such as stroke volume (SV), ejection fraction, contractility and work can be determined from the PV-loop. The SV is the difference between the end-diastolic volume and end-systolic volume, which is the amount of blood ejected per beat by the LV through the body, illustrated in figure 1B as the width of the PV-loop. The area within the loop is the ventricular work. The ejection fraction (EF) refers to the amount, or percentage, of blood that is pumped out of the ventricles with each contraction. Another parameter determined from the PV-loop is the end-systolic pressure volume relation (ESPVR, figure 1C), which is a measure of ventricular contractile state that is independent of heart rate and, more importantly, loading conditions within the physiological range.

The heart has the capability of matching the amount of blood ejected (SV) to the demand of the body. During exercise, the demand for oxygen and nutrients rises and the heart reacts to this increased demand by increasing its output through an increased beating frequency as well as an increase in SV (figure 1C). The increases in heart rate and SV are regulated by the sympathetic nervous system, which is reviewed in Chapter 2 of this thesis. Sympathetic activation increases cardiac output through the release of catecholamines (adrenaline and noradrenaline). In addition to sympathetic activation of the heart, cardiac performance is regulated via the so-called Frank-Starling mechanism, which is the ability of the heart to adjust its force development (or contraction) to changes in ventricular filling (end-diastolic volume). The cellular mechanism underlying the Frank-Starling mechanism is the length-dependent activation (LDA) of the myofilaments. Both the sympathetic activation as well as the Frank-Starling mechanism are the crucial factors regulating cardiac performance, and both processes are regulated at the level of the individual cardiac muscle cell, the cardiomyocyte.
1.3 Contractile function at the cardiomyocyte level

Multiple cellular processes need to take place for myocardial contraction to occur, such as electrical activation (excitation) of the cardiomyocytes, intracellular Ca\(^{2+}\) elevation and adenosine triphosphate (ATP) hydrolysis. The coupling between cardiomyocyte excitation and contraction is known as cardiac excitation-contraction coupling (EC-coupling)\(^2\). Automatic depolarization of the pacemaker cells in the SA node cells generate action potentials that travel throughout the atria and subsequently through the ventricles to quickly depolarize all cardiomyocytes. When the myocyte is depolarized, Ca\(^{2+}\) enters the cell through activated L-type Ca\(^{2+}\) channels located on the external sarcolemma and t-tubules. This relatively small inflow of Ca\(^{2+}\) is sensed by the calcium-release channels (ryanodine receptors) triggering a much larger Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\)-storage organelle, the sarcoplasmic reticulum (SR). This release increases intracellular Ca\(^{2+}\) concentrations 100-fold, from about 10\(^{-7}\) to 10\(^{-5}\) M\(^2,8\), allowing cross-bridge formation (i.e. interactions between the actin thin-filament and myosin thick-filament) to take place. This results in force development and shortening of the sarcomere\(^9\).

The main components of the cardiac sarcomere are roughly classified in the thick myosin and thin actin filaments and titin (figure 2). Contraction is achieved by interaction of thick and thin filament proteins, leading to movement of the Z-discs towards each other. The thick filament includes cardiac myosin binding protein C (cMyBP-C) and myosin. Myosin consists of two myosin heavy chains (MHC), which both have two myosin light chains (MLC1 (encoded by the MYL3 gene) and MLC-2 (encoded by the MYL2 gene)). The thin filament consists of actin, the troponin complex (i.e. troponin C, I and T) and tropomyosin (TM). Titin is the largest protein of the human body. It consists of two main isoforms, namely, the more stiff isoform N2A and the compliant isoform N2BA\(^10\). Titin has a central role in regulation of the sarcomeric viscoelasticity and it may modulate actomyosin interaction by a titin-based alteration of the distance between myosin heads and actin and thereby modulate active force development\(^11\). In addition, titin keeps the thick filaments centered in the sarcomere, resulting in optimal active force generation; titin is important for the assembly of the sarcomeres, is involved in mechano-sensing signaling and may be a key player in length-dependent activation of the contractile machinery, which underlies the Frank–Starling mechanism\(^12\).

1.4 Cardiomyocyte force production and relaxation

In the 1970's the cardiomyocyte contraction and the cross-bridge formation was described based on two stages\(^13\). First, in the absence of Ca\(^{2+}\), TM blocks the myosin-bindings sites on actin and when the intracellular Ca\(^{2+}\) rises myosin heads bind to actin and induce force generation. In the 1990's the 3-state model was proposed, indicating that the binding of myosin to actin does not occur in one single step, but in two steps\(^14\). Based on the position of TM, the thin filament can be in three different states termed: the blocked (B-state), closed (C-state) and open (M-state).
states. At low Ca\textsuperscript{2+}, TM blocks the myosin binding sites on actin (B-state). The rise in intracellular Ca\textsuperscript{2+} induces conformational changes of the TM-troponin complex, resulting in availability of the myosin binding sites on actin. Weakly-bound cross-bridges populate the C-state\textsuperscript{14, 15}. During the M-state ATP is hydrolysed and strong-binding cross-bridges are formed that induce additional movement of tropomyosin, resulting in myofilament contraction and sliding.

In order to initiate myocardial relaxation at cellular level, the cytosolic Ca\textsuperscript{2+} should be removed. This occurs mostly by Ca\textsuperscript{2+}-reuptake into the SR and to a smaller extent by removal from the cell by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX). Ca\textsuperscript{2+}-reuptake into the SR is performed by the SR Ca\textsuperscript{2+}-ATPase (SERCA), which is modulated by phospholamban (PLN). This modulation occurs in a phosphorylation-dependent manner. A small amount of Ca\textsuperscript{2+} is taken up by the mitochondria and removed from the cell by the sarcolemmal Ca\textsuperscript{2+}-ATPase pump\textsuperscript{2}.

1.5 Cardiomyocyte protein phosphorylation
Phosphorylation of myofilament and Ca\textsuperscript{2+} handling proteins regulates the function of the cardiomyocyte \textsuperscript{16}. Several kinases have been described in the heart, such as protein kinase A (PKA), protein kinase C (PKC) and CaMKII. Upon activation of β-adrenergic receptor (β-AR), PKA phosphorylates the myofilament proteins cTnI, cMyBP-C, titin and proteins involved in Ca\textsuperscript{2+} handling, PLN, the ryanodine receptor (RYR2) and the L-type Ca\textsuperscript{2+} channel. PKA-mediated protein phosphorylation induces positive inotropic (i.e. contractility) and lusitropic (i.e. relaxation) responses at the cardiomyocyte level. The phosphorylation of cTnI reduces myofilament Ca\textsuperscript{2+}-sensitivity\textsuperscript{17}, while cMyBP-C phosphorylation accelerates the cross-bridge cycling. Both changes enhance relaxation of cardiomyocytes\textsuperscript{18, 19}. In addition, PKA phosphorylation of titin reduces the resting tension of cardiomyocytes, and thereby contributes to relaxation of cardiomyocytes\textsuperscript{20}. Phosphorylation of PLN relieves its inhibitory effect on SERCA, inducing a faster Ca\textsuperscript{2+} re-uptake and sufficient levels of Ca\textsuperscript{2+} for the next activation cycle. Combined, these cellular changes allows the cell for proper relaxation, which is an important determinant of diastolic function of the heart.

1.6 Cardiac myosin binding protein C
Although the function of most of the myofilament proteins has been known for some time, cMyBP-C’s function has proven to be more elusive. Cardiac myosin binding protein C is localized in the C-zones of the A band of the sarcomere, forming transverse stripes of 43 nm apart in the cross-bridge bearing region. It is a large protein (150 kDa) consisting of immunoglobulin and fibronectin consisting domains (figure 3). cMyBP-C interact with many other proteins in the sarcomere, such as actin and myosin S2 in the N-terminal of cMyBP-C, and titin and myosin light meromyosin at the C-terminal of cMyBP-C. In vivo and ex vivo studies have shown that cMyBP-C acts as a physical restrain on the myosin heads, keeping them near the thick filament backbone and thereby reducing the probability of myosin heads to interact with actin during diastole. Modulation of thin filament activity by cMyBP-C is supported by in vitro motility assays\textsuperscript{21, 22}. cMyBP-C binds at low intracellular Ca\textsuperscript{2+} with the thin filament, displacing TM toward its high Ca\textsuperscript{2+} position, indicating that the myofilaments become activated. However, at high Ca\textsuperscript{2+} the binding of cMyBP-C induces a load on the thin filament, resulting in a lower rate of sliding. When cMyBP-C is dephosphorylated in the M-domain, the C1–M–C2 domains are strongly bound to myosin S2 and actin. This interaction prevents cross-bridge formation. cMyBP-C has many phosphorylation sites, with serine (Ser) 273, 282 and 302 located in the M-domain and Ser-133 in the Proline-Alanine rich domain\textsuperscript{23, 24}. When cMyBP-C is phosphorylated at Ser 273, 282 and/or 302 it releases its interaction with myosin S2. This allows myosin S2 to get ordered and extended to the thin filaments, resulting in tight myosin-actin interactions\textsuperscript{25}. Thus, cMyBP-C acts as a structural constraint limiting cross-bridge formation, and phosphorylation of the regulatory M-domain accelerates cross-bridge kinetics which is required for enhanced rates of relaxation and force development in diastole and systole, respectively\textsuperscript{26}.

Figure 2. Schematic representation of striated muscle components. Illustration of one single sarcomere containing thick and thin filament components. Adapted with permission from Sequeira et. al\textsuperscript{1}
The majority of HCM patients develop asymmetrical LV thickening indicative for haploinsufficiency. Recently, Semsarian et al. reported that a genetic cause may be even more common with an estimated prevalence of 1 per 500 individuals in the general population. Hypertrophic cardiomyopathy (HCM) is the most frequent occurring inherited cardiac disorder, with a prevalence of 1 per 500 individuals in the general population. Recently, Sensmian and co-workers reported that a genetic cause may be even more common with an estimated prevalence of 1/200. The majority of HCM patients develop asymmetrical LV thickening of particularly the interventricular septum (IVS). About 25% of HCM patients develop LV outflow tract obstruction, caused by contact between the mitral valve leaflet and the hypertrophied interventricular septum in systole. HCM mutations are frequently associated with diastolic dysfunction, which will lead to reduced ventricular filling, causing an increase in LV end diastolic pressure and LA pressure. Impaired diastolic function has been detected in mutation carriers, preceding other signs of myocardial contraction and relaxation abnormalities and LV hypertrophy. Other hallmarks of HCM are myocyte disarray, small-vessel disease and deposition of interstitial fibrosis. HCM is a heterogeneous disease with a wide range of genetic gene mutations and clinical presentation. The clinical course of HCM is characterized by a large inter-and intra-familial variability, ranging from cardiac arrhythmia and sudden cardiac death (SCD) in young individuals to asymptomatic mutation carriers.

2.2 Mechanism of HCM disease development

HCM is considered as a disease of the sarcomere. Mutations in the genes encoding the thick filament proteins cMyBP-C and MHC account for the majority of the cases. It has been reported that ~40% of all HCM genotyped cases are located in the MYBPC3 gene encoding cMyBP-C. Mutations in the MYBPC3 gene are truncating or missense mutations. Interestingly, in the myocardium of HCM patients with heterozygous truncating MYBPC3 mutations, truncated proteins were never detected and the amount of wild-type (WT) cMyBP-C was about 30% lower compared to non-failing donor tissue indicative for haploinsufficiency. Haploinsufficiency is probably the main disease mechanism. Human studies revealed that regardless of the disease-causing mutation, cardiac tissue from patients with obstructive HCM exhibits reduced maximal force generation and increased Ca\(^{2+}\) sensitivity of myofilaments. The increase in myofilament Ca\(^{2+}\) sensitivity perturbs the diastolic properties and length-dependent activation of the cardiomyocytes. It seems that increased myofilament Ca\(^{2+}\) sensitivity is a common characteristic of HCM. The high myofilament Ca\(^{2+}\) sensitivity was normalized for almost all samples after exogenous PKA treatment, indicating that post-translational protein modification may occur secondary in response to cardiac disease, while several sarcomere mutations have been identified as a direct cause of the high myofilament Ca\(^{2+}\) sensitivity.

2.3 Reduced beta-adrenergic receptor signaling in HCM

Activation of the sympathetic system and release of catecholamines, with subsequent downstream activation of the \(\beta\)-ARs, represents a powerful mechanism whereby cardiac contractility and relaxation of contractions increase to preserve perfusion of the body during increased stress (e.g. exercise). Moreover, cardiac performance is tightly regulated by the parasympathetic and sympathetic nervous systems. In diseased heart the contractile performance of the heart is reduced and it results in a lower blood supply to the body. It is important to note that chronic \(\beta\)-AR stimulation initiates a cascade of alterations at the cellular level, resulting in a diminished contractile performance of the heart. So, this mechanism is beneficial in the acute phase, but is detrimental upon chronic stimulation. The cellular alterations include reduced \(\beta\)-AR sensitization and decreased numbers of \(\beta\)-AR, which both might lead to a diminished \(\beta\)-AR signalling. Consequently, the downstream target, which is PKA, is less activated. It has been implicated that the phosphorylation state at the PKA sites of the myofilament proteins cTnI and cMyBP-C are lower in HCM patients compared to healthy non-failing donors. In chapter 2 we review the consequences of chronic \(\beta\)-AR stimulation for myocardial processes and function in diseased heart. Moreover, to investigate if and how \(\beta\)-AR signalling is impaired at an early stage of HCM development, we studied the...
response to β-AR stimulation and phosphorylation of PKA target proteins in a HCM mouse model harbouring a gene mutation (G to A transition on the last nucleotide of exon 6) in Mybpc3 encoding cMyBP-C in chapter 3.

2.4 Effects of exercise in HCM
As a mentioned earlier, the β-AR signalling is an essential mechanism during exercise, which becomes activated in order to maintain perfusion of the body. SCD is the most common cause of death in HCM, predominantly affecting young adults and athletes. The rate of annual SCD is less than 1%, however within subgroups of the HCM population the incidence is much higher. According to the European Society of Cardiology, people diagnosed with HCM are recommended not to participate in competitive sports. We investigated the effect of β-AR activation upon exercise training in a HCM-associated mouse model. We exposed heterozygous HCM mice to a 8 weeks voluntary exercise protocol. The myofilament protein alterations and force development were determined in sedentary mice and mice exposed to exercise (chapter 4).

2.5 Sex-differences in HCM
SCD is significantly more likely to occur in male than in female athletes. Multiple studies have shown that sex differences become more prominent in cardiovascular pathology. It has been reported that female HCM patients were underrepresented, suggesting females are less likely to develop symptoms of HCM. Moreover, at disease onset females were on average 9 years older than males. Another study in patients with obstructive HCM revealed that interventricular septum thickness is lower in females compared to males. Research in MYBPC3 mutation carriers showed larger atrial and ventricular dimensions and lower fractional shortening (FS) in males than in females. The pathophysiological mechanisms, which underlie the differences between males and females remain to be investigated. Therefore, in chapter 4 we investigate whether sex affects pathophysiological mechanisms in heterozygous MYBPC3-targeted knock-in mice.

3. AIM AND OVERVIEW OF THIS THESIS

3.1 Aim of the thesis
3.1.1 Part 1
The β-AR signalling pathway is a powerful signalling cascade to adapt cardiac output to high need of the body. However, this signalling pathway is disturbed in HCM, leading to hypophosphorylation of the downstream PKA targets. In chapter 2 of this thesis we review the β-AR signalling pathway in diseased heart, especially in HCM. As it remained undefined if all PKA targets are affected similarly by diminished β-AR signaling in HCM, phosphorylation of proteins involved in calcium handling and myofilament function were in chapter 3 using a HCM mouse model.

Ample clinical studies reported sex-related differences in HCM disease onset and severity. However, the pathophysiological cellular mechanisms, which underlie the differences between males and females remain to be investigated. Moreover, to investigate whether activation of β-AR signalling upon exercise induce a sexual dimorphic response, we exposed the HCM-associated mice to an exercise protocol, which is discussed in chapter 4.

3.1.2 Part 2
To better understand the complex interplay between calcium handling proteins, myofilament proteins and the β-AR mediated phosphorylation events in health and disease, we need a system to measure function in loaded intact cardiomyocytes. In Part 2 of this thesis we aimed to develop a novel method to mimic the cardiac cycle in single isolated cardiomyocytes. In chapter 5 we demonstrate that, by anchoring a single intact cardiomyocyte to our force transducer, it is possible to drive a feedback control loop that adapts the length of the cell to control the force it exerts. This approach can be used to functionally approximate the cardiac PV relationship at the cellular level by modulating cardiomyocyte length using feedback based on force level. This method (the Myostretcher) allows us to measure the external work performed and the power generated by a single intact cardiomyocyte under physiological conditions. This novel technique is validated by measuring the cardiomyocyte performance of the heterozygous (HET) RNA binding motif protein 20 (RBM20) deficient rats. RBM20 is an important modulator of gene splicing in the heart. Mutations in the splicing RBM20 result in low RBM20 levels and expression of large, highly compliant titin isoforms. In chapter 6, this model is used to validate the Myostretcher.

It has also been reported that expression of compliant titin isoform in RBM20 animals resulted in a lower Ca²⁺ sensitivity and force production of the cardiomyocytes. It seems that titin and Ca²⁺ play a significant role in active force development. Based on this, we hypothesized that titin together with diastolic Ca²⁺ might pre-activate the cardiomyocyte during diastole and thereby represents a major determinant for the level of active force production in the subsequent systolic phase. In chapter 6 we discuss the importance of pre-activation of the myofilament with Ca²⁺ on active force development in heterozygous RBM20 rats.

3.2 Thesis outline

Part 1. Altered myofilament function induced by post-translation modifications
In chapter 2 we give an overview of the β-AR pathway and the possible disturbances in this route might occur. Since β-AR activation diminishes with failing heart, an important mecha-
nism to restore this pathway and prevent further deterioration is supplying patients with β-blockers. Besides the regular available therapies, we also touch upon the future therapeutic opportunities to prevent or cure patients with a diseased heart. Patients with heart disease have more often a hampered β-AR signalling, which might result in a diminished activation of PKA and phosphorylation of target proteins.

In chapter 3 we investigate the effect of β-AR stimulation in HCM-associated mouse model. It is known from previous data that the myofilament protein cTnI is less phosphorylated at PKA target sites in HCM. To determine whether this disturbances in PKA-mediated phosphorylation is localized to the myofilament proteins, we exposed the isolated cardiomyocytes to β-AR stimulus (i.e. isoprenaline). We performed intact as well as membrane-permeabilized cardiomyocyte mechanical force measurements with isoprenaline and exogenous PKA incubation, respectively. We also determined the phosphorylation state of the myofilament protein cTnI and calcium handling protein PLN.

In chapter 4 describes the sex-related cardiac response upon exercise in Mybpc3-targeted HCM mice. It has been reported that women with HCM are underrepresented, however they are older at the time of diagnosis and more symptomatic. In this study we performed mechanical as well as molecular analysis to determine the pathophysiological mechanism underlying sex-specific differences in HCM.

Part 2. Mimicking the cardiac cycle in single isolated cardiomyocytes

In chapter 5 we validate a novel method to mimic the cardiac cycle in single isolated cardiomyocytes. To determine with current techniques the effects of hemodynamic conditions, researchers often rely on multicellular heart tissue, such as cardiac muscle strips. However, due to the absence of the extracellular matrix, the direct effect of the muscle remained unclear. Another disadvantage is that the multicellular muscle strips suffer from diffusion constrains which may limit availability of oxygen and nutrients. To overcome this we measured in single isolated cardiomyocytes the maximal work development at physiological conditions.

In chapter 6 we determined the hemodynamic alterations in HET RBM20 deficient rats. It has been established that mutations in the splicing factor RBM20 results in low RBM20 levels and expression of large, highly compliant titin isoforms. Expression of compliant titin isoforms has been linked to ventricular enlargement, dilated cardiomyopathy, arrhythmia and extensive fibrosis. By performing force-sarcomere length loops in single isolated cardiomyocytes from HET RBM20 and WT rats, we examined the maximal work development in HET as well as WT single cardiomyocytes. We further elucidated the role of compliant titin and the level of diastolic Ca$^{2+}$ in active force development of the cell by exposing the membrane-permeabilized cardiomyocytes to various levels of diastolic [Ca$^{2+}$]. In chapter 6 we investigate whether pre-activation of myofilaments by increasing the diastolic Ca$^{2+}$ levels may affect the active force development of the permeabilized isolated RBM20 cardiomyocytes.
4. REFERENCES


Altered myofilament function induced by post-translation modifications
β-adrenergic receptor signalling and its functional consequences in the diseased heart

Aref Najafi
Vasco Sequeira
Diederik WD Kuster
Jolanda van der Velden

ABSTRACT

Background
To maintain the balance between the demand of the body and supply (cardiac output), cardiac performance is tightly regulated via the parasympathetic and sympathetic nervous systems. In heart failure, cardiac output (supply) is decreased due to pathologic remodeling of the heart. To meet the demands of the body, the sympathetic system is activated and catecholamines stimulate β-adrenergic receptors (β-ARs) to increase contractile performance and cardiac output. Although this is beneficial in the acute phase, chronic β-ARs stimulation initiates a cascade of alterations at the cellular level, resulting in a diminished contractile performance of the heart.

Materials and methods
This narrative review includes results from previously published systematic reviews and clinical and basic research publications obtained via PubMed up to May 2015.

Results
We discuss the alterations that occur during sustained β-AR stimulation in diseased myocardium, and emphasize the consequences of β-AR overstimulation for cardiac function. In addition, current treatment options as well as future therapeutic strategies to treat patients with heart failure to normalize consequences of β-AR overstimulation are discussed.

Conclusions
The heart is able to protect itself from chronic stimulation of the β-ARs via desensitization and reduced membrane-availability of the β-ARs. However, ultimately this leads to an impaired downstream signalling and decreased protein kinase A (PKA)-mediated protein phosphorylation. β-blockers are widely used to prevent β-AR overstimulation and restore β-ARs in the failing hearts. However novel and more specific therapeutic treatments are needed to improve treatment of HF in the future.

Keywords
β-adrenergic receptor, heart failure, hypertrophic cardiomyopathy, Protein kinase A (PKA), A-kinase anchoring protein (AKAP)

INTRODUCTION

The heart is the center of the circulatory system [1]. The heart is exquisitely able to match cardiac output to the body’s demands, which it achieves by processing input from the sympathetic and parasympathetic nervous systems. The parasympathetic system, through its effector acetylcholine, lowers cardiac output by decreasing heart rate through its effect on sinoatrial cells, with limited effect on ventricular cardiomyocytes. Sympathetic activation increases cardiac output through the release of the catecholamines epinephrine and norepinephrine [2]. The sympathetic nervous system acts on both heart rate (through direct action on sinoatrial cells) and on contractility of individual cardiomyocytes. This is achieved by the local release of, mainly, norepinephrine through postganglionic fibers innervating the whole heart and systemic release of mainly epinephrine from the adrenal medulla.

Both epinephrine and norepinephrine cause ventricular cardiomyocytes to increase contractile force. Catecholamine effects on cardiomyocytes are mediated by β-adrenergic receptor (β-AR) activation [3]. β-ARs are members of the G-protein-coupled receptors (GPCRs) family [4]. GPCRs consist of two main domains: a seven-transmembrane-spanning receptor and an intracellular heterotrimeric G-protein complex (Gαβγ). Binding of a ligand to a GPCR induces a conformational change of the receptor, promotes exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) via guanine nucleotide exchange factors leading to dissociation of the G-proteins into activated Gαs and Gαi [5] (Fig. 1). The downstream effects of GPCR activation are largely determined by the type of Gα subunit that is coupled to the receptor. There are four different types of Gα subunit: Gαs, Gαi, Gαq, and Gα12/13 [6].

There are three subtypes of β-ARs: β1-AR, β2-AR and β3-AR. β1-AR is the main receptor on healthy heart muscle cells, with a ~4 times higher expression level compared to β2-ARs (ratio β1-AR to β2-AR 4:1) [7]. The expression of β1-AR is very low in the unstressed heart, but it is upregulated in heart failure [8]. In the failing heart β1-AR decreases, while the expression of β2-AR increases, reaching a ratio of 3:2 [9]. Both β1 and β2 receptors are coupled to Gαs (stimulatory) protein, that leads to increased contractility once activated, as will be discussed below. However, persistent β1-AR receptors activation can cause coupling of the receptor to the Gαi (inhibitory) pathway under the influence of G-protein-coupled receptor kinase (GRK2 and/or GRK5) and protein kinase A and/or C phosphorylation [10]. This might oppose the positive inotropic effects mediated via Gαs, which in turn, activates a cell survival pathway [11]. A negative inotropic effect has also been reported via activation of β2-ARs. β1-AR activation increases the activity of protein kinase G (PKG) via cyclic guanosine monophosphate (cGMP)-dependent signalling and thereby modulates both vascular and muscular function of the heart [12]. In failing hearts, a 2-to 3-fold increase in β1-AR expression, compared to non-failing heart has been found. The negative inotropic effects of β1-AR stimulation was mildly reduced in HF,
while the positive inotropic response was reduced by 75% in ex vivo myocardial samples from failing human hearts, compared with non-failing cardiac tissue. This implicates the importance of β₁-AR signalling and its attenuation of positive inotropic response in the failing hearts [12].

Downstream β-AR signalling

Binding of agonists to β₁- and β₂-ARs causes the formation of cyclic adenosine monophosphate (cAMP) production from adenosine triphosphate (ATP) via adenylyl cyclase (AC) [13]. The primary function of cAMP is activation of protein kinase A (PKA), which subsequently phosphorylates a set of regulatory proteins in cardiac muscle cells, leading to increased lusitropy (increased relaxation) and positive inotropy (increased contractility) [14, 15].

The increased lusitropic and inotropic effects in cardiac muscle cells following β₁/β₂-AR stimulation are mediated via PKA-phosphorylation of target proteins at multiple subcellular localizations. PKA-phosphorylation of myofilament proteins plays a key role in the enhanced lusitropic response. PKA-phosphorylation of cardiac troponin I (cTnI) reduces myofilament localizations. PKA-phosphorylation of myofilament proteins plays a key role in the enhanced lusitropic response. The PKA-RII subunit is predominantly located in the cytosol, with only a small percentage anchored to AKAPs [28], while the majority of PKA-RII is bound to AKAPs [28]. Another major difference between the PKA regulatory subunits is the sensitivity to cAMP. PKA-RII is activated with cAMP concentrations ranging from 200 to 400 nM, while PKA-RI has a higher cAMP sensitivity, with activating concentrations in the range of 50-100 nM of cAMP [29]. However

Figure 1. G protein coupled receptor (GPCR) activation. Binding of a β-adrenergic receptor (β-AR) agonist promotes exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), leading to dissociation of the G proteins into activated Gᵢ and Gₛ. Binding of Gₛ to adenylyl cyclase (AC) catalyzes the formation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), resulting in cAMP-dependent activation of protein kinase A (PKA), which is localized to various compartments of the cell via PKA-A-kinase anchoring proteins (AKAP) complexes. Activation of PKA is regulated via breakdown of cAMP by phospho-diesterase (PDE).

This multitude of effects at different locations within the cardiac muscle cells occurs within 30 seconds from the start of β-AR agonist exposure, as shown using a fluorescence resonance energy transfer sensor for cAMP and PKA activity [23]. The well-orchestrated changes in inotropic and lusitropic states of cardiac muscle cells are partly mediated through A-kinase anchoring proteins (AKAPs) [24]. AKAPs are a family of scaffold proteins that tether PKA as well as other signalling molecules to different sites within the cell [25], thereby providing spatial and temporal regulation of the PKA signalling event [24]. In the present review we will briefly discuss the pathophysiological alterations in the β₁-β₂-AR pathway as well as potential therapeutic approaches for the diseased heart.

Compartmentalization of PKA

PKA is a tetrameric complex, consisting of two genetically distinct regulatory subunits (PKA-RI and PKA-RII) and two catalytic subunits. Activation of PKA occurs via binding of cAMP to PKA-RI and PKA-RII, which reduces the affinity of the regulatory subunits for the catalytic subunits. This causes release of catalytic subunits, which are then able to phosphorylate substrate proteins that are in close proximity [26]. It has previously been shown that PKA-RI and PKA-RII are localized to spatially distinct subcellular compartments [27].

The PKA-RI subunit is predominantly located in the cytosol, with only a small percentage anchored to AKAPs [28], while the majority of PKA-RII is bound to AKAPs [28]. Another major difference between the PKA regulatory subunits is the sensitivity to cAMP. PKA-RII is activated with cAMP concentrations ranging from 200 to 400 nM, while PKA-RI has a higher cAMP sensitivity, with activating concentrations in the range of 50-100 nM of cAMP [29]. However
intranuclear activation of the different PKA regulatory subunits is complex. β-AR stimulation in rat cardiomyocyte resulted in activation of PKA in the PKA-RII compartment over PKA-RI [27]. Local control of cAMP production and breakdown can influence which PKA regulatory subunit may become activated. cAMP (and cGMP) levels are tightly regulated by the cyclic nucleotide degrading enzymes phosphodiesterases (PDEs). PDEs are enzymes that breakdown cyclic nucleotide phosphodiesterase, such as cAMP and cGMP. They are categorized based on their amino acid sequence homology, substrate specificities, and pharmacological properties [30]. PDE isoforms differ in their substrate specificity to breakdown cAMP or cGMP. In addition, PDE4 and PDE8 are specific for cAMP degradation, while PDE1, PDE2, PDE3 can hydrolyze both cAMP as well as cGMP [30]. PDE5 catalyzes the hydrolytic degradation of cyclic GMP, but not cAMP. Recently, the importance of cGMP, modulating cAMP levels via PDE's has been reported in cardiac myocytes [31]. Moreover, cGMP levels in the cardiac muscle cells may affect the cAMP pool that activates PKA-RI and PKA-RII [31]. The specific local effect of cGMP on cAMP levels depends on the mechanism through which cGMP content is raised and requires local PDEs [31].

It is important to note that a general increase in cAMP levels is not enough for the specific regulation of proteins and PKA phosphorylation of its targets. Therefore, localization of PKA in close proximity to its substrate is required to ensure spatial regulation of PKA phosphorylation [32]. It has been reported that PKA-RI and PKA-RII respond to distinct, spatially cAMP signals generated by β-AR agonists, which were regulated by unique subset of cAMP-degrading PDEs [27]. Although PKA-RI isoform has a higher cAMP sensitivity, an in vitro study has shown that catecholamines generate a restricted pool of cAMP that selectively activates PKA-RII isoform [27]. Besides the cAMP pools, to facilitate PKA-mediated activation and phosphorylation in a specific and efficient manner, PKA needs to be localized to distinct intracellular compartments by AKAPs.

More than 70 different AKAPs have been identified with each AKAP showing specificity for different subcellular areas [24]. AKAPs are a group of proteins which vary in molecular weight and do not share sequence homology, with the exception of an amphipathic helical region that binds to the regulatory subunits of PKA [24]. Several AKAPs have been identified in cardiac muscle cells [33]. The thin filament protein cardiac troponin T has been reported as a sarcomeric AKAP, which provides a pool of PKA that can quickly phosphorylate myofilament proteins upon activation [34]. Another myofilament AKAP, myomegalin, was found to be involved in phosphorylation of cMyBP-C as well as Ctni [35]. AKAP185 has been identified as the anchoring protein responsible for PKA-mediated regulation of SR Ca\(^{2+}\)-cycling, through phosphorylation of phospholamban (PLN) and RyR [33, 36]. The importance of AKAP79 to form a complex with AC has also been reported [37]. This AC-AKAP complexes might induce compartmentalization of cAMP-PKA signalling, which adds another dimension to the classical cAMP pathway. Taken together, localization of PKA-AKAP and AC-AKAP complexes at various compartments of the cardiac muscle cell may play an important role in modulation of cardiac protein phosphorylation and function.

**DISTURBED B-AR SIGNALLING IN CARDIAC DISEASES**

**Heart Failure**

Heart failure is a syndrome characterized by decreased cardiac output, through deficits in contractility, relaxation or, both. At the early stage of diminished cardiac function, an increase in sympathetic activity preserves cardiac output. As cardiac function further deteriorates, sympathetic activity will increase in an attempt to compensate for the progressive loss of cardiac function [38]. Chronic exposure of the heart to high levels of catecholamines may lead to further pathological changes in the heart, including continued progressive deterioration in cardiac structure and function [39, 40]. Sustained β-AR stimulation has been shown to correlate with LV dysfunction and mortality [41]. Physiologically, this phenomenon could be explained by chronic Ca\(^{2+}\) overload, which might cause cardiac muscle cell death [42, 43].

To prevent the adverse effects of chronic adrenergic receptor stimulation, a protective mechanism is in place to prevent further deterioration of cardiac function [44]. Long-term exposure to catecholamines induces attenuation of receptor responsiveness, a process termed desensitization [45]. This dampening process is initiated by phosphorylation of β-ARs by PKA, protein kinase C (PKC) and GRKs; predominantly GRK2 and GRK5 [46, 47]. Other kinases are also known to regulate GPCRs physiological responses upon phosphorylation, such as Akt/protein kinase B (PKB), caseine kinase (CK)-1 and CK-2 [48]. Desensitization of β\(_1\) - and β\(_2\) -ARs on the plasma membrane of the cardiac muscle cells particularly occurs via PKA, PKC and GRK phosphorylation. PKA (activated by G\(_{s}\)) and PKC (activated by G\(_{s}\)) mediated phosphorylation of β-ARs directly alter receptor conformation, which impairs the interaction with G-proteins and attenuates further signal transduction [10]. This type of process is known as heterologous desensitization or non-agonist-specific desensitization, which indiscriminately phosphorylates both stimulated and unstimulated receptors [5, 49]. In contrast, the “agonist-specific” desensitization of β-ARs occurs via phosphorylation of six family members of GRKs [5, 50]. GRK’s are roughly divided into three subfamilies: rhodopsin kinases (GRK1 and 7); β-AR kinases (GRK2 and 3) and the GRK4 subfamily (GRKs 4, 5 and 6) [51]. Importantly, GRK2, 3 and 5 are present in the human myocardium, with GRKs 2 and 5 the most abundant expressed in the heart [52, 53]. The carboxy domain of GRK5 has a high affinity for phospholipids, allowing for its preferential membrane association, while GRK2 and 3 are mainly detected in the cytoplasm. A study in GRK2 knockout mice revealed that the mice die at days 12-15 of the embryonic period, which seems to be a consequence of hypoplasia and heart failure [54]. In contrast, GRK3 and GRK5 knockout animals are viable [55]. Contrary to GRK2 and GRK5 overexpression, overexpression of
GRK3 was associated with unaltered β-AR signalling and preserved hemodynamic responses to isoproterenol. Also functional responses to angiotensin II receptor stimulation was preserved, while myocardial thrombin signalling was disturbed in GRK3 overexpressed animals [56]. GRK2, also known as β-AR kinase 1, and GRK5 are translocated to the agonist-occupied β-AR and phosphorylate β-AR upon binding with activated Gs subunit [46, 47]. GRKs interaction with lipids and membrane proteins that control the subcellular localization of GRKs is mediated by its C-terminus [7]. The C-terminus of GRK contains a pleckstrin homology domain, which interacts with phosphatidylinositol 4,5-bisphosphate and free Gs subunits. Following these interactions, GRK translocates to the plasma membrane, where it phosphorylates activated β-ARs. GRK phosphorylates the C-terminal tail of β-AR, which allows binding of the cytosolic protein β-arrestin. β-AR phosphorylation via both agonist (i.e. via GRKs) as well as non-agonist-specific (i.e. via PKA and PKC) promotes binding of β-arrestin [6]. Binding of β-arrestin to GPCRs induces uncoupling of their G-proteins and prevents further activation of Gs [6]. In addition to β-arrestin's role in GPCR desensitization, it also acts as an adapter protein that targets GPCRs to clathrin-coated vesicles for endocytosis (Fig. 2). The internalization of the receptor is facilitated by β-arrestin binding, which has specific binding domains for clathrin and adaptor protein 2 (AP2). For an efficient receptor internalization the interaction of β-arrestin with other endocytic proteins such as: N-ethylmaleimide-sensitive fusion protein (NSF), ADP ribosylation factor 6 (ARF6) and its guanine nucleotide exchange factor ARNO is required [57]. The interaction between β-arrestin and the E3 ubiquitin ligase has shown to induce ubiquitination of β-arrestin. Moreover, ubiquitinated β-arrestin shows enhanced clathrin binding, GPCR interaction as well as scaffolding of mitogen-activated protein kinases, while nonubiquitinated β-arrestins are impaired in each of these functional interaction [58]. The internalization of GPCR is followed by post-endocytic sorting of internalized receptors for recycling or lysosomal degradation. Besides the role of β-arrestin in receptor desensitization and internalization, it has a role in modulating signalling directly as will be discussed below.

After short-term activation of the β-AR, dephosphorylation of the internalized receptor by protein phosphatase 2A (PP2A) might occur [59], leading to recycling of the internalized vesicles to the plasma membrane. In case of chronic AR stimulation, the internalized β-ARs will be degraded in the lysosomes leading to less β-ARs on the plasma membrane (Fig. 2) [60]. In HF β-AR levels decrease up to ~60% [9], while β-AR kinase 1 activity increases, reflecting severely diminished β-AR signalling [6, 47]. Another mechanism by which cells respond to sustained β-AR stimulation is up-regulation of β2-AR signalling through Gi, resulting in attenuation of the positive inotropic effects of β-AR stimulation [2]. Additionally, GRK activity is up-regulated in failing human hearts [61]. The second messenger cAMP and the catalytic subunit of PKA seem to be unaltered in human heart failure, while the expression of regulatory subunits was decreased [62]. In addition, in comparison to healthy individuals, a reduction of 40% of RI and approximately 30% of RII expression have been reported in the failing hearts [62]. Due to the fixed stoichiometry of PKA (2 regulatory-2 catalytic complex), the levels of regulatory subunit in a particular subunit will determine the local concentration of the catalytic subunit and subsequently phosphorylation of its targets. Since >75% of PKA-RII is targeted to certain intracellular compartments through its association with AKAPs [63], the reduction of RII in HF might affect PKA compartmentalization and phosphorylation [62]. Apart from the mismatch between the regulatory and catalytic PKA subunits in HF, major changes in PKA-AKAP association occur when the heart transforms from a healthy to failing state [64]. The expression of several AKAPs has been reported to be altered in the failing myocardium [64]. For example, AKAP1 which is involved in cell survival via B-cell lymphoma 2 (Bcl-2)-associated death promoter (BAD, i.e. regulator of programmed cell death) phosphorylation is decreased in the failing heart, while the relative population of AKAP18δ (targeting PKA to phospholamban), was significantly higher in failing heart [64, 65]. These alterations in AKAP expression might in turn affect PKA-mediated protein phosphorylation in the failing heart.

Another mechanism, which has been reported in patients with HF is alteration in PDEs levels. It has recently been demonstrated that PDE2 is almost twofold higher expressed in the failing myocardium [66]. This high levels of PDE2 was also observed in chronic β-AR stimulated rats, where it resulted in ~4 fold increase in cAMP hydrolytic activity and a diminished inotropic response upon catecholamine exposure [66]. Interestingly, a consequence of the down-regulation of β-AR signalling is the low PKA-mediated phosphorylation of inhibitor-1 (I-1). I-1 is a ~19 kDa protein that is widely expressed in mammalian tissue. I-1 inhibits protein phosphatase-1 (PP1) activity only when it is phosphorylated by PKA at Thr-35; this inhibition does not occur during reduced β-AR signalling [67, 68]. PP1 is able to dephosphorylate various myofilament proteins as well as Ca2+-handling proteins [17, 67]. In HF, a 77% decrease in I-1 phosphorylation as well as 55% reduction of I-1 protein level have been reported, indicating activation of PP1 [69]. Taken together, low β-AR signalling and activated protein phosphatase activity in response to chronic exposure to catecholamines may lead to a reduced downstream PKA-mediated protein phosphorylation, which affects myocardial inotropic, lusitropic and chronotropic function of the heart.

**Structural and metabolic alterations in HF**

For the maintenance of cardiac performance, the heart needs to produce high-energy phosphates. FFA β-oxidation by mitochondria provides more than 70% of total ATP, which is needed for proper cardiac performance [70]. The harmful effects of catecholamines on mechanical and biochemical properties of the heart were already known in the late 70’s [71]. Isolated rat hearts were exposed to a high dose of epinephrine, which resulted in high oxygen utilization for the same amount of work, and finally in a diminished myocardial ATP content in treated animals [71]. Most experimental and clinical studies suggest that metabolic remodelling occurs in the failing heart as a response to chronically altered workload and substrate bioavailability [72].
Fatty acid utilization is substantially down-regulated, whereas glucose oxidation is significantly accelerated in HF patients [73]. It has been reported that insulin and β-adrenergic stimulation share the same downstream signalling components, such as $G_\text{i1}$ [74], β-arrestin [75] and GRK2 [76]. Moreover, β$_2$-AR stimulation plays an important role in overall glucose homeostasis by acting on pancreatic islet hormone secretion, liver and muscle glucose transport mechanism [77]. Recently, a study by Qin Fu and co-workers reported that insulin can directly impair β-adrenergic signalling (via insulin receptor-β$_2$-AR signalling complex) in mice hearts [78]. Another studies in cardiomyocytes as well as skeletal muscles have shown that stimulation of β$_2$-ARs induce a significant increase in glucose uptake [77]. Noteworthy, long as well as short term stimulation of β$_2$-ARs have been associated with altered cardiac energy metabolism [77]. An important factor causing metabolic perturbation in HF is myocardial structural remodelling, such as fibrosis [79] and reduced capillary density [80], which may affect sufficient delivery of oxygen and substrates to the mitochondria. In addition, structural changes are responsible for impaired oxygen diffusion to the cardiac muscle cells. The low oxygen availability contributes to a decreased glucose and FFA oxidation in the failing heart, resulting in an insufficient supply of ATP. Sustained hyperactivated heart has also been associated with coronary vasoconstriction and elevated plasma FFA [81], which in time may interfere with glucose utilization and thereby cause oxygen wastage and formation of reactive oxygen spices (ROS) [82]. Chronic stimulation of β$_2$-ARs may lead to elevation of NADPH oxidase activity, which is accompanied by a greater ROS production, as well as p38MAPK phosphorylation (i.e. involved in cell migration, proliferation, differentiation an apoptosis). Moreover, β$_2$-AR activation has been shown to be involved in development of cardiac dilatation and dysfunction as well as augmented pro-inflammatory and pro-fibrotic signalling, while antioxidant treatment protected hearts against these abnormalities. This implies that ROS have detrimental effects upon chronic β$_2$-AR stimulation, which form a potential therapeutic target in cardiac failure [83].

**Therapeutic opportunities of HF**

Since the first landmark studies on treatment of HF patients with β-blockers in the 1970s [84] and the clinical trials in the 1990s [85], blockers of the β-AR system have become a mainstay in the treatment of HF. Regarding to the European Society of Cardiology, β-blockers therapy may leads to substantial improvement in ejection fraction. In addition, β-blockers are anti- ischaemic, are more effective in reducing the risk of sudden cardiac death and overall mortality [86]. The first generation of β-blockers were non-selective, meaning that they block both β$_1$ and β$_2$ adrenergic receptors. The second generation was designed to be more cardioselective, exhibiting greater affinity for β$_1$ - than β$_2$-AR. The third generation β-blockers are designed for patients with hypertension; these drugs are non-selective, as they also induce vasodilatation [87]. Multiple meta-analyses of β-blocker trials (table 1) have shown that β-blocker treatment consistently reduced mortality by 30%, lowered hospitalization to 40% and reduced sudden cardiac death by 38% [88, 89]. The difference between β-blockers might be caused by mechanism of function, including receptor affinity and hemodynamic properties of the β-blockers. For example, Carvedilol has a lower affinity for β$_1$-ARs than for β$_2$-ARs (affinity ratio of β$_1$ / β$_2$-ARs for Carvedilol =0.6), while Metoprolol and Nebivolol have a higher affinity for β$_1$-ARs than β$_2$-ARs (affinity ratio of β$_1$ / β$_2$-ARs for Metoprolol=6.0 and for Nebivolol=40.6). Bisoprolol has the highest β$_1$-AR affinity with an affinity ratio of β$_1$ / β$_2$=103 [90, 91]. Both Nebivolol and Carvedilol cause vasodilatation via nitric oxide, whereas Carvedilol also exerts antiproliferative and antioxidative effects and limits myocardial damage via gene inhibition [92]. However, there are a number of adverse side-effects reported with β-blocker therapy; for example:

**Figure 2.** Schematic representation of β-AR desensitization and degradation. β-AR phosphorylation via protein kinase A (PKA), protein kinase C (PKC) or G protein coupled receptor kinases (GRKs) promotes binding of β-arrestin to β-AR. The phosphorylated β-AR will be internalized. Function can be restored upon dephosphorylation via protein phosphatase 2A (PP2A). With sustained presence of β-AR agonist, the internalized β-AR remains internalized and will be degraded in the lysosome.
bradycardia, heart block, bronchospasm, fatigue, muscle cramps and sleep disturbances [93]. New drugs that specifically target the detrimental effects of β-AR hyperactivation are therefore highly needed. For example: ivabradine decreases heart rate to reduce myocardial oxygen consumption and increases myocardial perfusion via prolongation of the diastolic phase [94].

An alternative way of protecting the heart by modulating the β-AR pathway is through biased signalling [57]. According to the classical paradigm, β-AR is activated by ligand binding that leads to downstream signalling through Gαs, or this signalling is turned off by interaction of the receptor with β-arrestin, i.e. desensitization. However, recently the concept of biased signalling of β-AR (or GPCR in general) has come to the fore. It was shown that β-arrestins do not merely shut down signalling of the β-AR, but, have multiple downstream effector pathways, independent of G-protein signalling, in response to agonist stimulation [95-97]. β-arrestins function as adaptor proteins, linking β-AR to downstream signalling pathways, such as MAPK and Akt (reviewed in [57]). The binding of β-arrestins to β-AR is regulated by phosphorylation of the receptor, as discussed earlier. The protective role of the interaction between β-arrestin-β-AR has been shown in a transgenic mouse study overexpressing mutant β2-AR that is not able to recruit β-arrestins. These mice showed marked cardiomyocyte apoptosis and LV dilatation under sustained catecholamine exposure [98]. Several β-blockers such as alprenolol, bucindolol, nebivolol and carvedilol have been reported to induce β-arrestin stimulation, rather than inhibit G-protein coupled signalling [95, 99, 100]. Nobles and co-workers demonstrated that β-arrestin-biased ligand carvedilol, induced a different phosphorylation pattern (promoted by GRKs) of β2-AR than that of an unbiased, β2-AR agonist, isoproterenol [101]. Moreover, it has been shown that different GRK subtypes phosphorylate β-AR at different serine/threonine residues and that this differential phosphorylation pattern has functional implications. This mechanism is also known as the receptor phosphorylation barcode hypothesis. A recent study has also shown that carvedilol mediated β-arrestin1 stimulation leads to the induction of a specific miRNA profile, by interfering in miRNA processing [102]. This might also contribute to the additional cardioprotective effects of biased ligands in cardiac disease.

There are also promising data available for targeting directly the contractile machinery of the cardiomyocyte. For example, Omecartiv Mercarbil treatment revealed an increased contractility in rat isolated cardiomyocytes, without affecting the calcium handling of the cell [103]. This agent has the ability to modulate both the enzymatic as well as mechanical property of cardiac myosin. Although this drug showed significant contractile improvement, the increase in ATP turnover reported in this study and myocardial energetic state of the failing heart is however underestimated, since failing myocardium is an engine out of fuel (nicely reviewed by Stefan Neubauer [104]). Overall and taking into count the metabolic derangement in HF patients, Omecartiv Mercarbil is a promising therapeutic agent (which is now at phase 2 trial) to treat patients with or without β-blocker therapy.

Another drug that targets directly the contractile apparatus is Levosimendan. This drug is mainly regarded as calcium sensitizer in failing human myocardium, but it can potentially cause positive inotropic effects through PDE3 inhibition [105, 106]. A study in failing human ventricular muscle strips showed an increase of ventricular contraction to 40% of its basal values in a concentration-dependent manner [106]. This phenomenon was also demonstrated for lusitropic responses to Levosimendan, reported as reduction in relaxation time upon Levosimendan treatment. Interestingly, this agent shifted the concentration–response curve of isoprenaline to lower concentrations, indicative for sensitization of the β-AR response [106]. Clinical trials using Levosimendan in patients with HF have shown to increase cardiac output, decrease pulmonary wedge pressure and improve symptoms. Importantly, this agent provides a survival advantage in comparison to conventional treatment [107]. Overall, this drug has ample promising properties which can be used for management of HF.

Physical activity or exercise training has also been used as a therapeutic tool to treat patients with (mild) HF. Exercise training reduced circulating catecholamine levels [108]. Importantly, the peak VO2 (maximal oxygen consumption), which is a surrogate marker for the maximal cardiac output, improved with exercise training in HF patients, promoting a reduction of 28-35% hospitalization and mortality [109]. Regarding to ACCF/AHA guidelines, exercise training or regular physical activity is recommended as safe and beneficial treatment strategy for patients with mild cardiac failure [110].

Another attractive therapeutic target is inhibition of β-Adrenyl cyclase-5 (AC5). A higher life expectancy and improved cardiac function have been reported in AC5 knock-out mice [111]. Lowering GRK expression or activity in HF prevents and/or reverses functional as well as morphological impairments as has been reported in a mouse model with severe HF [7, 112]. GRK2−/− mice, with 50% less GRK2 expression in their hearts, exhibit increased inotropic reserve after isoproterenol stimulation compare to wild-type [113]. Moreover, adreno-associated virus serotype-6 (AAV6) was used to deliver βARKct (peptide that reduces GRK2 activity) in a post-myocardial infarction (MI) pig model, which resulted in sustained amelioration of cardiac function [114]. Similar results were obtained with long-term AAV6-βARKct delivery to a HF rat model, in which βARKct therapy was superior to β-blocker treatment [115]. Targeting the Gs subunit interaction with GRK2, which has been reported to be involved in both β1- and β2-AR desensitization and downregulation [116], prevented ventricular dysfunction after chronic βAR stimulation [117]. Finally, the importance of AKAPs and their interactions in several cellular processes and their dysregulation in disease, make AKAP complexes potential drug targets, specifically the PKA-AKAP interaction. Inhibition of PKA-AKAP interaction (with the HT31 peptide), using adenviral gene transfer in control Sprague-Dawley rats. The authors demonstrate a significant lower LV contractility in HT31 expressing animals, compared to non-infected control rats. This difference however, was abolished after isoprenaline (ISO, a non-selective β-AR
agonist) treatment [118]. Similarly, end-diastolic pressure in Ht31 rats was significantly reduced in Ht31-expressing animals, which normalized to control levels after ISO. Interestingly, the ejection fraction was unchanged at baseline, but the increase in EF after ISO administration was higher in Ht31 animals, compared to control [118]. In addition, Inhibition of PKA-AKAP interaction resulted in no changes in cTnI, PLN and ryanodine receptor phosphorylation in Ht31-expressing rat hearts at baseline compared to controls, while administration of a high dose of ISO resulted in an attenuated phosphorylation of these proteins in Ht31-expressing animals, compared to controls. [118]. Besides the increase in LV stroke volume as well as LV ejection fraction with high dose of isoproterenol in Ht31-expressing animals, a significant increase in LV contractility, which was similar to control values was detected in treated animals. It is important to mention that chronic exposure to catecholamines with inhibition in PKA-AKAP interaction needs to be elucidated in future studies. Taken together, β-blocker therapy is the first-line treatment for patients suffering from HF, while selectively targeting the specific actions of β-blocker including ACS, GRK2 inhibition and modifying PKA-AKAP interaction are promising future molecular approaches for treatment of HF.

HYPERTROPHIC CARDIOMYOPATHY

Hypertrophic cardiomyopathy (HCM) is an inherited cardiac disease, which is clinically defined as LV (asymmetrical, mainly septal) hypertrophy, which cannot be explained by abnormal loading conditions [119]. HCM has a prevalence of 0.2% in the general population [120]. The hypertrophied septum may narrow the blood flow from the LV to the aorta, referred to as “LV outflow tract obstruction (LVOTO)” [119]. Mutations in genes encoding sarcomeric proteins cause HCM. Similar to HF, in HCM a desensitized cardiac β-AR signalling pathway has been identified; however, the plasma catecholamine concentration remained unaltered in HCM patients [121]. A reduced number of β-receptor binding sites have been reported in patients suffering from HCM [122]. As a consequence, lower PKA-mediated protein phosphorylation was reported in myocardium from HCM patients [123], which is similar to observations in HF patients [62, 65, 124].

A recent study in 12-month old HCM mice harboring a R21C mutation in the TNNI3 gene (encoding cTnI), showed an attenuated lusitropic response upon ISO administration, [125]. This mutation prevents PKA-mediated phosphorylation of serine (Ser) 23/24 of cTnI, the main PKA phosphorylation target sites on cTnI. A blunted response to ISO has also been reported in transgenic mice carrying TPM1 and MYH7 mutations, encoding tropomyosin and myosin heavy chain, respectively [126, 127]. These studies suggest that an impaired β-adrenergic signalling cascade is a common feature in HCM. As discussed above, defects in PKA-mediated protein phosphorylation are known to impair Ca++-handling and contractile function of the myofilaments, which are important modifiers of disease progression [128-130]. However, in HCM little is known about physiological alterations, which might cause the disturbed β-AR signalling, leading to perturbed PKA phosphorylation of its targets. More research is necessary to investigate whether all PKA targets are equally affected by hampered β-AR activation in HCM. Also further research is needed to elucidate the precise role of AKAPs contribution in cardiac disease.

Treatment of HCM

Based on the European Society of Cardiology guidelines, non-vasodilating β-blockers, titrated to the maximum tolerated dose, are recommended as first-line therapy to improve symptoms in HCM patients with resting or provoked LVOTO [119]. High myofilament Ca++-sensitivity represents a central cellular pathomechanism in the initiation and progression of HCM, which may contribute to impaired diastolic function. Therefore, calcium desensitizers are attractive agents to improve myocardial relaxation and diastolic function in HCM [131]. Besides the calcium desensitizers, ion channel therapy (i.e. sodium, potassium and Ca++ channel blockers) and metabolic treatment have been reported to improve haemodynamics in HCM with resting or provoked LVOTO, who are intolerant or have contraindications for β-blockers [131]. One of the most performed invasive procedures to treat HCM patients with LVOTO is surgical ventricular myectomy. Taking together, HCM is an inherited cardiac disease, which is associated with disturbed β-AR signalling leading to reduced PKA-mediated phosphorylation and perturbed contractile function of the myocardium, which can be treated with β-blockers, Ca++- desensitizers, ion channel blockers and surgical myectomy operation.

CONCLUSION

Activation of the sympathetic system and release of catecholamines, with subsequent downstream activation of the β-ARs, represents a powerful mechanism whereby cardiac contractility and relaxation of contractions increase to preserve perfusion of the body during increased stress (e.g. exercise). Chronically high levels of catecholamines may lead to pathologic changes in the heart, including continued elevation of sympathetic tone and a progressive deterioration in cardiac structure and function. Moreover, the heart is able to protect itself from chronic stimulation of the β-ARs via desensitization and reduced availability of mainly β1-ARs. These processes may lead to diminished downstream signalling and consequently decreased PKA-mediated target protein phosphorylation. The beneficial effects of β-blockers have been attributed to their protective effect against chronic catecholamine stimulation of β1-β2-ARs, which results in “re-sensitization” of the β-AR signalling pathway. Besides the β-blockers, also other novel, but preclinical therapeutic treatments, such as ACS inhibitors, lowering GRK2
expression and targeting PKA-AKAP interaction may be of relevance to treat heart diseases in the future.

Acknowledgements
We acknowledge support from the Netherlands organization for scientific research (NWO; VIDI grant 91711344), the 7th Framework Program of the European Union (“BIG-HEART”, grant agreement 241577), and from ICIN-Netherlands Heart Institute.

Conflict of interest
None to be declared.

Contributions
A. N., V.S., D.K. and J.v.d.V. designed the manuscript. A. N., V.S., D.K. and J.v.d.V. wrote the manuscript. All authors discussed the review and commented on it.

REFERENCES
15 Sutherland EW and Rall TW. Formation of adenosine-3,5-phosphate (cyclic adenylyl) and its relation to the action of several neurohormones or hormones. Acta Endocrinol Suppl (Copenh) 1960;34(Suppl 50):171-4.


Insulin inhibits cardiac contractility by inducing a Gi-biased transduction of the beta-adrenergic receptor kinase: evidence that the kinase may act on multiple adenylate cyclase-coupled receptors. Proc Natl Acad Sci U S A 1986;83:6362-6.


Zakhary DR, Moravec CS, Stewart RW and Bond M. Protein kinase A (PKA)-dependent troponin-I phosphorylation and PKA regulatory subunits are decreased in human dilated cardiomyopathy. Circulation 1999;99:505-10.


86 McMurray JJ, Adamopoulos S, Anker SD, Auricchio A, Bohm M, Dickstein K et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 2012;33:1787-847.


102 Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K et al. Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. J Pharmacol Exp Ther 2010;335:572-9.


CHAPTER 2


Selective phosphorylation of PKA targets after β-adrenergic receptor stimulation impairs myofilament function in Mybpc3-targeted HCM mouse model

Aref Najafi
Vasco Sequeira
Michiel Helmes
Ilse AE Bollen
Max Goebel
Jessica A Regan
Lucie Carrier
Diederik WD Kuster
Jolanda van der Velden

CHAPTER 3

ABSTRACT

Aims
Hypertrophic cardiomyopathy (HCM) has been associated with reduced β-adrenergic (β-AR) receptor signaling, leading downstream to a low protein kinase A (PKA) mediated phosphorylation. It remained undefined whether all PKA targets will be affected similarly by diminished β-AR signaling in HCM. We aimed to investigate the role of β-AR signaling on regulating myofilament and calcium handling in an HCM mouse model harboring a gene mutation (G>A transition on the last nucleotide of exon 6) in Mybpc3 encoding cardiac myosin-binding protein C (cMyBP-C).

Methods and Results
Cardiomyocyte contractile properties as well as phosphorylation state were measured in left ventricular (LV) permeabilized and intact cardiomyocytes isolated from heterozygous (HET) or homozygous (KI) Mybpc3-targeted knock-in mice. Significant higher myofilament Ca\(^{2+}\)-sensitivity and passive tension was detected in KI mice, which were normalized after PKA treatment. Loaded intact cardiomyocyte force-sarcomere length (SL) relation was impaired in both HET and KI mice, suggesting a reduced length-dependent activation. Unloaded cardiomyocyte function revealed an impaired myofilament contractile response to isoprenaline (ISO) in KI, while the calcium handling response to ISO was maintained. This disparity was explained by an attenuated increase in cardiac troponin I (cTnI) phosphorylation in KI, while the increase in phospholamban (PLN) phosphorylation was maintained to wild-type (WT) values.

Conclusions
These data provide evidence that in the KI HCM mouse model β-AR stimulation leads to preferential PKA phosphorylation of PLN over cTnI, resulting in an impaired inotropic and lusitropic response.

Keywords
Calcium handling, contractility, Hypertrophic cardiomyopathy, β-adrenergic signaling

1. INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most frequently occurring inherited cardiac disorder, with a prevalence of 1:200 in the general population.\(^1\) HCM is characterized by (asymmetrical) left ventricular (LV) hypertrophy, diastolic dysfunction, cardiomyocyte disarray and cardiac fibrosis.\(^1,2\) Mutations in genes encoding sarcomeric proteins cause HCM.\(^2,4\) with mutations in genes MYH7 and MYBPC3 encoding myosin heavy chain (MHC) and cardiac myosin binding protein C (cMyBP-C) respectively being the most frequent cause of HCM.\(^5\) A desensitized cardiac β-adrenergic receptor (β-AR) signaling pathway,\(^6\) which is a hallmark of the failing heart has been identified in patients with HCM.\(^7\) Besides desensitization of the β-AR pathway, a reduced number of β-receptor binding sites have been reported in patients suffering from HCM.\(^8\) As a consequence, diminished downstream β-AR signaling may occur, leading to a lower protein kinase A (PKA)-mediated protein phosphorylation.\(^9,10\) PKA-mediated phosphorylation of myofilament proteins (e.g. cardiac troponin I (cTnI) and cMyBP-C) and Ca\(^{2+}\)-handling proteins (e.g. phospholamban (PLN)) mediate positive inotropic and lusitropic cardiac effects.\(^11,12\) In addition, phosphorylation of cTnI reduces myofilament Ca\(^{2+}\)-sensitivity and cMyBP-C phosphorylation accelerates cross-bridge cycling, both of which lead to enhancement of relaxation rate.\(^13,14\) The phosphorylation of PLN increases the activity of SR Ca\(^{2+}\)-adenosine triphosphatase (ATPase)-2 (SERCA2a) pump and thereby the rate of cardiac relaxation.\(^15\) Due to increased Ca\(^{2+}\) uptake upon β-AR stimulation, a higher SR Ca\(^{2+}\) content will be reached, which is available for the subsequent contraction, leading finally to an enhancement of the contractile performance.\(^16\)

The myofilament proteins cMyBP-C and cTnI are relatively highly phosphorylated in snap-frozen cardiac tissue from healthy individuals, while phosphorylation level is lower in heart tissue from patients with HCM.\(^5,18\) Similarly, low PLN phosphorylation levels have been reported in transgenic HCM mouse models and in patients with heart failure (HF).\(^14,16\) The functional consequences of disturbed β-AR signaling and reduced PKA-mediated phosphorylation are shown in the blunted increase in contractile response following isoproterenol administration, a β-AR agonist.\(^16\) β-AR mediated increases in inotropy and lusitropy requires the concerted phosphorylation of targets at multiple subcellular localizations. Spatial and temporal intracellular targeting of PKA is regulated through a set of A-kinase anchoring protein complexes (AKAPs).\(^17\)

We hypothesized that this coordinated PKA-mediated phosphorylation is disturbed in HCM disease pathology. Therefore, we investigated the effect of β-AR stimulation on myofilament and Ca\(^{2+}\)-handling in a HCM-associated mouse model. The experiments were performed in a Mybpc3-targeted knock-in mouse model, which carries a heterozygous (HET) or a homozygous (KI) Mybpc3 point mutation (G>A transition) on the last nucleotide of exon 6.\(^18\) The single mutation resulted in low levels of mRNA and less cMyBP-C protein expression,\(^19\) similar to earlier findings.

SELECTIVE PHOSPHORYLATION OF PKA TARGETS IN HCM

18
in humane HCM myocardial tissue. This mutation is associated with a severe HCM phenotype and poor prognosis in humans. The HET mice are comparable to pre-hypertrophic mutation carriers, with some degree of diastolic impairments, whereas the HCM phenotype (hypertrophy) is most evident in KI mice.

2. METHODS

2.1 Animal model
An expanded version of the methods section can be found in the online supplemental information. Experiments were performed in accordance with the Guide for the animal care and use committee of the VU University Medical Center (VUmc), and with approval of the Animal Care Committee of the VUmc (DEC-number FYS 11-02 and FYS 12-03), conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. In total, 58 mice (age 13 weeks at time of experiment) of both sexes were included in the study: Wild-type (WT, N=21), HET (N=19) and KI (N=18) Mybpc3-targeted knock-in mice on a Black Swiss genetic background.

2.2 Force measurements on single membrane-permeabilized myocytes
Cardiomyocytes were chemically permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and washed between a force transducer and a piezoelectric motor as described previously.

2.3 Titin isoform composition and phosphorylation
Titin isoform composition was analyzed in mice LV homogenates, using a vertical Hoefer SE600 gel system (Hoefer inc., USA) with a 1% agarose gel, as described previously. Changes in isoform composition were calculated as a ratio of N2BA/N2B. Human soleus (containing the ~3.7 mDa N2A isoform) was used as a standard. To determine phosphorylation of titin, the gel was stained with ProQ Diamond phosphostain (Molecular Probes) for 60 minutes, washed and subsequently stained with SYPRO Ruby (Molecular Probes). Titin phosphorylation is expressed as phosphorylated titin (i.e. summed N2B and N2BA values) over total titin protein level.

2.4 Myosin heavy chain isoform composition
Relative myosin heavy chain (MHC) content was determined in LV tissue from WT, HET and KI mice, as described previously. Briefly, MHC isoforms were separated on a 6% acrylamide resolving gel and a 3% stacking gel using a SE600 Hoefer gel system at 32 mA constant current. Human atrial homogenates, containing both α and β-MHC, were used as a standard. Subsequently, the gels were stained using SYPRO® Ruby and MHC isoforms were determined.

2.5 Intact cardiomyocyte isolation
Intact cardiomyocytes were isolated as described previously.

2.6 Force-sarcomere length relation
Intact cardiomyocytes were glued on a ~60° angled short glass fiber which was placed on a force transducer and piezo motor (long fiber). Once the cell was attached to the glass fibers, the cardiomyocyte was lifted up. Preload was applied by stretching the cardiomyocyte using the piezo motor, resulting in diastolic as well as systolic force-sarcomere length (FSL) relation. Finally, to normalize the slope of FSL, we calculated the ratio of the systolic and diastolic FSL relation, which is the amount of force produced per unit increase in sarcomere length (SL).

2.7 Cardiomyocyte shortening and Ca²⁺-handling
Unloaded cardiomyocyte measurements were performed as described previously. To investigate the β-AR stimulated signaling response of the cardiomyocyte, we stopped the continuous perfusion with Hepes buffer (HB) and immediately started perfusion with isoprenaline (ISO;100 nmol/L in HB, Sigma Aldrich; Supplementary material online, Table S1).

2.8 Protein phosphorylation analysis
Cardiac TnI phosphorylation status of frozen heart tissue samples as well of the isolated cardiomyocytes from baseline (BL) and ISO conditions were analyzed using one-dimensional SDS polyacrylamide-bound Mn²⁺-phos-tag gel electrophoresis and Western blotting, as described previously. It is important to note that LV frozen protein homogenates are from tissue which was immediately collected upon excision, while intact cardiomyocytes were enzymatically isolated and subsequently part was stored for protein homogenization and phos-tag analysis. Different patterns in cTnI phos-tag analysis between frozen LV and isolated cardiomyocytes might be caused by loss of phosphorylation during the enzymatic isolation procedure.

Western blotting analysis of site-specific phosphorylation of PLN at the serine (Ser)-16 site, PKA regulatory subunit II (PKA-RII) as well as catalytic (PKAcat) subunit expression and cMyBP-C Ser-302 phosphorylation levels were performed using antibody against PLN phospho Ser-16 (Badrilla, A010-12), PKA-RII (Abcam, ab38949), PKAcat subunit (Enzo Life Sciences, P22694), and Ser-302 phospho antibody (gift from Dr. Sakthivel Sadayappan, Loyola University Chicago), respectively. The signals were normalized for α-actinin (Sigma Aldrich, A7811), α-tropomyosin (Sigma Aldrich, T9283) and cMyBP-C (Santa Cruz Biotechnology, 137180 Clone E7). The pentameric as well as monomeric isoform of PLN were detected using the antibody against phosphorylated Ser-16 site. Both pentameric and monomeric isoforms of PLN signals were separately normalized for total PLN (Abcam, ab86930) as well as α-actinin levels and finally summed as one PLN phosphorylated Ser-16 value. The phosphorylation of threonine (Thr)-17 of PLN (Badrilla, A010-13AP) was also analyzed, which was normalized for total PLN expression.
PKA-RII, PKAc and cMyBP-C expression signals were normalized to α-actinin levels, while Ser-302 cMyBP-C phosphorylation level was corrected for α-tropomyosin.

2.9 Data Analysis
Data analysis and statistics were performed using Prism version 6.0 (Graphpad Software, Inc., La Jolla, CA). Data are presented as mean±SEM of all single cardiomyocytes per mouse group. Data were tested for normality by Kolmogorov-Smirnov normality test. When data were distributed normally and in case of testing one variable in more than 2 groups, samples were compared using 1-way ANOVA or in case of two or more variables, the data was compared using a 2-way ANOVA. If a significant value in 2-way ANOVA was detected, a Holm-Sidak’s multiple comparison post-hoc test was performed to identify the significance within the multiple groups. Significance was accepted when p<0.05.

3. RESULTS

3.1 Higher myofilament Ca\(^{2+}\)-sensitivity in KI permeabilized cardiomyocytes
To assess myofilament Ca\(^{2+}\)-sensitivity of force, force-Ca\(^{2+}\) relations were performed at SL 1.8 µm (Figure 1A) for all frozen LV samples. Myofilament Ca\(^{2+}\)-sensitivity was significantly higher in KI (i.e. lower EC\(_{50}\)) than in WT mice (Figures 1A, 1B and Table I), while EC\(_{50}\) value in HET mice failed to reach levels of statistical significance (p=0.06). As PKA-mediated phosphorylation of cTnI exerts a dominant regulatory role in the reduction of myofilament Ca\(^{2+}\)-sensitivity,\(^{21}\) phos-tag gel analyses were performed to study the distribution of phosphorylated cTnI forms (un-(0P), mono-(1P) and bis-(2P); Figure 1C) in LV frozen samples. As illustrated in Figure 1D, a significant reduction in 2P form of cTnI was detected in both HET and KI samples as well as a higher DP band particularly in KI mice. To investigate the underlying mechanism of hypophosphorylated cTnI in HCM mice, Western blot analysis was performed to detect PKA-RII and PKAc subunit expression. Interestingly, we found a significantly higher in PKA-RII expression in KI mice (Supplementary material online, Figure S1A), while PKAc expression did not differ among groups (Supplementary material online, Figure S1B). Consequently, PKA-RII/PKAc ratio was significantly higher in KI, compared to WT and HET counterparts (Figures 1E and 1F).

In order to confirm that the lower EC\(_{50}\) in KI cardiomyocytes is due to cTnI hypophosphorylation, permeabilized cardiomyocyte measurements were performed following incubation with exogenous PKA. PKA normalized the force-Ca\(^{2+}\) relation in KI to WT levels (Figures 1G and 1H). It is noteworthy that cells treated with exogenous PKA showed a higher maximal force (F\(_{\text{max}}\)) than untreated cells in all groups (Table I). In line with previous study,\(^{18}\) we also confirmed a significant reduction of cMyBP-C expression in KI mice, compared to WT and HET mice (Figures 1I and 1J).

3.2 Higher passive tension in permeabilized KI cardiomyocytes
Diastolic dysfunction is a early finding in our HCM-associated mouse model,\(^{12}\) which recapitulates the situation in both mutation carriers\(^{25}\) and manifest HCM patients.\(^{26}\) As passive stiffness of cardiomyocytes may underlie diastolic dysfunction, passive tension (F\(_{\text{pas}}\)) was assessed in membrane-permeabilized LV tissue at SL ranging from 1.8 to 2.4 µm. F\(_{\text{pas}}\) over the entire range of SL was significantly higher in KI cardiomyocytes than WT and HET (Figure 2A). To determine whether alterations in titin isoform composition may contribute to the higher F\(_{\text{pas}}\) in KI, the N2BA/N2B ratio was determined. No significant difference between the groups was found (Figure 2B). As phosphorylation of titin by PKA reduces F\(_{\text{pas}}\) analysis of full titin phosphorylation over total titin expression was performed using ProQ Diamond and SYPRO Ruby staining. No differences in total titin phosphorylation were found between the groups (Figure 2C). However exogenous PKA treatment normalized F\(_{\text{pas}}\) in KI cardiomyocytes to WT and HET levels (Figure 2D), indicating that PKA-mediated titin phosphorylation plays an important role in modulating F\(_{\text{pas}}\) in KI cardiomyocytes.

3.3 Length-dependent activation of the myofilaments
Length-dependent activation (LDA) of sarcomeres was determined by measuring Ca\(^{2+}\)-dependent myofilament force production at 1.8 and 2.2 µm SL (Figures 3A and 3B). An increase in SL resulted in a significantly higher F\(_{\text{pas}}\) and Ca\(^{2+}\)-sensitivity (Figures 3C, 3D and Table I) in all groups. Calculating the difference in EC\(_{50}\) between SL 1.8 and 2.2 µm (ΔEC\(_{50}\)) showed a lower LDA increase in myofilament Ca\(^{2+}\)-sensitivity in KI (ΔEC\(_{50}\)=0.38 ± 0.08 µmol/l) than in WT (ΔEC\(_{50}\)=0.56 ± 0.11 µmol/l), although the difference was not significant (p=0.3). Myofilament Ca\(^{2+}\)-sensitivity in KI cardiomyocytes normalized to WT levels at both SL after treatment with PKA (Figures 3E, 3F and Table I). Finally, stretching the cardiomyocyte from SL 1.8 to 2.2 µm revealed (before and after PKA treatment) a decrease in nH coefficient in all groups (Table I). No changes were detected in the maximal rate of tension redevelopment at saturating [Ca\(^{2+}\)*] (max k\(_{\text{tr}}\)) within mice groups (1.8 vs. 2.2 µm) and between the groups (Table I).

3.4 Reduced intact cardiomyocyte LDA response in KI mice
In addition to LDA measurements in membrane-permeabilized cardiomyocytes, we examined the LDA response in intact cardiomyocytes from WT, HET and KI mice. Glass fibers were used to attach an intact cardiomyocyte, stretch the cell and measure force (Figure 4A). In individual cardiomyocytes the force-SL (FSL) relationship was determined, using the slope of diastolic as well as systolic force against SL (i.e. force produced per unit increase in SL during rest and peak contraction; Figures 4B and 4C). The diastolic slope of FSL relation was comparable between the groups (Figure 4D), while the systolic slope of the FSL response was significantly reduced in both HET and KI mice (Figure 4E). Consequently, the systolic/diastolic ratio was significantly lower in both HET and KI groups, indicating a reduced LDA response in intact HET and KI cardiomyocytes.
3.5 Blunted cardiomyocyte response upon ISO treatment in KI mice

Exogenous PKA normalized the deficits in myofilament function in the membrane-permeabilized cardiomyocytes (Figures 1 and 3), indicative for disturbed β-AR signaling in our HCM-associated mouse model. To reveal the disturbed β-AR signaling and the contractile response upon β-AR stimulation, isolated intact cardiomyocyte function and phosphorylation were investigated by exposing the cells to the β-AR agonist ISO. Cardiomyocyte contractile function and Ca\(^{2+}\)-transients were monitored before and after ISO perfusion (Figure 5A, 5B and Table 2). Diastolic SL was significantly lower in KI compared to WT (Figure 5A and Table 2) as described previously. The maximal relengthening velocity, which is the maximum speed reached from peak shortening to baseline SL, was lower in HET mice compared to WT (Table 2), while other contractile and Ca\(^{2+}\)-handling parameters were similar among groups at BL.

Perfusion with ISO resulted in an increased relative cell shortening in all groups (Figure 5C and Table 2). Interestingly, cell shortening increased significantly in KI ISO-treated cardiomyocytes compared to untreated cells; however, the increase after ISO in KI was smaller than that in WT cardiomyocytes, indicating reduced inotropic response in KI upon ISO treatment (Figure 5C and Table 2). Similarly, an attenuated response to ISO was also seen in other contractile parameters of KI cardiomyocytes, such as cell shortening and maximal relengthening velocity (Figures 5D, 5E, 5F).
Table 1. Effects of exogenous protein kinase A (PKA) and sarcomere length (SL) on myofilament function.

<table>
<thead>
<tr>
<th></th>
<th>No PKA</th>
<th>With PKA</th>
<th>P&lt;sub&gt;interaction&lt;/sub&gt;</th>
<th>P&lt;sub&gt;influence&lt;/sub&gt;</th>
<th>P&lt;sub&gt;genotype&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>SL 1.8µm SL 2.2µm</td>
<td>SL 1.8µm SL 2.2µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;max&lt;/sub&gt; (kN/m²)</td>
<td>11.3 ±0.7</td>
<td>16.9 ± 1.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µmol/l)</td>
<td>3.8 ± 0.2</td>
<td>3.2 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nH</td>
<td>3.2 ± 0.2</td>
<td>2.6 ± 0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>max k&lt;sub&gt;t&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.1 ± 0.2</td>
<td>4.1 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HET</td>
<td>SL 1.8µm SL 2.2µm</td>
<td>SL 1.8µm SL 2.2µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;max&lt;/sub&gt; (kN/m²)</td>
<td>10.6 ± 1.0</td>
<td>14.8 ± 1.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µmol/l)</td>
<td>3.4 ± 0.1*</td>
<td>3.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nH</td>
<td>3.3 ± 0.2</td>
<td>2.6 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>max k&lt;sub&gt;t&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>4.1 ± 0.3</td>
<td>4.4 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>SL 1.8µm SL 2.2µm</td>
<td>SL 1.8µm SL 2.2µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;max&lt;/sub&gt; (kN/m²)</td>
<td>9.5 ± 1.0</td>
<td>17.7 ± 1.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µmol/l)</td>
<td>2.9 ± 0.1*</td>
<td>3.8 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nH</td>
<td>2.9 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>max k&lt;sub&gt;t&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3.9 ± 0.4</td>
<td>3.6 ± 0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P<sub>interaction</sub> F<sub>max</sub> (kN/m²) EC<sub>50</sub> (µmol/l) nH max k<sub>t</sub> (s<sup>-1</sup>)
No PKA 0.313 <0.0001 0.303 0.606
With PKA 0.313 0.040 0.121 0.081

WT, Wild-type (Number of mice (N)=9, number of cardiomyocytes (n)=28); HET, heterozygous knock-in mice (N=9, n=26); KI, homozygous knock-in mice (N=8, n=26); F<sub>max</sub> maximal generated tension; EC<sub>50</sub>, Ca<sup>2+</sup>-sensitivity (i.e. [Ca<sup>2+</sup>]<sub>50</sub>) at which 50% of F<sub>max</sub> is reached; nH, steepness of the force-Ca<sup>2+</sup> relations; max k<sub>t</sub>, maximal rate of tension redevelopment at saturating [Ca<sup>2+</sup>]; *p<0.05 vs. corresponding WT; #p<0.05 vs. corresponding HET; §p<0.05 vs. corresponding SL 1.8 µm in two-way ANOVA; ‡p<0.05 vs. corresponding untreated mice in two-way ANOVA.
**Figure 3.** Myofilament length-dependent activation. (A, B) Cardiomyocyte force development as a function of [Ca\(^{2+}\)] at sarcomere length (SL) 1.8 and 2.2 µm in wild-type (WT; Number of mice (N)=9, number of cardiomyocytes (n)=28) and homozygous knock-in (KI; N=8, n=26). (C) A similar increase in maximal force (F\(_{\text{max}}\)) as WT was found after myofilament stretch from 1.8 to 2.2 µm in both heterozygous knock-in (HET; N=9, n=26) and KI mice. (D) The length-dependent increase in Ca\(^{2+}\)-sensitivity of force development was slightly lower in KI mice. (E) At both SL F\(_{\text{max}}\) was higher in protein kinase A (PKA) treated cells compared to untreated cells in all groups (WT: N=9, n=29; HET: N=9, n=26; KI: N=8, n=24). (F) No differences in myofilament Ca\(^{2+}\)-sensitivity were present in PKA treated cells from the 3 groups. *p<0.05 vs. corresponding WT in two-way ANOVA; #p<0.05 vs. corresponding HET in two-way ANOVA; §p<0.05 vs. corresponding SL 1.8 µm in two-way ANOVA; ‡p<0.05 vs. corresponding untreated SL in two-way ANOVA.

3.6 Phospholamban, but not cTnI phosphorylation increased with ISO treatment in KI
As ISO treatment in KI led to a similar increase Ca\(^{2+}\)-transients as in WT, but had a blunted effect on cardiomyocyte function, we tested whether β-AR stimulation resulted in similar increases in phosphorylation of cTnI and PLN in the three experimental groups (Figures 6A-6F). The distribution of phosphorylated cTnI in untreated intact isolated cardiomyocytes did not differ between the groups (Supplementary material online, Figure S2). After ISO treatment the bisphosphorylated forms of cTnI increased significantly in WT and HET cardiomyocytes, while in KI mice the levels were not significantly different from untreated cells (Figures 6A and 6B). Similarly, relative cMyBP-C Ser-302 phosphorylation in WT mice increased significantly upon ISO exposure, while no alterations were detected in HET and KI cardiomyocytes with ISO treatment (Figures 6C and 6D). In contrast, PLN Ser-16 analysis revealed a significant and similar increase in PLN phosphorylation upon ISO in all groups (Figures 6E and 6F; p\(_{\text{treatment}}\)<0.05 in 2-way ANOVA). This finding is in line with our observation that Ser-16 phosphorylation of PLN in frozen LV tissue was not different between the groups (Supplementary material online, Figures S3A and S3B). Finally, no changes in Thr-17 phosphorylation, which is a Ca\(^{2+}\)-calmodulin kinase II (CaMKII) target was similar between groups before and after ISO treatment (Figures 6F and 6G).

**DISCUSSION**

PKA-mediated phosphorylation of several myofilament and Ca\(^{2+}\) handling proteins is an important event upon β-AR stimulation, mediating positive inotropic and lusitropic cardiac effects.\(^{11,12}\) A reduced phosphorylation of PKA targets has been reported in mouse models and patients with manifest HCM.\(^{10,14,15}\) Moreover, low cTnI and cMyBP-C phosphorylation has been reported in myocardium of human HCM patients,\(^{9,10}\) while recent research has shown that PLN phosphorylation is preserved in patients and a HCM-associated mouse model.\(^{22,28}\) It is therefore not completely known whether a general down-regulation of PKA activity, or changes in PKA localization occur in HCM. Our main findings showed: 1) High myofilament Ca\(^{2+}\) sensitivity and passive tension in membrane-permeabilized KI cardiomyocytes which were normalized to WT values after treatment with exogenous PKA; 2) Lower phosphorylated forms of cTnI, but preserved PLN phosphorylation in KI compared to WT hearts; 3) a blunted increase in cardiomyocyte contractility, but a preserved increase in Ca\(^{2+}\)-transients in KI compared to WT treated with ISO and 4) failure to increase cTnI phosphorylation in KI upon ISO treatment.
Table 2. Blunted myofilament response upon β-AR stimulation in KI cardiomyocytes.

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>ISO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic SL</td>
<td>1.73 ±0.01</td>
<td>1.78 ±0.01</td>
</tr>
<tr>
<td>Shortening velocity (µm/s)</td>
<td>-3.6 ±0.3</td>
<td>-2.1 ±0.2</td>
</tr>
<tr>
<td>Amplitude shortening (µm)</td>
<td>0.06 ±0.01</td>
<td>0.03 ±0.01</td>
</tr>
<tr>
<td>Sarcomere shortening (%</td>
<td>4.6 ±0.4</td>
<td>3.3 ±0.4</td>
</tr>
<tr>
<td>Time to peak shortening (s)</td>
<td>0.053 ±0.009</td>
<td>0.056 ±0.002</td>
</tr>
<tr>
<td>Time to 50% relengthening (s)</td>
<td>0.122 ±0.018</td>
<td>0.143 ±0.009</td>
</tr>
<tr>
<td>Max. Relengthening velocity (µm/s)</td>
<td>2.24 ±0.37</td>
<td>0.86 ±0.17</td>
</tr>
<tr>
<td><strong>HET</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic SL</td>
<td>3.6 ±0.3</td>
<td>1.77 ±0.01</td>
</tr>
<tr>
<td>Shortening velocity (µm/s)</td>
<td>-8.5 ±0.7</td>
<td>-6.9 ±0.8</td>
</tr>
<tr>
<td>Amplitude shortening (µm)</td>
<td>0.20 ±0.07</td>
<td>0.16 ±0.07</td>
</tr>
<tr>
<td>Sarcomere shortening (%</td>
<td>9.8 ±0.6</td>
<td>11.2 ±0.6</td>
</tr>
<tr>
<td>Time to peak shortening (s)</td>
<td>1.46 ±0.06</td>
<td>1.32 ±0.10</td>
</tr>
<tr>
<td>Time to 50% relengthening (s)</td>
<td>0.143 ±0.011</td>
<td>0.24 ±0.08</td>
</tr>
<tr>
<td>Max. Relengthening velocity (µm/s)</td>
<td>1.70 ±0.27</td>
<td>0.86 ±0.17</td>
</tr>
<tr>
<td><strong>KI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic SL</td>
<td>1.65 ±0.01</td>
<td>1.65 ±0.01</td>
</tr>
<tr>
<td>Shortening velocity (µm/s)</td>
<td>-3.3 ±0.3</td>
<td>-2.5 ±0.5</td>
</tr>
<tr>
<td>Amplitude shortening (µm)</td>
<td>0.09 ±0.03</td>
<td>0.06 ±0.03</td>
</tr>
<tr>
<td>Sarcomere shortening (%</td>
<td>5.7 ±0.4</td>
<td>5.7 ±0.4</td>
</tr>
<tr>
<td>Time to peak shortening (s)</td>
<td>0.57 ±0.04</td>
<td>0.57 ±0.04</td>
</tr>
<tr>
<td>Time to 50% relengthening (s)</td>
<td>0.117 ±0.009</td>
<td>0.122 ±0.018</td>
</tr>
<tr>
<td>Max. Relengthening velocity (µm/s)</td>
<td>2.61 ±0.35</td>
<td>0.86 ±0.17</td>
</tr>
</tbody>
</table>

WT, Wild-type (Number of mice (N)=11, number of cardiomyocytes (n)=56); HET, heterozygous knock-in (N=9, n=55) mice; KI, homozygous knock-in mice (N=10, n=51); BL, baseline; ISO, isoprenaline treated cardiomyocytes; SL, sarcomere length; Max., maximal; *p<0.05 vs. corresponding WT; #p<0.05 vs. corresponding HET in two-way ANOVA; ‡p<0.05 vs. corresponding untreated mice in two-way ANOVA.

Figure 4. Reduced Length-dependent increase in force development per unit increase in SL in both homozygous knock-in (KI) as well as heterozygous knock-in (HET), compared to wild-type (WT) mice. (A) Intact cardiomyocytes were glued between a force transducer and a piezomotor. (B) By stretching a WT cardiomyocyte, we detect a linear force-SL (FSL) relation during diastole as well as systole. (C) An increase in both diastolic and systolic FSL was observed upon stretch. (D) No differences were found in diastolic slope of force-SL relation among groups. (E) In contrast, the systolic slope of FSL was significantly reduced in both HET (Number of mice (N)=9, number of cardiomyocytes (n=55)) and KI (N=10, n=51) compared to WT (N=11, n=113) cells. (F) As a consequence, the ratio of systolic over diastolic slope of the FSL relation was significantly lower in both HET and KI than in WT mice. *p<0.05 vs. WT in one-way ANOVA.
CHAPTER 3

SELECTIVE PHOSPHORYLATION OF PKA TARGETS IN HCM

while PLN phosphorylation increased similar to WT. Overall, our study shows that in KI cardiomyocytes β-AR stimulation preferentially phosphorylates PLN over cTnI resulting in a blunted myofilament inotropic and lusitropic response.

4.1 Myofilament Ca\(^{2+}\)-sensitivity and \(F_{\text{pas}}\) corrected after PKA

Due to improved genetic screening, sarcomere mutations can be detected in the early stage of manifest HCM as well as in family members carrying the mutation. Diastolic dysfunction has been found in mutation carriers even before the onset of hypertrophy in humans.\(^{22}\) A study in the same HCM mice carrying the same mutation as in the present study reported diastolic dysfunction both in HET and KI mice.\(^{22}\) At the myofilament level, diastolic dysfunction can be caused by increased Ca\(^{2+}\)-sensitivity and/or increased \(F_{\text{pas}}\) both of which were observed in KI mice in the present study (Figures 3D and 2A, respectively). In contrast to previous observation in PKA pre-treated trabeculae,\(^{22}\) the higher myofilament Ca\(^{2+}\)-sensitivity in KI in the present study was restored to WT level after PKA treatment (Figure 1D and Table I), indicating that the difference in Ca\(^{2+}\)-sensitivity between KI and WT was mainly due to hypophosphorylation of the sarcomeric proteins rather than the sarcomeric mutation itself. In the present study, the distribution of phosphorylated forms of cTnI was significantly lower in both HET and KI mouse hearts (Figure 1D). It is well established that phosphorylation of cardiac sarcomeric proteins, in particular cTnI, by PKA reduces myofilament Ca\(^ {2+}\)-sensitivity.\(^ {29}\) PKA pre-treatment corrected the myofilament Ca\(^{2+}\)-sensitivity particularly in KI cardiomyocytes to values observed in WT cells. PKA had no additional effect in WT cells (Figure 1H) which can be explained by the relatively high level of bisphosphorylated cTnI (Figures 1C and 1D) in WT hearts. Wijnker et al.\(^ {30,31}\) revealed that the PKA-mediated decrease in myofilament Ca\(^ {2+}\)-sensitivity was maximal at ~55% bisphosphorylated cTnI. Similar findings were observed in PKA-treated cardiomyocytes obtained from HCM patients who underwent myectomy surgery as well as studies in transgenic HCM mouse models.\(^ {9,12}\) PKA pre-treatment also normalized the high \(F_{\text{pas}}\) in KI to the WT values, which might indicate a lower PKA-mediated phosphorylation of titin in KI mice. It is known that PKA (as well as protein kinase G) phosphorylation of titin reduce the resting tension, while protein kinase C titin phosphorylation predominantly increases passive tension.\(^ {27}\) A drawback of the current technique is that we could not distinguish between different phosphorylation sites and we therefore cannot exclude that compensatory changes (e.g. more PKC and less PKA phosphorylation of titin) might contribute to changes in passive stiffness in our mouse model (Figure 2C). A possibly explanation for the reduced myofilament phosphorylation and consequently higher myofilament Ca\(^ {2+}\)-sensitivity and \(F_{\text{pas}}\) may reside in impaired β-AR signaling cascade. We have previously reported the increased myofilament Ca\(^ {2+}\)-sensitivity in patients with HCM in which the majority developed less force compared with donor.\(^ {9,32,33}\) In the present study we showed a higher passive tension and high basal activation at low calcium levels in KI cardiomyocytes, which might affect myocardial relaxation and reduces the Frank-starling reserve. The low resting SL in intact KI cardiomyocytes before and after ISO treatment is.

Figure 5. Impaired cardiomyocyte function in homozygous knock-in (KI) upon isoprenaline (ISO). (A, B) Cardiomyocyte shortening as well as Ca\(^ {2+}\) transients at baseline (BL) and during ISO treatment are illustrated. (C) Less cell shortening was detected in KI (Number of mice (N)=10, number of cardiomyocytes (n)=45) compared to wild-type (WT; N=11, n=56) after ISO perfusion. (D, E) The cardiomyocyte shortening as well as maximal (Max.) relengthening velocity were significantly slower in KI compared to WT after ISO. (F, G) The Ca\(^ {2+}\) amplitude as well as the Max. kinetics of Ca\(^ {2+}\) release and reuptake were comparable between the groups. (H) Similarly, the relaxation index tau, obtained from the Ca\(^ {2+}\) transient remained unchanged in both heterozygous knock-in (HET; N=9, n=42) and KI mice compared to WT. *p<0.05 vs. corresponding WT in two-way ANOVA; †p<0.05 vs. corresponding HET in two-way ANOVA; ‡p<0.05 vs. corresponding untreated mice in two-way ANOVA.
SELECTIVE PHOSPHORYLATION OF PKA TARGETS IN HCM

CHAPTER 3

Figure 6. Cardiomyocyte phospholamban (PLN), but not cardiac troponin I (cTnI) and cardiac myosin binding protein C (cMyBP-C) phosphorylation, increased with isoprenaline (ISO) treatment. (A) The distribution of phosphorylated forms of cTnI is illustrated using phos-tag acrylamide gel. (B) The relative fraction of bisphosphorylated form (2P) of cTnI was significantly increased in wild-type (WT; Number of mice (N)=10) and heterozygous knock-in (HET; N=9) upon ISO treatment, compared to untreated cardiomyocytes, which was set as 1. (C) Western blot analysis of cMyBP-C, normalized for the loading control α-tropomyosin was performed after ISO treatment. (D) A significant increase in serine (Ser)-302 phosphorylation of cMyBP-C was particularly found in WT (N=2; HET;N=2; KI;N=4) cardiomyocytes upon ISO treatment, compared to untreated cardiomyocytes, which was set as 1. (E) The phosphorylated isoforms of PLN Ser-16 in untreated cells and after ISO are separately normalized for total PLN and the loading control α-actinin and finally summed up as one PLN phosphorylation value per mouse. (F) In contrast, PLN Ser-16 phosphorylation in KI (N=9) was similar to the WT (N=12) and HET (N=8) values after ISO (p<0.05 in 2-way ANOVA). No changes in threonine (Thr)-17 phosphorylation has been found in KI (N=7) and HET (N=7) mice before and after ISO, compared to corresponding WT (N=11). ‡p<0.05 vs. corresponding untreated mice in two-way ANOVA.

indicative for high basal activation. A possible contributor to the high resting tension is residual cross-bridge attachment. In a cMyBP-C knock-out mouse model, the cross-bridge inhibitor 2,3-butanedione monoxime (BDM) was shown to increase diastolic SL, indicating that some residual cross-bridge attachment occurs in permeabilized cardiomyocytes at low Ca\textsuperscript{2+}. We have also recently shown that the cross-bridge component might be a more important component to resting or passive tension than previously thought. Noteworthy, the myofilament contraction does not seem to be affected by low basal SL in KI mice as it there was no correlation between resting SL and sarcomere shortening, demonstrated in the supplementary Figure S7.

In addition to KI mice, it should also be mentioned that despite reduced expression of cMyBP-C in KI mice, maximal ktr was similar in permeabilized cardiomyocytes from KI and WT mice. Reduced cMyBP-C expression will release the brake on myosin heads and increase the rate of force development. However, small disease-related changes in MHC-isoform may also alter the rate of force development. MHC isoform analysis (Supplementary material online, Figures S6A and S6B) revealed a significant increase in β-MHC (i.e. slow isoform of MHC) in KI mice, indicating that an effect of reduced cMyBP-C expression on speed of force redevelopment may be counterbalanced by the expression of slow β-MHC isoform in KI.

Normalization of myofilament Ca\textsuperscript{2+}-sensitivity and F\textsubscript{pas} after PKA incubation in KI mice indicates a relatively limited direct contribution of cMyBP-C in regulation of myofilament Ca\textsuperscript{2+}-sensitivity and passive tension. There is still much debate on the role that cMyBP-C plays in modulating Ca\textsuperscript{2+}-sensitivity. This is illustrated by the diverse effects seen in the different cMyBP-C protein null mouse models: Harris et al. showed that Ca\textsuperscript{2+}-sensitivity decreased in cMyBP-C KO; Fraysse et al. showed in a different model without functional cMyBP-C that Ca\textsuperscript{2+}-sensitivity increased, while Barefield et al. showed no change in Ca\textsuperscript{2+}-sensitivity. In a number of studies from our group, we have shown that in cardiac tissue from HCM patients, low levels of cMyBP-C do not directly mediate myofilament calcium sensitivity, but changes in Ca\textsuperscript{2+}-sensitivity
occur due to secondary remodelling. The most important determinant in the regulation of myofilament Ca\(^{2+}\)-sensitivity of force development is phosphorylation of myofilament targets, in particular cTnI.\(^{26}\) This is supported by our data that show that restoring the low cTnI phosphorylation level can normalize myofilament Ca\(^{2+}\)-sensitivity and that this overrules any role that cMyBP-C might play in modulating Ca\(^{2+}\)-sensitivity.

Noteworthy, our previous study in patients with a MYBPC3 missense mutation revealed a similar pattern of protein phosphorylation as the truncated MYBPC3 mutation group,\(^{41}\) indicating that the β-AR pathway might be affected to a similar degree, regardless of the type of mutation. Recent findings also seem to indicate that the distinction between missense and truncation mutations is not as black and white as previously thought. Even in patients with missense mutations, a reduced level of cMyBP-C was found, which might be caused by decreased stability of the mutant protein.\(^{45,46}\) Besides carrying different types of mutations, it is important to note that the protein expression level may contribute to the disturbed β-AR pathway. Previously, we provided evidence that mutant protein may impair sarcomere function at ~38% expression,\(^{6}\) which emphasizes the importance of studying the WT and mutant protein level at which myofilament protein phosphorylation and performance is impaired.

### 4.2 Reduced force-Sarcomere length relation in HET and KI cardiomyocytes

In KI mice the slope of diastolic FSL relation of the intact isolated cardiomyocytes seems to be unaltered (Figure 4D), while a shorter SL (Figure 5A) and a higher passive tension in these permeabilized cells was measured (Figure 2A), compared to WT. The difference in passive tension between KI and WT mice became apparent at SL ≥2.0 µm in permeabilized cardiomyocytes.

In loaded intact cells, we were able to determine the FSL relation at SL range ~1.7-1.9 µm. Another factor that might influence our measurements is the inability to normalize absolute force development for cross-sectional area in an intact cell. To circumvent this problem we used the ratio of systolic over diastolic slope of FSL relation.

We calculate the slope of diastolic and systolic FSL relations obtained by varying the preload (i.e. stretching the single intact cell). It has been previously reported that the FSL relation in mammals is near linear at SL 1.85-2.05 µm.\(^{21,42}\) Our loaded intact cardiomyocytes revealed a significantly lower systolic/diastolic ratio of FSL relation in both HET and KI than in WT mice within the range of studied SL, suggesting less tension production per unit increase in SL (Figure 4F). This finding in loaded intact cells is in contrast to our permeabilized cardiomyocyte data where an increase in SL (from 1.8 to 2.2 µm) elevated the maximal generated tension similarly in all groups (Figures 2C, 2E and Table 1). The difference between LDA response in intact and permeabilized cardiomyocytes could be explained by the presence of intracellular Ca\(^{2+}\)-handling machinery, while in permeabilized cardiomyocytes all organelles are removed and the [Ca\(^{2+}\)] is manually regulated. We observed in membrane-permeabilized cardiomyocytes a higher F\(\text{max}\) in KI isolated cells. However, the diastolic FSL relation in intact cardiomyocytes remained unchanged between the groups. Several factors, such as temperature, pH and experiment medium may explain differences between membrane-permeabilized and intact cardiomyocytes. Noteworthy, the SL range in intact cardiomyocytes was lower (~1.7-1.9 µm) compared to permeabilized cardiomyocytes (1.8-2.2 µm), which also might underline the discrepancy between intact and permeabilized cells. It is important to mention that in permeabilized cardiomyocytes of KI mice we observed that myofilaments were sensitized to Ca\(^{2+}\) ions (i.e. myofilament effect), while in intact cardiomyocytes we did not find changes in Ca\(^{2+}\)-handling (i.e. sarcoplasmic reticulum effect) compared to WT. This suggests that the Ca\(^{2+}\) handling function in KI is preserved, while there is an increased myofilament Ca\(^{2+}\) sensitivity. Taking together, our data indicate a mildly blunted increase in myofilament Ca\(^{2+}\)-sensitivity upon stretch in membrane-permeabilized cardiomyocytes from KI mice, while intact cardiomyocytes showed an impaired length-dependent cardiomyocyte activation in HET and KI mice.

### 4.3 Blunted cardiomyocyte response to ISO with preserved Ca\(^{2+}\) handling

β-AR receptor stimulation activates adenylyl cyclase, which catalyzes the synthesis of cyclic adenosine monophosphate (cAMP) from ATP. cAMP binds to regulatory subunits of the PKA holoenzyme complex, leading to conformational changes and release of the PKAcat subunit of PKA. Our results indicate blunted myofilament β-AR response to ISO in KI. However, the increase in Ca\(^{2+}\) transients after ISO was unchanged between groups. This suggests preferential β-AR signaling to the SR over the myofilament in KI. It is important to note that the baseline phosphorylation state was similar between KI and WT mice (Figure 6), indicating that the baseline inotropic state is probably comparable between the groups, and leading subsequently in KI with ISO treatment to an attenuated β-AR response.

A reduced response upon β-AR stimulation was also reported in hearts from cMyBP-C knock-out mice.\(^{45}\) Another study in Mybpc3 transgenic mouse model, using PKA-dependent back phosphorylation assay revealed a reduced cTnI as well as PLN phosphorylation.\(^{44}\) In the present study we found a preserved Ca\(^{2+}\)-handling despite an attenuated cTnI and Ser302 cMyBP-C phosphorylation after ISO treatment in KI mice. In our experiments, we did not see reduced PKAcat expression (Supplementary material online, Figure S1B), but observed selective targeting of PKA to the SR. Interestingly, our findings in KI mice regarding the attenuated myofilament response upon ISO did not appear to result in alterations in Ca\(^{2+}\)-transients of the cell. This might appear in contrast to a recent study that indicated that the inability to phosphorylate cTnI and lower myofilament Ca\(^{2+}\)-buffering following ISO treatment, results in a decreased Ca\(^{2+}\)-transient amplitude.\(^{45}\) The latter study was performed in mice carrying TnI which could not be phosphorylated by PKA. The lack of an effect on calcium transient in our KI model may be explained by the degree of cTnI phosphorylation, which may still be sufficiently high.
The differential response upon β-AR stimulation in the present study between the myofilament and Ca\(^{2+}\)-handling systems was confirmed by protein phosphorylation data (preserved PLN phosphorylation increase, attenuated cTnI phosphorylation increase).

### 4.4 Low myofilament phosphorylation upon ISO

Selective phosphorylation of PKA targets was also confirmed by our protein analyses. In LV frozen tissue, a significantly lower cTnI, but a preserved PLN phosphorylation was seen in KI compared with WT (Supplementary material online, Figures S3A and S3B). This suggests that phosphorylation of PLN is preferential over cTnI in vivo. Upon ISO treatment, cTnI bisphosphorylation and cMyBP-C Ser-302 failed to increase in KI cardiomyocytes, while PLN phosphorylation increased to an equal extent in all groups (Figures 6A-6F).

It should also be noted that cTnI phosphorylation in KI was significantly lower in LV frozen tissue compared with WT, which was not seen in the isolated cells at baseline. This experimental discrepancy is caused by loss of protein phosphorylation, particularly in WT cardiomyocytes, during the isolation procedure. This artefact is an important factor to keep in mind when performing isolated cardiomyocyte experiments. The LV frozen data in our opinion closely reflect the situation in the in vivo heart. The experiments performed in the intact isolated cells exposed to ISO, were used to assess the ability of β-adrenergic receptor stimulation to signal downstream to cTnI and PLN.

The selective signalling to one compartment (SR) over another (myofilaments) could be explained by changes in PKA localization. This localization of PKA is achieved by docking of the PKA complex to these AKAPs occurs through the regulatory subunits. Two major forms of PKA complexes have been described. These two types differ in their structure in the regulatory subunit of the PKA complex, termed as PKA-R1 (PKA regulatory subunit I) and PKA-R2,\(^{46}\) both of which bind to the same PKAcatal subunit. A higher PKA-R1, but unchanged PKAcatal subunit expression has been reported in failing human myocardial tissue,\(^{47}\) indicating that during HF PKA subunits may not be expressed in matched quantities. Similarly, in the present study a higher PKA-R2 and unchanged PKAcatal expression was detected in KI mice (Supplementary material online, Figure S1).

Another important phenomenon is high oxidative stress in patients with HCM,\(^{48}\) which may result in oxidation of many proteins, among which the regulatory subunit of PKA. A study by Brennan et al.\(^{49}\) showed that oxidation of the two regulatory RI units in response to H\(_2\)O\(_2\), may cause a subcellular translocation and activation of the kinase, resulting in phosphorylation of the target proteins. The translocation as the authors indicated is partially mediated by the oxidized form of the kinase having an enhanced affinity for alpha-myosin heavy chain, which serves as an AKAP and localizes PKA to the myofilament substrates. Taken together, selective phosphorylation of PLN over myofilament proteins and preserved Ca\(^{2+}\)-homeostasis in KI after ISO treatment, may possibly be caused by an maintained PKA localization towards the SR, resulting in a preserved PLN, but lower myofilament protein phosphorylation (Supplementary material online, Figure S4).

### 4.5 Conclusion and clinical implications

The HCM-associated mouse model carrying a G>A transition on the last nucleotide of exon 6 showed similar cellular changes as observed in previous studies in cardiac samples from HCM patients with a known mutation in the gene encoding cMyBP-C such as reduced expression of mutant protein, low PKA-mediated myofilament protein phosphorylation, increased Ca\(^{2+}\)-sensitivity and perturbed length-dependent activation.\(^{50}\) Our study confirmed an increased Ca\(^{2+}\)-sensitivity and reduced cardiomyocyte contractile performance in KI mice. It seems that a post-translational modification (such as reduced myofilament protein phosphorylation) in KI mice and HCM patients with MYBPC3 mutations is one of the major mechanisms of altered myocardial function. The data presented in this study show that PKA hypophosphorylation can explain the observed contractile dysfunction. However, simply increasing PKA phosphorylation by reduced desensitization or other strategies is likely not sufficient as the localization of PKA signalling needs to be addressed. AKAPs are important modulators in spatial and temporal control of cellular signalling. Alterations in AKAP interaction and/or expression are associated with cardiac pathologies.\(^{51}\) This suggests that complete understanding of AKAP-PKA complexes and their function in heart disease might be interesting targets for treatment of cardiomyopathy.\(^{52}\)

#### Funding

We acknowledge support from the Netherlands organization for scientific research (NWO; VIDI grant 91711344), the 7th Framework Program of the European Union (“BIG-HEART”, grant agreement 241577), and from ICIN-Netherlands Heart Institute.

#### Acknowledgments

We are grateful to Saskia Schlossarek for organizing and supplying us with Mybpc3-targeted KI mice and for valuable support. We thank Sakthivel Sadayappan from department of Cell and Molecular Physiology, Health Sciences Division, Loyola University Chicago for supplying us with cMyBP-C Ser-302 antibody.

#### Conflict of interest

None declared.
REFERENCES


CHAPTER 3

SELECTIVE PHOSPHORYLATION OF PKA TARGETS IN HCM


47. Han YS, Arroyo J, Ogut O. Human heart failure is accompanied by altered protein kinase a subunit expression and post-translational state. Arch Biochem Biophys. 2013;538:25-33


SUPPLEMENTAL INFORMATION

DETAILED METHODS

Experimental solutions for force measurements in permeabilized single cardiomyocyte

The composition of all solutions was calculated based on a computer program similar to that described previously. The pH of all solutions was adjusted to 7.1 at 15°C by KOH and ionic strength was adjusted to 180 mmol/L with KCl. The relaxing solution contained 2 mmol/L free Mg2+, 1 mmol/L MgATP, 20 mmol/L EGTA, 10 mmol/L BES and 14.5 mmol/L PCR (P7936, Sigma). Several activating solutions were prepared: 1) Ca2+-activating solution consisted of 2 mmol/L free Mg2+, 1 mmol/L MgATP, 20 mmol/L EGTA, 10 mmol/L BES and 32 μmol/L free Ca2+. Ca2+-activating solutions with lower free [Ca2+] were obtained by mixing of the Ca2+-activating and relaxing solutions and assuming an apparent stability constant of the Ca2+-EGTA complex of 10^7.31.) Isometric force measurements in permeabilized cardiomyocytes

Small left ventricular (LV) tissue sample was thawed in relaxing solution (5.95 mmol/L NaCl, 6.04 mmol/L MgCl2, 1 mmol/L EGTA, 139.6 mmol/L KCl, 10 mmol/L Imidazole, pH 7.0) and cardiomyocytes were mechanically isolated by tissue disruption. The cells were chemically permeabilized by incubation for 5 minutes in relaxing solution containing 0.5% (v/v) Triton-X100 and glued between a force transducer and a piezoelectric motor. Isometric force measurements were performed at maximal and submaximal [Ca2+] (ranging from 1 to 30 μmol/L) and at various sarcomere lengths (SLs) ranging from 1.8 to 2.4 μm. Passive force (FPas) was determined by shortening the myocyte in a relaxing solution (10−6 μmol/L) by 30% of its length. Maximal developed force (Fmax) was determined by activating the cardiomyocyte at saturating [Ca2+] (30 μmol/L), generating a total force value (Ftot): Fpass was obtained by subtracting Frest from Ftot (i.e. Frest=Ftot−Fpass). Maximal tension (in mN/m2) was calculated as Fmax normalized to cross-sectional area of the cardiomyocytes. Force-Ca2+ relations were fit to a modified Hill equation (nH) and myofilament Ca2+-sensitivity was denoted as EC50 ([Ca2+] at which half of Fmax was reached). A single exponential was fitted to the rate constant of force redevelopment at maximal actuation (Ktr). The length-dependent increase in myofilament Ca2+-sensitivity upon an increase in sarcomere length is based on the difference in EC50 at sarcomere lengths of 1.8 and 2.2 μm (ΔEC50). Additional force measurements were performed following exogenous protein kinase A (PKA) treatment of permeabilized cardiomyocytes for 40 minutes at 20°C in relaxing solution containing the catalytic subunit of PKA (100 U incubation, Sigma). It is important to note that for the passive force measurement we used separate cardiomyocytes for PKA treatment instead of re-using the myocyte used for baseline Fmax detection. This means that the passive force measurements (i.e. without and with PKA) in contrast to the length-dependent force data are unpaired, resulting in a small variation between cardiomyocytes without versus with PKA treatment.

Data analysis isometric force measurements

Force-Ca2+ relations were fit by a non-linear fit procedure to a modified Hill equation using KaleidaGraph version 3.6 (Synergy Software, Reading, PA) as follows:

\[ P(\text{Ca}^{2+}) / P_i = [\text{Ca}^{2+}]^{nH} / (K^{nH} + [\text{Ca}^{2+}]^{nH}) \]

Where \( P \) is steady-state force, \( P_i \) denotes the steady isometric force at either saturating [Ca2+], \( nH \) describes the steepness of the relationship, and \( K \) represents the [Ca2+] at which force is half-maximal (0.5 x \( P_i \)). Myofilament Ca2+-sensitivity is denoted as EC50.

Titin isoform composition analysis

Titin isoform composition analysis was performed in mice LV homogenates using a vertical Hoefer SE600 gel system (Hoefer Inc., USA) with a 1% agarose gel, as previously described. Samples were normalized against myosin heavy chain content and loaded in triplicates on a 1% agarose gel (1% w/v Sea Kem Gold agarose (BioWhittaker Cell Biology Products, USA), 30% v/v glycerol, 50 mmol/L Tris-base, 0.384 M glycine, and 0.1% w/v SDS). Samples were run at 30 mA constant current, stained with SYPRO® Ruby Protein Gel Stain (Invitrogen, USA) and visualized using a LAS3300 Imager (Fujifilm, Japan). The integrated optical density of T1 titin (full-length titin isoforms N2BA and N2B), T2 titin (degradation products) and myosin heavy chain were determined for each sample, using AIDA Image analyzer software version 4.21 (Raytest GmbH, Germany). Changes in isoform composition were calculated as a ratio of N2BA:N2B. Human soleus (containing the ~3.7 mDa N2A isoform) was used as a standard.

Isolation of intact ventricular cardiomyocytes

Mice were euthanized with isoflurane and hearts were quickly removed and rinsed in cold perfusion buffer (PB, online table 1) at pH 7.46. The heart was then cannuilated via the aorta to the Langendorff apparatus and perfused for 3 min with PB at 37°C. Thereafter, the heart was perfused with digestion buffer containing liberase blendzyme-2, trypsin and 50 μmol/L CaCl2, for a period of 8–11 min. The right ventricle and atria were removed, and the LV was cut into small pieces and triturated with a plastic Pasteur pipette for 3 min in stopping buffer solution containing bovine calf serum. CaCl2 was stepwise added to the cell suspension to a final concentration of 1.8 mmol/L. The stopping buffer was washed out by using Hepes buffer (HB) several times. Cardiomyocytes were used within the first 6h of isolation. All experiments were performed under approval of the Animal Care and Use Committee of the VU University.
To identify the phosphorylation state of the cardiomyocytes, we incubated the cardiomyocyte suspension for 20 min at room temperature with HB (i.e. baseline (BL)) and with 100 nmol/L isoprenaline (ISO). The cells were centrifuged at 3000 rpm for 30 seconds and the supernatant was removed. Finally, cardiomyocytes were stored at -80°C for protein analysis.

**Force-sarcomere length relation**
A ~60° angled short glass fiber was placed on a force transducer and piezo motor (long fiber). The long fiber was displaced by the force transducer (model 406A; Aurora Scientific) to 4-5 different lengths. Force was measured by calibrating force to glass fiber displacement, which was attached to the piezo motor. In addition, the displacement was corrected for piezo movement. Subsequently, the tip of the glass fibers was treated with IonOptix pre-coat. After 30 min, the tip of the glass fiber was shortly placed in the IonOptix MyoTak®. A rod shape cell on poly-HEMA (Sigma-Aldrich) coated coverslip (to prevent cell attachment to the bottom of the chamber) was selected. During 1Hz field stimulation and at room temperature, glass fibers were carefully lowered onto opposite ends of the cardiomyocyte. The cell was pressed between the coverslip and both glass fibers. Once the cell was attached to the glass fibers, the cardiomyocyte was lifted up. Preload was applied by stretching the cardiomyocyte using the piezo motor, resulting in diastolic as well as systolic force. Finally, to normalize the slope for cross-sectional area (CSA), we calculated the ratio of the systolic and diastolic force-sarcomere length (FSL) relation, which is the amount of force produced per unit increase in sarcomere length (SL).

**Unloaded Cardiomyocyte shortening and Ca²⁺-transient**
Unloaded cardiomyocyte measurements were performed as described previously. Cardiomyocytes were incubated in HEPES buffer (HB) containing 1 µmol/L Fura-2-AM (Life technologies) for 15 min and rinsed for 10 min in HB. Cardiomyocytes were placed into a 37 °C temperature-controlled chamber with platinum electrodes to electrically stimulate cells. Cardiomyocytes without spontaneous contraction were selected for analysis and perfused continuously with HB containing 1.8 mmol/L CaCl₂. Cells were monitored for 3-5 min to assess BL sarcomere shortening and Ca²⁺ transients upon field stimulation (1Hz, 4 ms, 20 V) using a video-based sarcomere length (SL) detection system (IonOptix corporation). The cells loaded with Fura-2-AM were excited at 340 and 380 nm with 510 nm emissions. The F340/F380 ratio was used as a measure of cytosolic [Ca²⁺]. To investigate the β-AR stimulated signaling response of the cardiomyocyte, we stopped the continuous perfusion with HB and immediately started perfusion with isoprenaline (ISO;100 nmol/L in HB, Sigma Aldrich; Online Table 1). 100 µmol/L ascorbic acid (Sigma, A5960) was added to the ISO solution to protect ISO from degradation.
Figure S3. Phospholamban (PLN) phosphorylation in left ventricular (LV) frozen tissue. (A) The monomeric as well as pentameric phosphorylation signal were normalized for both total PLN and α-actinin value. (B) Although in KI LV tissue an intense monomeric PLN phosphorylation signal was detected, after combining the pentameric isoform and normalizing the signal for total PLN and α-Actinin, we found no significant differences of serine (Ser)-16 phosphorylation of PLN between wild-type (WT; N=8), heterozygous knock-in (HET; N=10) and homozygous knock-in (KI, p=0.06; N=7) mice.

Figure S4. Schematic illustration of the selective phosphorylation of phospholamban (PLN). The calcium handling protein PLN is selectively phosphorylated over myofilament protein cTnI in HCM heart.
Figure S5. Phosphorylation of cardiac myosin binding protein C (cMyBP-C), cardiac troponin T (cTnT) and myosin light chain-2 (MLC2). (A) cMyBP-C, cTnT and MLC2 phosphorylation was investigated using ProQ diamond and SYPRO Ruby staining. (B) The total phosphorylation of cMyBP-C was unchanged in HET (N=4), compared to WT (N=4) mice, while no phosphorylation signal was detected for KI (N=5) mice. (C) cTnT as well as MLC2 (D) was similar in all groups.

Figure S6. Myosin heavy chain (MHC) isoform analysis in cardiac tissue from WT (N=10), HET (N=10) and KI (N=7) mice. (A) MHC isoforms were separated on a 6% acrylamide gel and a 3% stacking gel. (B) KI animals expressed significantly higher β-MHC isoform than WT and HET mice. *p<0.05 vs. WT in one-way ANOVA; #p<0.05 vs. HET in one-way ANOVA.

Figure S7. The relation between diastolic sarcomere length and sarcomere shortening in WT (A), HET (B) and KI (C) mice. A significant correlation was found in WT untreated cells and HET cardiomyocytes treated with ISO.
### SUPPLEMENTAL TABLE

<table>
<thead>
<tr>
<th></th>
<th>Perfusion Buffer (PB)</th>
<th>Hepes Buffer (HB)</th>
<th>Digestion Buffer</th>
<th>HB+ ISO</th>
<th>Stop Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (mM)</td>
<td>113</td>
<td>134</td>
<td>113</td>
<td>134</td>
<td>113</td>
</tr>
<tr>
<td>KCl (mM)</td>
<td>4.7</td>
<td>5.0</td>
<td>4.7</td>
<td>5.0</td>
<td>4.7</td>
</tr>
<tr>
<td>KH₂PO₄ (mM)</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄ (mM)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>NaHCO₃ (mM)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KHCO₃</td>
<td>10</td>
<td></td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES (mM)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine (mM)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄ (mM)</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>5.5</td>
<td>11.1</td>
<td>5.5</td>
<td>11.1</td>
<td>5.5</td>
</tr>
<tr>
<td>2,3-Butanedione mono-</td>
<td>5.0</td>
<td></td>
<td>5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl₂(mM)</td>
<td>1.8</td>
<td>0.05</td>
<td>1.8</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Isoprenaline (nM)</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liberase blendenzyme 2 (mg/ml)</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsine (mg/ml)</td>
<td>0.1</td>
<td></td>
<td></td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>Bovine calf serum (ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid (µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### SUPPLEMENTAL REFERENCES

Sexual dimorphic response to exercise in hypertrophic cardiomyopathy-associated MYBPC3-targeted knock-in mice

Aref Najafi
Saskia Schlossarek
Elza D van Deel
Nikki van den Heuvel
Ahmet Güçlü
Max Goebel
Diederik WD Kuster
Lucie Carrier
Jolanda van der Velden

Pflugers Arch. 2015;467:1303-17
ABSTRACT

Hypertrophic cardiomyopathy (HCM), the most common genetic cardiac disorder, is frequently caused by mutations in MYBPC3, encoding cardiac myosin binding protein C (cMyBP-C). Moreover, HCM is the leading cause of sudden cardiac death (SCD) in young athletes. Interestingly, SCD is more likely to occur in male than in female athletes. However, the pathophysiological mechanisms leading to sex-specific differences are poorly understood. Therefore, we studied the effect of sex and exercise on functional properties of the heart and sarcomeres in mice carrying MYBPC3 point mutation (G>A transition in exon 6) associated with human HCM. Echocardiography followed by isometric force measurements in left ventricular (LV) membrane-permeabilized cardiomyocytes were performed in wild-type (WT) and heterozygous (HET) knock-in mice of both sex (N=5 per group) in sedentary mice and mice that underwent a 8-week voluntary wheel running exercise protocol. Isometric force measurements in single cardiomyocytes revealed a lower maximal force generation ($F_{\text{max}}$) of the sarcomeres in male sedentary HET (13.0 ±1.1 kN/m$^2$) compared to corresponding WT (18.4 ±1.8 kN/m$^2$) male mice. Exercise induced a higher $F_{\text{max}}$ in HET male mice, while it did not affect HET females. Interestingly, a low cardiac troponin I bisphosphorylation, increased myofilament Ca$^{2+}$-sensitivity and LV hypertrophy were particularly observed in exercised HET females. In conclusion, in sedentary animals contractile differences are seen between male and female HET mice. Male and female HET hearts adapted differently to a voluntary exercise protocol, indicating that physiological stimuli elicit a sexually dimorphic cardiac response in heterozygous MYBPC3-targeted knock-in mice.

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiac disorder, affecting 1:500 individuals in the general population [19, 30]. It is characterized by (asymmetrical) left ventricular (LV) hypertrophy, cardiomyocyte disarray, cardiac fibrosis, diastolic dysfunction and ventricular arrhythmias [1, 19, 20]. Mutations are most frequently found in genes that encode sarcomeric proteins, which is why HCM has been referred to as disease of the sarcomere. Approximately 40% of identified HCM mutations are found in the MYBPC3 gene, which encodes the thick filament protein cardiac myosin binding protein-C (cMyBP-C) [1]. HCM is an autosomal-dominant disorder and most affected individuals carry a heterozygous mutation. Homozygous mutations are rare and are associated with a more severe phenotype [48]. Although the fact that mutations in sarcomeric genes cause HCM is well accepted, the genotype-phenotype relationship is not straightforward. Some mutation carriers remain without symptoms during their entire life, while others die of acute cardiac arrest or develop cardiac disease at relatively young age [31, 37].

HCM is the most frequent cause of sudden cardiac death (SCD) in young athletes [32], suggesting that the addition of high levels of exercise on an existing arrhythmogenic substrate is detrimental. According to the European Society of Cardiology, people diagnosed with HCM are recommended not to participate in competitive sports [45]. Strikingly, SCD is significantly more likely to occur in male than in female athletes [32], although the mechanism is unclear.

In various clinical studies sex-related differences in HCM disease onset and severity have been reported [26, 28, 41, 43]. In a HCM patient population females were underrepresented, suggesting females are less likely to develop symptoms [41]. Moreover, at disease onset females were on average 9 years older than males [41]. Another study in patients with obstructive HCM revealed that interventricular septum thickness is lower in females compared to males [28]. A recent study in MYBPC3 mutation carriers showed larger atrial and ventricular dimensions and lower fractional shortening (FS) in males than in females [43]. The pathophysiological mechanisms, which underlie the differences between males and females remain to be investigated.

In the present study we investigated the effect of sex and exercise on whole heart and sarcomere function in a MYBPC3-targeted knock-in (KI) mouse model, which carries a heterozygous MYBPC3 point mutation (G>A transition on the last nucleotide of exon 6) [15, 48, 59]. This mutation is associated with a severe HCM phenotype and bad prognosis in humans [48].
MATERIALS AND METHODS

Exercise protocol and echocardiography
Experiments were performed in accordance with the Guide for the animal care and use committee of the VU University Medical Center (VUMc), and with approval of the Animal Care Committee of the VUMc (DEC-number FYS12-03). In total, forty 5-week-old mice were included in the study. Wild-type (WT) and heterozygous (HET) MYBPC3-targeted KI mice, in Black Swiss genetic background of both sex were divided in sedentary and exercise groups (N=5 each group) [15, 48, 59]. The exercise groups were exposed to an 8-week voluntary wheel running protocol and the running distances were monitored in individual animals.

Sedentary and exercised mice (after the 8-week exercise protocol) of ~13 weeks of age were sedated with 4% isoflurane and ventilated with 0.25 l/min O2 and 0.45 l/min air. To maintain the anesthetized condition, mice were ventilated with ~2% isoflurane and identical O2 and air flow rates. Subsequently, 2D-echocardiographic recording (Siemens Acuson Sequioa) was performed of the LV short axis at mid-papillary level. LV end-diastolic as well as end-systolic lumen was determined from the M-mode images. Fractional shortening (FS) was calculated from the short axis M-mode images as 100% x (LV end-diastolic lumen – LV end-systolic lumen)/LV end-diastolic lumen. After the echocardiogram the LV (including the interventricular septum) and right ventricle (RV) were weighted and subsequently stored in liquid nitrogen (N2) [53].

Cardiomyocyte measurements
Cardiomyocytes from sedentary and exercised mice were mechanically isolated as described previously [3, 51, 56]. A small piece of the (frozen) LV was thawed in relaxation solution [5], cells were mechanically isolated and incubated for 5 minutes in relaxation solution with 0.5% (v/v) Triton X-100 to remove all membranes. Subsequently, the membrane-permeabilized myocytes were washed twice in relaxation solution, and a single cardiomyocyte was attached with silicone adhesive between a force transducer and a piezoelectric motor. The myocyte was transferred to a mechanical chamber and released in a Karlish solution. The chamber was then connected to a force transducer (Grass FT03) and the force redevelopment to a single exponential function [54]. Subsequently, the myocyte was moved to the relaxation solution, in which a second slack was performed to determine passive force development (Fpas). Maximal Ca2+-activated tension (Fmax) was calculated by subtracting Fpas from the total force at saturating [Ca2+]. All force values were normalized for myocyte cross-sectional area.

Phosphorylation of cardiac troponin T
The unphosphorylated and phosphorylated species of cardiac troponin T (cTnT) and myosin light chain 2 (MLC2) was determined using ProQ Diamond phosphostaining as described previously [64]. The phosphorylation signal is normalized to the corresponding SYPRO Ruby-stained protein signal.

Data analysis
Data analysis and statistics were performed using Prism version 6.0 (Graphpad Software, Inc., La Jolla, CA). The myofilament Ca2+-sensitivity (expressed as EC50, Fmax, Fpas, max ktr and the steepness of the force-Ca2+ relation (nH)) were presented as means±SEM of all single cardiomyocytes per mouse group to account for the variation between individual cardiomyocytes. Echocardiographical and protein analysis data are indicated as means±SEM per group. Data were tested for normality by using Komogorov-Smirnov normality test. Overall, the data showed a normal distribution and they were compared using 2-way ANOVA followed by a Holm-Sidak Post-hoc test. P<0.05 was considered significant.

RESULTS
No major cardiac remodelling in male and female HET mice at 13 weeks of age
Functional cardiac and myofilament parameters of sedentary male and female WT and HET mice are displayed in Table 1. In accordance with a previous study in male HET mice of 3 months of age [59], FS and LV dimensions and weights did not differ from WT groups in both male and female HET MYBPC3-targeted KI mice. A significantly lower heart rate (HR) was detected in
both HET male and female mice compared to corresponding WT group (Table 1; \( P < 0.05 \) in 2-way ANOVA).

Echocardiographic analysis revealed no changes in FS, but a significantly lower LV end-systolic and end-diastolic lumen in females compared to genotype-matched males (Table 1; \( P < 0.05 \) in 2-way ANOVA). In addition, female mice had lower LV and RV weight (normalized to tibia length; Table 1) compared to males of the same genotype. When heart weight was normalized to body weight, no differences were seen between the different groups (Supplemental Figures 1A, B).

**Figure 1.** Sarcomere function in WT and HET mice. Force-Ca\(^{2+}\) relations were measured at a sarcomere length of 2.2 µm in single skinned cardiomyocytes from WT and HET sedentary mice. Force at submaximal [Ca\(^{2+}\)] was normalized to the maximal force and plotted against [Ca\(^{2+}\)]. The relative force-Ca\(^{2+}\) relation was measured in male (A) and female (B) mice from WT and HET. A significantly different pattern in maximal generated tension (F\(_{\text{max}}\)) was observed in HET male and female (C, \( P < 0.05 \) in 2-way ANOVA). A higher myofilament Ca\(^{2+}\)-sensitivity of force development, i.e. lower EC50 value was detected in HET mice (D, \( P < 0.05 \) in 2-way ANOVA). *P<0.05 male HET vs. male WT; †P<0.05 female HET vs. male HET; ‡P<0.05 female HET vs. male WT; #P<0.05 in 2-way ANOVA.

**Table 1.** Cardiac characteristics of sedentary mice

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>HET</td>
</tr>
<tr>
<td>FS (W)</td>
<td>33 ± 2</td>
<td>31 ± 0.5</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>580 ±13</td>
<td>507 ± 18'</td>
</tr>
<tr>
<td>LV weight (mg/cm)</td>
<td>64 ± 1</td>
<td>62 ± 1</td>
</tr>
<tr>
<td>RV weight (mg/cm)</td>
<td>16 ± 1</td>
<td>15 ± 0.4</td>
</tr>
<tr>
<td>ESL (mm)</td>
<td>2.7 ± 0.2</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>EDL (mm)</td>
<td>4.0 ± 0.2</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>ED posterior wall (mm)</td>
<td>0.88 ± 0.04</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>F(_{\text{max}}) (kN/m(^2))</td>
<td>18.4 ± 1.8</td>
<td>13.0 ± 0.9'</td>
</tr>
<tr>
<td>F(_{\text{pas}}) (kN/m(^2))</td>
<td>1.8 ± 0.2</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>EC(_{50}) (µmol/l)</td>
<td>2.9 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>nH max k(_{\text{tr}}) (s(^{-1}))</td>
<td>3.2 ± 0.3</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>max k(_{\text{tr}}) (s(^{-1}))</td>
<td>6.2 ± 0.5</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

WT, Wild-type; HET, heterozygous KI mice; FS, fractional shortening; HR, heart rate; bpm, beats per minute; LV, left ventricular; RV, right ventricular; cardiac weights were corrected by tibia length (in cm); LV ESL, left ventricular end-systolic lumen; LV EDL, left ventricular end-diastolic lumen; ED posterior wall, end-diastolic posterior wall thickness; F\(_{\text{max}}\), maximal generated tension; F\(_{\text{pas}}\), passive tension; EC\(_{50}\), Ca\(^{2+}\)-sensitivity (i.e. [Ca\(^{2+}\)] at which 50% of F\(_{\text{max}}\) is reached); nH, steepness of the force-Ca\(^{2+}\) relations; max k\(_{\text{tr}}\), maximal rate of tension redevelopment at saturating [Ca\(^{2+}\)]. N, number of mice; n, number of cardiomyocytes. *P<0.05 HET vs. corresponding WT; †P<0.05 female vs. corresponding male in post-test analyses.
Reduced maximal force generating capacity in male HET sedentary mice

Force was measured at various \([Ca^{2+}]\) in single cardiomyocytes, normalized to maximum force and fitted to the Hill equation (Figures 1A, B). Cardiomyocytes from male HET mice developed lower \(F_{\max}\) than male WT mice (Figure 1C). This drop in \(F_{\max}\) was not seen in female HET mice. Consequently, \(F_{\max}\) was significantly lower in HET males than in females (Figure 1C). In addition, while no differences were found in \(F_{\max}\) between WT and HET groups, a significantly higher \(F_{\max}\) was observed in females compared to male counterparts (Table 1, \(P_{sex}<0.05\) in 2-way ANOVA).

Myofilament \(Ca^{2+}\)-sensitivity (expressed as \(EC_{50}\); Figure 1D), \(nH\) and max \(k_{tr}\) did not differ between male and females (Table 1). Cardiomyocytes from HET mice were more sensitive to \(Ca^{2+}\) than from WT mice as illustrated by a small leftward shift of the force-\(Ca^{2+}\) relation (Figures 1A, B) and a lower \(EC_{50}\) (Figure 1D, \(P_{sex}<0.05\) in 2-way ANOVA). No difference was found in \(nH\) between WT and HET groups, while max \(k_{tr}\) tended to be lower in HET compared to WT male and females (Table 1).

Sex-dependent effects of exercise on cardiac contractile performance and remodelling

We evaluated the effects of 8-week voluntary wheel running in male and female mice on in vivo heart performance as well as sarcomere function. A significantly longer distance was run by females than males (Supplemental Figures 2A, B, \(P_{sex}<0.05\) in 2-way ANOVA), which was mostly ascribed to the significantly higher distance ran by HET females. The longer distance in HET females was detected from day 6 of the exercise protocol (Supplemental Figures 2E, \(P_{sex}<0.05\) in 2-way ANOVA).

Cardiac contractile performance and dimensions were altered in male and female mice following the 8-week exercise protocol (Figure 2; Table 2). Female HET mice showed a hypertrophic response to the exercise protocol, as indicated by the 15% increase in LV weight (normalized to tibia length) compared with sedentary HET females. This LV hypertrophy was even more pronounced when normalized to body weight (Supplemental Figure 1B). A similar increase (13%) was found in RV weight in exercised HET female mice compared to corresponding sedentary mice (Table 2). This response to exercise was not seen in WT females (Figure 2A, C; Table 2). In contrast to the exercise-mediated hypertrophy in HET females, no significant changes in LV and RV weights were detected between exercised and sedentary male mice (Figures 2A, C; Table 2). In addition, end-diastolic posterior wall thickness remained unchanged in all groups (Tables 1, 2).

In addition, FS was differentially affected in male and female HET mice (Figure 2D, \(P_{sex}<0.05\)); while FS tended to increase in exercised HET males (P=0.06 in Post-hoc test) compared with sedentary HET males, no remarkable changes in FS were detected in females after exercise (Figure 2D).

Increased \(Ca^{2+}\)-sensitivity after exercise in both sexes

At the sarcomere level, exercise caused a marked increase in myofilament \(Ca^{2+}\)-sensitivity in WT male and female mice compared to their sedentary counterparts (Figure 3A), while \(F_{\max}\), \(F_{\text{pas}}\), \(nH\) and max \(k_{tr}\) did not differ between exercised and sedentary WT animals (Figures 3B; Table 2). In HET mice, exercise was associated with a sex-dependent change in sarcomere function: only in females did exercise increase myofilament \(Ca^{2+}\)-sensitivity (Figure 3C), while in males \(F_{\max}\) (Figure 3D) and \(F_{\text{pas}}\) (Table 2) were significantly higher after exercise. No significant differences were observed in \(nH\) and max \(k_{tr}\) of exercised WT and HET mice (Table 2).
Table 2. Cardiac characteristics of exercised compared to sedentary mice

<table>
<thead>
<tr>
<th></th>
<th>Male&lt;sub&gt;sex&lt;/sub&gt;</th>
<th>Female&lt;sub&gt;sex&lt;/sub&gt;</th>
<th>P&lt;sub&gt;interaction&lt;/sub&gt;</th>
<th>P&lt;sub&gt;exercise&lt;/sub&gt;</th>
<th>P&lt;sub&gt;sex&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS (%)</td>
<td>33 ± 2</td>
<td>36 ± 3</td>
<td>0.45</td>
<td>0.39</td>
<td>0.95</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>580 ± 13</td>
<td>575 ± 20</td>
<td>0.82</td>
<td>0.56</td>
<td>0.91</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28.1 ± 0.6</td>
<td>21.0 ± 0.1</td>
<td>20.9 ± 0.6</td>
<td>0.04</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LV weight (mg/cm)</td>
<td>64 ± 1</td>
<td>49 ± 2</td>
<td>0.01</td>
<td>0.74</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RV weight (mg/cm)</td>
<td>16 ± 1</td>
<td>11 ± 1</td>
<td>0.15</td>
<td>0.26</td>
<td>0.0001</td>
</tr>
<tr>
<td>ESL (mm)</td>
<td>2.7 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>0.07</td>
<td>0.30</td>
</tr>
<tr>
<td>EDL (mm)</td>
<td>4.0 ± 0.2</td>
<td>3.5 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>0.003</td>
<td>0.06</td>
</tr>
<tr>
<td>ED posterior wall (mm)</td>
<td>0.88 ± 0.04</td>
<td>0.98 ± 0.13</td>
<td>0.90 ± 0.10</td>
<td>0.52</td>
<td>0.29</td>
</tr>
<tr>
<td>F&lt;sub&gt;max&lt;/sub&gt; (kN/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>18.4 ± 1.9</td>
<td>17.5 ± 1.4</td>
<td>19.2 ± 2.3</td>
<td>0.78</td>
<td>0.15</td>
</tr>
<tr>
<td>F&lt;sub&gt;pass&lt;/sub&gt; (kN/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>1.8 ± 0.2</td>
<td>2.8 ± 0.5</td>
<td>2.7 ± 0.3</td>
<td>0.24</td>
<td>0.42</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt; (µmol/l)</td>
<td>2.9 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>2.3 ± 0.1</td>
<td>0.95</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>nH</td>
<td>3.2 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.3</td>
<td>0.36</td>
<td>0.15</td>
</tr>
<tr>
<td>max ktr (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>6.2 ± 0.5</td>
<td>5.9 ± 0.8</td>
<td>6.0 ± 0.4</td>
<td>0.60</td>
<td>0.67</td>
</tr>
</tbody>
</table>

As a result of the exercise-mediated increase of F<sub>max</sub> and F<sub>pass</sub> in HET males and higher Ca<sup>2+</sup>-sensitivity in WT mice and HET females, the total generated force as a function of [Ca<sup>2+</sup>] was higher in all exercised compared to sedentary groups (Figures 4A-D). The effect of exercise on force generating capacity of the sarcomeres was largest in HET male mice (Figures 4C).

### Sex-related changes in myofilament protein phosphorylation

Sarcromeric protein phosphorylation is a key modulator of myofilament function. Phos-tag gel analysis was performed to study the distribution of un- (0P), mono- (1P) and bis- (2P) phosphorylated cTnI (Figures 5A, B). In WT male and female, as well as in HET male mice we observed only minor non-significant changes in the phosphorylation pattern of cTnI after exercise (Figures 5C, D). Post-hoc analysis revealed a significant decrease in the cTnI biphosphorylation in exercised compared to sedentary female HET mice (Figure 5D). Phospho-specific ProQ staining was used to measure changes in sarcomeric protein phosphorylation levels. No changes in MyBP-C phosphorylation were seen between WT and HET mice (Figures 6A, B). The phosphorylation state of cTnT seemed to increase in WT exercised mice, however it did not reach levels of statistical significance (Figure 6C). No remarkable changes were observed in phosphorylation level of cTnT in HET mice (figure 6D). A sex-specific response in MLC2 phosphorylation in exercised WT mice was detected (figure 6E, P<sub>interaction</sub> <0.05 in 2-way ANOVA), while no changes were detected in HET mice (Figure 6F).
Figure 3. Effect of exercise on myofilament force generating capacity and Ca\(^{2+}\)-sensitivity. Myofilament Ca\(^{2+}\)-sensitivity was significantly higher in exercised male and female WT mice compared to sedentary WT groups (A, \(P_{\text{sex}} < 0.05\) in 2-way ANOVA). Maximal generated tension remained unchanged in exercised WT compared to sedentary groups (B). A significantly higher Ca\(^{2+}\)-sensitivity was also found in exercise compared to sedentary HET females, while EC50 values in HET male remained unchanged (C, \(P_{\text{sex}} < 0.05\) and \(P_{\text{interaction}} < 0.05\) in 2-way ANOVA). Fmax was significantly higher in exercised HET male mice, compared to sedentary counterparts (D, \(P_{\text{sex}} < 0.05\) and \(P_{\text{sex}} < 0.05\) in 2-way ANOVA). †\(P<0.05\) female vs. corresponding male; §\(P<0.05\) exercised vs. corresponding sedentary mice; #\(P_{\text{interaction}} < 0.05\) in 2-way ANOVA.

Figure 4. Total generated tension in sedentary and exercised mice. Total force as a function of calcium concentration measured at sarcomere length 2.2 \(\mu\)m in sedentary and exercised male (A, C) and female (B, D) mice. Higher force generating capacity of sarcomeres was found in exercised compared to sedentary male and female mice. 1P<0.05 female vs. corresponding male; 2P<0.05 exercised vs. corresponding sedentary mice.
Figure 5. Phosphorylation state of cardiac tropinin I. Phos-tag gel analysis (A, B) was performed to investigate if exercise was associated with changes in the cTnI phosphorylation pattern (C, D). Exercise resulted in higher levels of un-phosphorylated (0P) cTnI and a lower level of bisphosphorylated (2P) cTnI in exercised compared to sedentary HET females (D, Pinteraction <0.05 in 2-way ANOVA). *P<0.05 HET vs. corresponding WT; †P<0.05 female vs. corresponding male; §P<0.05 exercised vs. corresponding sedentary mice; #Pinteraction <0.05 in 2-way ANOVA.

DISCUSSION

In this study we investigated the sex-related differences in cardiac and sarcomere performance in sedentary and exercised mice harbouring the HCM-associated MYBPC3 mutation. Our data show sex-dependent changes in cardiac and sarcomere properties in particular in exercised mice harbouring a MYBPC3 mutation. The main findings were that 1) sedentary HET males, but not females, have a lower $F_{max}$ than WT; 2) exercise enhanced $F_{max}$ solely in HET males; 3) exercise resulted in hypertrophy, lower cTnI phosphorylation and increased myofilament Ca$^{2+}$-sensitivity only in HET female mice.

Human HCM patients are typically heterozygous carriers for the disease causing mutation. To study the early phases of the disease, multiple mouse models have been generated in which heterozygous MYBPC3 truncation mutations were introduced [15, 34, 59] or one allele of MYBPC3 was knocked-out [6, 7, 17]. Although these mice did not develop overt hypertrophy [2, 6, 7, 17, 34] until advanced age [6, 34] they did show various degrees of dysfunction at the myofilament level. The different models share certain parameters of cardiac dysfunction, whilst others seem to be more model-specific and therefore perhaps less likely to be translatable to the human disease. An increased Ca$^{2+}$-sensitivity of force development is seen in almost all models (Fig 1D) [2, 15]. Also, in a recent paper by Cheng et al. an increased force production at low calcium was found in cMyBP-C heterozygous null mice compared to controls, indicating increased Ca$^{2+}$-sensitivity, although pCa$^{50}$ was not significantly changed [7]. Another study by Harris et al. in heterozygous cMyBP-C knock-out mice showed no change in Ca$^{2+}$-sensitivity [17]. The reduction of maximum force seen in males (Fig 1C) is seen in some [2], but not in other models [7, 15]. The human studies are done on hypertrophied tissue from patients with obstructive HCM (typically tissue obtained after myectomy operation), and represent a more advanced stage of disease than the early disease stage that is assessed in many of the mouse models. This caveat notwithstanding, they are the most important benchmark to which the findings in mouse must be compared. Reduced force production and increased Ca$^{2+}$-sensitivity of force development are seen in HCM patients with MYBPC3 mutations [55-57]. It should be noted that the increased Ca$^{2+}$-sensitivity could be normalized to donor heart levels by pre-treatment of the skinned cardiomyocytes with PKA [55, 56] and are therefore not likely to be a direct consequence of the mutation but rather the low phosphorylation level of cTnl and cMyBP-C. Taken together, these findings suggest that myofilament dysfunction seen in hypertrophied human myocardium is already seen in most heterozygous MYBPC3 truncation/null mouse models before the onset of cardiac remodelling.

Sex-specific changes in mice with a MYBPC3 mutation

Several studies in mice have reported sex-specific differences in HCM. A study using a transgenic mouse model of a HCM-associated cardiac troponin T (TNNT2; cTnT) mutation (cTnT-
trunc) showed that only female mice developed hypertrophy upon angiotensin II infusion [29]. Interestingly, adrenergic stimulation with two agonists resulted in sudden cardiac death of all male but not female mutant animals, which may suggest altered adrenergic responsiveness between male and female mice carrying an HCM-associated mutation [29]. Another HCM mouse model (harboring heterozygous α-myosin heavy chain (MHC) missense mutation) revealed sex-specific electrophysiological abnormalities [4]. During ventricular stimulation, 28% of females and 62% of males developed inducible ventricular tachycardia [4]. McKee et al. showed a sexual dimorphic response to cardiac remodeling, myofilament function and phosphorylation state of the sarcomeric proteins in HCM mice harboring a mutant (R403Q) MHC [35]. LV mass as well as cardiac trabeculae Ca\(^{2+}\)-sensitivity were higher in HCM females than male counterparts. The lower Ca\(^{2+}\)-sensitivity in heterozygous males compared to corresponding females was explained by higher cTnI phosphorylation in the males. The authors hypothesized that the inability of the myofilaments to respond to adrenergic stimulation could underlie HCM disease progression in male, but not in female HCM mice [33]. In contrast, another study in mice carrying a single R403Q missense allele for cardiac α-MHC, showed that Ca\(^{2+}\)-sensitivity was lower in female HCM compared to corresponding HCM males. The underlying mechanisms are not clear; however, the data suggest no contribution of myosin isoform profile or cTnI phosphorylation. The difference could be explained as authors indicated by accelerated development of HCM by male sex hormones and/or by absence of female sex hormones [44]. In contrast to these findings, we found no differences in Ca\(^{2+}\)-sensitivity between sedentary male and female mice. Also we did not observe the changes in cTnI phosphorylation in male mice [35]. These discrepancies might be caused by differences in mutated protein (cMyBP-C versus MHC) and/or age of the mice.

Our study in male and female mice showed no remarkable changes in FS and ventricular weight in both male and female HET groups compared to WT. Although no cardiac remodelling was evident at the age of 13 weeks in sedentary males and females (Figure 1), sarcomeric functional properties were different between HET and WT male animals suggesting that sarcomere deficits precede the development of hypertrophy. A higher myofilament Ca\(^{2+}\)-sensitivity was observed in both male and female HET mice compared to WT counterparts. Our observations are in line with previously published data in MYBPC3-targeted HET knock-in male mice [15], which showed significant diastolic dysfunction in the absence of ventricular remodeling [15, 59]. Diastolic function of individual cardiomyocytes is partly determined by Ca\(^{2+}\)-sensitivity of force development and passive stiffness of the myofilaments. Males and females reacted differently on exercise, with Ca\(^{2+}\)-sensitivity being increased only in the females. Furthermore, our force measurements revealed a significantly higher $F_{\text{pas}}$ in females at sedentary state. In comparison to sedentary counterpart, a significantly higher $F_{\text{pas}}$ was detected in exercised HET males, while in HET females the cardiomyocyte passive tension remained unchanged. Also no remarkable changes in $F_{\text{pas}}$ were observed in WT male and female mice at both sedentary and exercised conditions. Clinical studies reported diastolic dysfunction in mutation carriers even

Figure 6. Sarcomeric protein phosphorylation levels. SDS-polyacrylamide gel of heart lysates stained with phospho-specific ProQ. No changes in cMyBP-C phosphorylation was seen in WT and HET mice (A, B). A minor increase in cTnI phosphorylation was detected in exercised WT males and females, while no response was observed in HET mice (C, D). A different MLC2 phosphorylation pattern between male and females was found in WT mice (E, $P_{\text{interaction}}<0.05$), however MLC2 phosphorylation in HET mice remained unchanged (F).
before the onset of hypertrophy in humans [20, 37, 46]. Diastolic dysfunction may be partly explained by high myofilament Ca\(^{2+}\)-sensitivity of force development as it has been observed in MYBPC3-targeted knock-in mice and human HCM patients with MYBPC3 mutations [21, 22, 51, 55, 56]. The present study suggests that the higher Ca\(^{2+}\)-sensitivity is independent of sex, while myofilament passive tension at sedentary state is sex-dependent.

In addition, the generated tension over the entire range of calcium concentrations was lower in HET compared to WT sedentary males, which resulted in a reduction of the force generating capacity of the sarcomeres at physiologic (submaximal) [Ca\(^{2+}\)]\(_{\text{max}}\) (Figure 4A, C; open symbols). A reduction in maximal force generating capacity may be caused by post-translational modifications of sarcomeric proteins such as phosphorylation and oxidative modifications and/or cellular remodeling and reduced myofibrillar density [16]. Our recent study in cardiomyocytes from HCM patients with MYBPC3 mutations revealed that low cardiomyocyte F\(_{\text{max}}\) in these patients was largely explained by cellular hypertrophy and reduced myofibrillar density [63]. As no significant cardiac hypertrophy was evident in HET male mice at 13 weeks of age, cellular hypertrophy and reduced myofibrillar density most likely do not explain the significant drop in F\(_{\text{max}}\) observed in the male HET mice. Studies in male as well as in female mice with a R403Q mutation in the gene encoding MHC indicate that male and female mice were hypercontractile (indicated as increase in +dp/dt/ΔLV-pressure) at 4 months of age [42]. However, the increase in +dp/dt/ΔLV-pressure was more pronounced in female than in male HCM mice, compared with age-matched WT mice [42]. The cellular contractile dysfunction observed in sedentary HET compared to WT male may in part explain the earlier onset of symptoms in male HCM patients compared with females.

The recently developed technique of engineered heart tissue (EHT) can be used to study early changes in HCM. EHT generated from MYBPC3 HET mice showed increased expression of hypertrophic markers and alterations in contractile response to external Ca\(^{2+}\) [52]. This model could give valuable insight in sex differences in HCM.

Exercise increases FS in WT (15% increase) and HET (21% increase) male mice, while no change was observed in exercised females. A study in exercised male and female WT rats, reported higher contractile performance (increased stroke work) in males, but not females, compared with sedentary sex-matched rats [49]. In the present study, exercise did not increase cardiac weight in males, while a significantly higher LV weight was found in HET females (15%, Figure 2C) and a minor higher LV weight in female WT (7%, Figure 2A). No correlation between LV weight and distance was detected in male as well as female WT and HET exercised mice (supplemental figure 2D). Likewise, previous studies in WT rat and mice showed a more prominent increase in heart weight after exercise in females compared to males [9, 14, 24]. The higher heart weight in females was attributed to increased hypertrophic signaling [24]. In contrast to the higher LV weight in exercised female HET mice, end-diastolic posterior wall thickness (Tables 1, 2) remained unchanged. This finding may indicate that the exercise induced hypertrophy is eccentric rather than concentric in nature [38]. This is supported by the so-called Morganroth hypothesis, which states that endurance exercise elicits eccentric hypertrophy of the heart [38].

The exercise-mediated increase in FS was higher in HET male mice (Figure 2D) and may in part be explained by the greater maximal force generating capacity of cardiomyocytes from exercised HET male compared to sedentary counterparts (Figure 3D). Similarly, a study in post-myocardial infarction mice, exposed to an 8-week voluntary exercise protocol, showed a significant increase in FS after exercise [10]. Moreover, the significant increase in maximal generated force after exercise may in part contribute to the higher FS in exercised post-MI mice [10]. In addition, FS fails to increase upon exercise in transgenic mice with HCM. Also other parameters such as HR and cardiac output remain unchanged in exercised transgenic mice, indicating lack of physiological response to exercise in HCM mice [39]. In contrast, a study in HCM male mice showed a significant increase in heart weight after 2 months of exercise, while no changes in heart weight were detected in non-transgenic mice [23]. Overall, this study indicates that exercise may prevent and reverse cardiac disease phenotypes in these HCM mice [23]. Another study in HCM mice demonstrated less cardiac growth upon exercise in mice with a mutation in the gene encoding cTnT associated with HCM [18]. Interestingly, a higher energy cost was found in transgenic mice, which did not change after exercise [18]. Another striking observation in our present study and at the sarcomere level was the relatively large decrease in E\(_C\)\(_{\text{p}}\), i.e. increase in Ca\(^{2+}\)-sensitivity in exercised WT males and females compared to corresponding sedentary mice (Figures 3A, C). Increased Ca\(^{2+}\)-sensitivity was also reported in rat models after treadmill exercise [11, 62]. The cellular and molecular mechanisms for the increase in Ca\(^{2+}\)-sensitivity in exercised rats is not fully known. It is well known that protein kinase A phosphorylation of cTnI may lead to a lower myofilament Ca\(^{2+}\)-sensitivity. In our study, analysis of cTnI phosphorylation in sedentary as well as exercised mice revealed less of the bisphosphorylated form of cTnI in HET sedentary male (43 ±0.08%) than in WT counterparts (54 ±0.07%). However, no changes in bisphosphorylation of cTnI were detected in exercised WT and HET male mice. In contrast to males, exercise resulted in a significantly lower cTnI bisphosphorylation in HET females, while no changes were detected in WT counterparts. Our previous study in end-stage failing cardiomyocytes revealed that bisphosphorylation of cTnI is needed to reduce Ca\(^{2+}\)-sensitivity of the myofilament force [61]. Furthermore, we found that Ca\(^{2+}\)-sensitivity decreased maximally at ~55% bisphosphorylated cTnI [61]. It should be noted that the other myofilament proteins (cMyBP-C and cTnT) did not show changes in phosphorylation level; however, a significant different MLC2 phosphorylation pattern was detected in WT mice. In comparison to sedentary counterparts, MLC2 phosphorylation was higher in WT exercised males, while in female WT exercised mice MLC2 phosphorylation was reduced (Figure 6E). The importance of MLC2 phosphorylation for the adaptation of the heart to stress has recently...
been described by Warren et al [60]. Phosphorylation of MLC2 is typically reduced following pathological hypertrophy, for instance a ~40% lower MLC2 phosphorylation was detected in WT pressure-overloaded mice hearts, which was predominantly due to myosin light chain kinase degradation. Another study indicated a significantly higher MLC2 phosphorylation in exercised rats compared to sedentary counterparts [13]. In the present study we found a sex-effect on MLC2 phosphorylation upon exercise in WT mice, with MLC2 phosphorylation going up in males and down in females after exercise. Our CtNl and CMyBP-C phosphorylation in sedentary HET mice, compared to the corresponding WT confirmed the data from the previous study in this HCM mouse model [15].

In the present study, exercise did not alter myofilament Ca\textsuperscript{2+}-sensitivity in HET males, while a higher $F_{\text{max}}$ was observed. As a consequence, the total force generating capacity in HET males increased from 14.6 in sedentary to 24.8 kN/m\textsuperscript{2} (Figure 4C) in exercised mice at maximal activation. Overall our data indicate that exercise exerts a similar effect on sarcomere function in male and female WT mice, while the effect in HET mice is sex-dependent.

The exercise-induced decrease in $E_{\text{ca}}$ indicates that the myofilaments become more sensitive to Ca\textsuperscript{2+} and therefore less Ca\textsuperscript{2+} is needed to produce the same amount of force. It is well accepted that mutations in MYBPC3 gene are associated with increased Ca\textsuperscript{2+}-sensitivity in HCM [55]. Although high myofilament Ca\textsuperscript{2+}-sensitivity may be favorable for contractile function of the heart, sensitized myofilaments may represent a substrate for impaired relaxation and cardiac arrhythmias. Increased myofilament sensitivity to [Ca\textsuperscript{2+}] may induce cross-bridge interaction at low diastolic calcium level, leading to resting cardiac tone and impaired relaxation and compliance of the heart [27]. Patients with HCM are also prone to cardiac arrhythmias and SCD [12, 36]. In addition, it has been reported that a family history of SCD is less often present in females carrying a MYBPC3 gene mutation [8]. However, this study indicates that females suffer more from palpitations than males [8]. Moreover, SCD in HCM has been associated with physical activity [33, 40]. A study in a HCM mouse model expressing mutant troponin T demonstrated a risk of tachycardia, which was directly proportional to the degree of Ca\textsuperscript{2+}-sensitization [3]. Another study in the same mouse model indicated that an important pro-arrhythmic consequence of increased Ca\textsuperscript{2+}-sensitivity is a pause-dependent potentiation of Ca\textsuperscript{2+}-release, action potential prolongation and triggered activity [50], leading to arrhythmia. The increase in Ca\textsuperscript{2+}-sensitivity, which might function as a source for arrhythmia indicated by Baudenbacher et al. [3], was further increased especially in exercised female HET mice. Interestingly, we observed a lower $E_{\text{ca}}$ value (indicating an increase in Ca\textsuperscript{2+}-sensitivity) in both male and female exercised WT mice, while Ca\textsuperscript{2+}-sensitivity remains unchanged in exercised HET male mice. It is important to mention that male HET mice have a lower $E_{\text{ca}}$ at sedentary condition, compared to the WT counterparts, as indicated by figure 1D. Moreover, the high Ca\textsuperscript{2+}-sensitivity in mice at sedentary state and especially in females after exercise may deteriorate cardiac relaxation and increase the risk of ventricular arrhythmia in HCM. Noteworthy, the majority of SCD cases in HCM are males, while in the present study the exercised HET male mice have no changes in Ca\textsuperscript{2+}-sensitivity following exercise, while female HET mice do show an increase of Ca\textsuperscript{2+}-sensitivity. It has to be noted that high Ca\textsuperscript{2+}-sensitivity is only one of the mechanisms through which arrhythmias arise. Described risk factors for SCD are higher level of hypertrophy and increased interstitial fibrosis. Also changes in Ca\textsuperscript{2+}-handling proteins are likely to be important contributors to arrhythmia development. The higher occurrence of SCD in male HCM patients might be explained by these non-myofilament changes. A study by Varnava et al. demonstrated a high level of fibrosis at sedentary state particularly in male HCM patients [58]. Similarly, a study in HCM mouse model showed more fibrosis in male HCM mice, compared with corresponding females [29]. Interestingly, Regitz-Zagrosek et al. indicated that stressed female hearts are more protected against calcium overload, which is especially mediated by female hormones [47]. Thus, besides the sex-specific myofilament response upon exercise, which is illustrated in the present study, other mechanisms might be involved to occurrence of SCD in HCM.

**STUDY LIMITATIONS**

The mutation of the mouse model is based on HCM patients with a founder mutation, which has been associated with a severe phenotype and poor prognosis [15, 59]. HCM patients are typically heterozygous for a sarcomeric gene mutation. However, LV function and geometry in 13-week-old HET mice were apparently normal, which makes the translation to manifest HCM patients difficult. Interestingly, the HET mice might be comparable to pre-hypertrophic mutation carriers, as the sarcomere changes (high Ca\textsuperscript{2+}-sensitivity) may underlie diastolic dysfunction, which has been reported at early stages of HCM. The increase in Ca\textsuperscript{2+}-sensitivity may create susceptibility to arrhythmia, which can lead to SCD in HCM. Noteworthy, most of the SCD cases are manifest HCM patients; however, the HET mice recapitulate the situation in mutation carriers with no LV hypertrophy. Thus, the stage of the disease seems to play also an important role in SCD risk. The increased risk on SCD in HCM patients following exercise is determined in part by the intensity of the exercise. High intensity exercise such as football is discouraged, while low-intensity aerobic exercise in HCM patients is considered reasonable [45]. Our model of voluntary wheel running is likely to be low-intensity and therefore unlikely to elicit arrhythmias, which were also not the focus of the current study.

Finally, it should be noted that the voluntary exercise protocol is difficult to regulate. The distance ran by female and male WT mice did not differ, while female HET mice ran significantly more than the male counterpart. Interestingly, female HET mice ran significantly more from week 1 of the exercise protocol (Supplemental Figure 2E). The longer distance ran in female
HET mice did not explain the increase in LV mass, as no correlation was found between distance ran and LV weight (Supplemental Figure 2D).

CONCLUSION

Our study revealed functional and structural differences between male and female mice both in sedentary and exercise groups. The female mice demonstrated a slightly higher FS compared to males, lower LV weight and showed no change in cardiomyocyte passive stiffness at sedentary state. In contrast, exercise increased hypertrophy solely in females, and improved fractional shortening and sarcomere contractile function without an increase in LV mass in particular in male mice. Overall our data indicate contractile differences between sedentary male and female HET mice. The heart of the male and female HET mice adapt differently to the voluntary exercise protocol, indicating that physiological stimuli elicit a sexually dimorphic cardiac response in heterozygous MYBPC3-targeted knock-in mice.

Acknowledgment

We are grateful to Nicky Boontje for valuable technical support and laboratory assistance.

Grant

We acknowledge support from the Netherlands organization for scientific research (NWO; VIDI grant 91711344), the 7th Framework Program of the European Union (“BIG-HEART”, grant agreement 241577) and from ICIN-Netherlands Heart Institute.

REFERENCES


54. van Deel ED, de Boer M, Kuster DW et al. (2011) Exercise training does not improve cardiac function in compensated or decompensated left ventricular hypertrophy induced by aortic stenosis. J Mol Cell Cardiol 50:1017-25 DOI


57. van Dijk SJ, Boonjte NM, Heymans MW et al. (2013) Preserved cross bridge kinetics in human hypertrophic cardiomyopathy patients with MYBPC3 mutations. Pfluegers Arch


Mimicking the cardiac cycle in single isolated cardiomyocytes
Mimicking the cardiac cycle in intact cardiomyocytes using diastolic and systolic force clamps; measuring power output

Michiel Helmes
Aref Najafi
Bradley M. Palmer
Ernst Breel
Niek Rijnveld
Davide Iannuzzi
Jolanda van der Velden
ABSTRACT

Aims
A single isolated cardiomyocyte is the smallest functional unit of the heart. Yet, all single isolated cardiomyocyte experiments have been limited by the lack of proper methods that could reproduce a physiological cardiac cycle. We aimed to investigate the contractile properties of a single cardiomyocyte that correctly mimic the cardiac cycle.

Methods and Results
By adjusting the parameters of the feedback loop, using a suitably engineered feedback system and recording the developed force and the length of a single rat cardiomyocyte during contraction and relaxation, we were able to construct force-length relations analogous to the pressure-volume relations at the whole heart level. From the cardiac loop graphs, we obtained, for the first time, the power generated by one single cardiomyocyte.

Conclusion
Here, we introduce a new approach that by combining mechanics, electronics and a new type optical force transducer, can measure the force-length relationship of a single isolated cardiomyocyte undergoing a mechanical loop that mimics the pressure-volume cycle of a beating heart.

Key Words
Cardiomyocyte function, Microtechnology, Force-length relation

1. INTRODUCTION

The functional properties of a beating heart are typically captured by analyzing how the pressure-volume (PV) relationship evolves during a cardiac cycle. This approach is widely recognized as an invaluable tool in cardiovascular (patho)physiology and pharmaceutical research. To study the effects of various hemodynamic conditions and disease states at the tissue level, researchers often rely on direct measurements of the force-length (FL) loop of multicellular cardiac muscle strips. The interpretation of multicellular muscle strip experiments is complicated by the presence of the extracellular matrix, which makes it difficult to disentangle the properties of the cardiac muscle cells from those of the milieu. Moreover, multicellular muscle strip experiments suffer from diffusion constraints that limit oxygenation and metabolic work. This can be overcome with the use of ultra-thin trabeculae, however that is a skill few laboratories possess. Both problems can be solved by replacing the multicellular strip with a single isolated cardiomyocyte. However, the forces generated by a single intact cardiomyocyte under physiological conditions are 3 orders of magnitude smaller than those recorded in multicellular muscle preparations. It is thus not surprising to find that none of the force transducers presented in the literature has been able to achieve the required sensitivity and responsiveness (i.e. reaction speed) to establish FL cycles in a single isolated cardiomyocyte experiment that could correctly mimic the behavior of cells in the heart. To perform such measurements a feedback control system would be needed that could accurately measure the force developed by the cell and, in real time, change its length to control force development. Here, we solve this longstanding problem by introducing a sensor that improves sensitivity and responsiveness by an order of magnitude over the current state-of-the-art. We demonstrate that, by anchoring a single cardiomyocyte to our force transducer, it is indeed possible to drive a feedback control loop that adapts the length of the cell to control the force it exerts. We show that this approach can be used to functionally approximate the cardiac PV relationship at the cellular level by imposing a ‘pre-load’ and ‘after-load’ by modulating cardiomyocyte length using feedback based on force level. This allows measuring the external work performed and the power generated by a single intact cardiomyocyte under physiological conditions.

2. METHODS

The animal experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.
2.1 Force transducer design

Intact isolated cardiomyocytes at 37°C produce 10-20% of the maximum forces measured in membrane-permeabilized cardiomyocytes at equivalent sarcomere lengths (SLs) at room temperature. To measure the force generated by a single intact cardiomyocyte with sufficient sensitivity, responsiveness and stability to allow force control, a new type of force transducer was developed. For any force control to be meaningful, the base-line drift has to be low, which was targeted at constant temperature <0.1 μN/minute. As most of the baseline drift arises from the air-water interface (buoyance, surface tension) the only practical solution was to design a force probe that could be fully submersed.

In addition to having to function submerged in an aqueous solution, the force transducer was required to sense forces on the scale of μN with a resolution of nN and with a resonance frequency >1 kHz to enable feedback control of force. To match these requirements, we designed an optical force transducer where we use the traditional deflection of a cantilever as an indicator of force, though bending of the cantilever was measured via laser interferometry (Fabry-Perot type interferometer) as already used for applications in other research fields (Figures 1A and 1B). Briefly, laser light is delivered to the cantilever through a standard 125 μm diameter optical fiber (Figure 1B). The light reflected from the fiber-to-liquid interface and from the cantilever travels back through the same optical fiber towards its distal end, creating an interference signal whose amplitude depends on the position of the cantilever end relative to the fiber. Before the start of the experiment, the wavelength of the laser is adjusted to put the interferometer in quadrature condition, i.e. at the point where the sine that describes the amplitude of the interference signal has maximum derivative. Under this condition, the linear range of the signal (i.e. <5% error) extends over a range of at least 100 nm in either direction, with a resolution of 1 nm (over 20 kHz bandwidth). Stiffness of the manufactured probes can easily be varied between 5 and 100 N/m, which will affect the force resolution accordingly. Because the spring constant of the cantilever used for these experiments was equal to 37 N/m, the force had a force resolution of 37 nN on a 3.7 μN range in either direction with a noise band of 1.5 nm which equals 56 nN. Baseline drift was <0.1 μN/minute at constant temperature. None of the traces shown in this paper are filtered; they all show the raw output of the read-out. As the cantilever is just a very small rectangular sheet of gold coated glass (1200 μm x 400 μm x 40 μm), it has a high resonance frequency of approximately 7 kHz. This provides sufficient responsiveness to allow force control. For details on the manufacture and calibration of the probe see the online methods section.

2.2 System set-up

The work loop data collected for this paper were generated with a system built around a standard ionoptix set-up designed for calcium and contractility measurements in cardiac myocytes.
the proportional correction) could be modified via a newly designed module that was added to the Ionoptix software and could be adjusted interactively during the experiment.

Excitable cardiomyocytes are normally between 100-140 μm in length and 15-30 μm in width. Under experimental conditions at 37°C, unloaded cardiomyocytes, excited with electrical stimulation, can shorten up to 15 μm with the fastest rates of shortening in the order of 500 μm/s (10 μm within a 20 ms time period). To follow this contraction, our setup relies on a closed-loop direct drive piezo-translator that has 50 μm range and that can complete a 1 μm step in less than 1 ms (Mad City Labs, Madison, WI). To determine the SL, the sarcomere length acquisition module from Ionoptix (Milton, Ma) in combination with a high speed camera (Myocam-5, Ionoptix) was used to acquire SL at a rate of 250Hz. The algorithm does a frequency analysis of the region of interest (FFT) from which the SL is determined.

### 2.3 Attachment of single cardiomyocyte

Cardiomyocytes were glued to 35 micron glass needles that were attached to the cantilever of the force transducer and to the piezo translator (Figure 1C). The needles were between 0.5 and 1.5 mm long, and were sufficiently stiff to prevent bending during the contractions. The gluing procedure is similar to the method described by Prosser et al.18 The tips were coated with an aluminum silicate suspension (Ionoptix pre-coat). The pre-coat was air-dried after which they were dipped in MyoTak (Ionoptix). In a well-attached cell we could do force measurements for up to an hour in a temperature controlled chamber (Figure S1) at 37°C without significant rundown of the preparation.

### 2.4 Work loop algorithm

To mimic the cardiac PV relationship at the cellular level with an analogous FL relationship, we implemented a feed-back control system that, by modulating the cardiomyocyte length, controls the force generated by the cardiomyocyte between a predefined pre-load and after-load. Pre-load and after-load when used in this paper refer to the target force levels of the feedback control during the diastolic and systolic phase of the myocyte contraction, respectively.

In our method, the four phases of the cardiac cycle are defined as follows (Figures 2A and 2B): Phase I, which is analogous to the isovolumic contraction of a ventricle, starts immediately after electrical stimulation and encompasses a brief period of time during which the cell generates a force without substantially changing its length. Phase II, which is analogous to the ejection phase of the ventricle after the aortic valve opens, refers to the period of time when the pre-programmed after-load force is maintained constant by shortening the cell via the feed-back loop. This phase ends when the force measured by the transducer drops below the after-load value. The reversal of the piezotranslator from shortening to stretching, when it tries to maintain the force level, triggers the exit of phase II. Phase III, which is analogous to the isovolumic relaxation, is characterized by a natural decrease of force at constant length.
Phase IV starts as soon as the force decreases below the pre-programmed pre-load force. In this last phase, which simulates the filling of the heart in diastole, the feedback loop stretches the cell to maintain the pre-load force value until the start of the next cycle with the next electrical stimulus. For the implementation of the algorithm, we used FPGA, which digitizes in real-time the force signal and the prescribed pre-load and after-load values (programmed and communicated via the software). The output from the FPGA drives the piezoelectric translator in Phase II and Phase IV to maintain the force exerted by the cell constantly equal to the prescribed values.

The precision of the force control can be tweaked with two parameters, the frequency of the iteration loop and multiplier of the proportional correction when a mismatch between the set-point and the actual is measured. We set the frequency of the iteration loop as close as possible to the update frequency of the force transducer (20 kHz). During test runs the multiplier was increased to the point where oscillations started to occur and then used half that value for the remainder of the experiments. This resulted in an overall response frequency of the feedback system in the order of 100 Hz. At room temperature this was fast enough to achieve practically square loops (Figure S2). At 37°C however this still left significant overshoot of the set-points. This can probably be solved with more sophisticated feed-back algorithms, but that was beyond the scope of this study. Figures 2A and 2B show exemplary force and length signals acquired during one of these cycles. Figure 2C further shows, by way of example, the FL curves obtained for different pre-load and after-load values in a series of mechanical loops with the myocyte beating at 4 Hz. As expected from whole heart experiments, the slope of the loop curve at the end-systolic point is relatively constant throughout the entire maneuver.

2.6 Experimental protocol
To set the feedback control parameters, a rat ventricular myocyte was electrically stimulated and stretched until a minimum force development of 0.3-0.5 μN was recorded. The forces at 10% and 30% of the developed force (i.e. of the difference between the maximum and minimum forces registered from isometric contractions) were used to designate the initial preload and afterload values, respectively. With these initial preload and afterload values set, we engaged the force clamping algorithm. When control of the cardiac cycle appeared stable the actual protocol was started (see Figure S3 for an example of a stable recording). At each pre-load condition, the after-load was varied using a ramp function that would rise over several electrical stimulations by 0.75 μN and then decline back by 0.45 μN. The preload was then raised by 0.3 μN and, thereafter, by another 0.3 μN (Figure S4). This protocol was repeated for 1, 2, 4, 6 and 8 Hz pacing frequencies in the presence of Tyrode or Tyrode with 100 nmol/L isoprenaline (ISO, Sigma Aldrich). More than 90% of the attempts to attach a cell was successful. Because of the elaborate protocol, the full protocol could be completed in 3 to 5 cells per experimental day.

2.7 Data presentation and analysis
For data analysis, we imported the force- and length-data into the pressure-volume-loop module from LabChart 7.0 (AD instruments, Australia). The analysis calculated the external work value and the end-systolic and end-diastolic force values for each loop. Further analysis of these loop data was done in a Microsoft Excel spreadsheet and Prism version 6.0 (Graphpad Software, Inc., La Jolla, CA). All the force and length measurements were differential, i.e. initial force was not set to zero, which explains why the forces do not go to zero as would be expected in some of the graphs. Length always refers to movement of the piezo motor, not to cell length.

3. RESULTS
3.1 Optimal cardiomyocyte isolation
Stretching intact cardiomyocytes has been possible for a long time using carbon fibers15, but the force bearing capacity was limited. The development of a glue specific for intact myocytes16 greatly improved the ability to stretch intact cardiomyocytes. In rat cardiomyocytes we can now measure forces of up to 3-4 μN, which is a 5-to 10-fold improvement over the carbon fiber method.13 To achieve these relatively high levels of force, cell isolation had to be optimized for mechanical experiments. We attempted two different digestive enzymes, Liberase TM (0.16 mg/mL; Roche) and Worthington type II. While cardiomyocyte yield was highest with Liberase digestion, the Worthington type II digested cells were more sticky and would bear forces that were 2-3 times higher before cells detached. We also found that we could not add protease to the digestion procedure.17 Protease is used to make cardiomyocytes more accessible for patch clamping. We found however that adding protease led to cells that were very prone to arrhythmias when stretched.

3.2 Workloops
We were able to control force development by the myocyte by modulating cell length in a real-time feed-back loop. Figure S3 shows an example of a stable recording where the data show that force can be successfully modulated. Figure S3 also shows over- and under-shoot of the targeted pre- and after-load at 37°C. Feed-back is often set up using Proportional-, Integral-, and Derivative (PID) response. Our algorithm only has a proportional response. This works well for the slow changes at room temperature, but proved to be inadequate at 37°C; the rapid rate of force development in early systole and early diastole could easily be controlled at the set-point by setting the multiplier of the P (Proportional) very high, but this resulted in oscillations when the relaxation later in diastole slowed down. The lesser of two evils was accepting an over- and undershoot. The relative over/undershoot will vary with the rate of force development; the higher the pre-load and the closer the afterload is to the pre-load, the more over- and undershoot. This does not materially affect the experiments as the end-systolic
and end-diastolic values are still controlled and correct. To remove the over- and undershoot, the feed-back response has to be differentiated with respect to the rate of force change. To do this properly however, it should probably also include a time-varying after-load to better mimic the cardiac cycle. The electronics infrastructure allows for this and will be the subject of a next study.

Signal generators built into the software allowed us to pre-program changes in the pre- and afterload. Figure 2C shows the results of a typical protocol. The data traces show that both pre- and after-load can indeed be varied in a controlled manner. The end-diastolic and end-systolic force relation are well described with a linear line in the region studied.

Furthermore, calculating the area within a FL cycle (or, in other words, by integrating the force loop as a function of length over a cardiac cycle), we can obtain the total mechanical work produced by the cardiomyocyte during the contraction-relengthening process (Figure 3A). This method allows us to study how the mechanical work may vary as a function of pre-load, after-load and stimulation frequency. It is expected that, for very low or very high after-load values, the cycle would be predominantly isotonic or isometric, respectively, producing virtually no external mechanical work (Figure 3A). Therefore, for each value of pre-load force, there must exist an after-load force value for which the external mechanical work produced is maximal. To find the peak work, the work versus after-load relation was fitted with a 2nd order polynomial. Figure 3B proves that our method is indeed capable to capture this feature (for more examples see on-line supplement Figure S2). Knowing the pacing frequency, we can then quantify the power generated by a single cardiomyocyte, as shown in Figure 3C. The average peak power was 55.2±20.5 μW (n=10). Maximum work per loop was achieved at 4 Hz for 8 out 10 cardiomyocytes, peak power was achieved at 6 Hz for 7 out of 10 cells. The maximal power generated by a single cardiomyocyte is thus achieved at physiological heart rates.

We tested our approach by exposing a single cardiomyocyte to a β-adrenergic receptor stimulus by measuring the FL relation before (Figure 4A) and after steady-state exposure to 100 nmol/L ISO (Figure 4B). The ISO increased the developed force as expected, and lowered the slope of the end-diastolic force-length (EDFL) relation (Figures 4A and 4B). The combination of increased force development, illustrated by the higher end-systolic force-length (ESFL) relation, and reduced EDFL led, in this example, to a fourfold increase in the work performed per cycle (Figure 4C).

Another example is shown in Figure 5 and online video S2, where pacing frequency is switched abruptly from 8Hz to 1Hz. In the build-up of frequency from 1 Hz (not shown) to 8 Hz, there is little difference in the maximum amount of work per loop; the increase in peak systolic force at 8 Hz due to higher systolic calcium is cancelled out by impaired relaxation. The post-rest potentiation effect due to the systolic effect of high sarcoplasmic reticulum calcium load after 8Hz pacing and the improved relaxation in diastole leads to a 5-fold increase in the work performed per loop (Figure 5C).

The method is repeatable. In a number of cells (n=8) we repeated the 4 Hz pacing frequency after completing the protocol from 1 to 8 Hz pacing frequencies (Figure S6A). Returning to a lower frequency after pacing at 6 and 8 Hz leads to a small reduction in the maximum work vs. end-diastolic sarcomere length relation, which may be indicative of some run down (Figure S6B-I), but a Bland-Altman plot shows that the repeated runs at the 4 Hz pacing frequency are well in agreement with each other (Figure S7A and S7B; n=8). This indicates that the method presented in this paper is suitable for repeated measures, for example to test pharmaceutical compounds.

3.3 Sarcomere length range
In the experiments described in the present study, the ceiling of the end-diastolic SL at the highest pre-load level was frequently between 2.0 and 2.1 μm, but almost never exceeded 2.1 μm (supplemental Figure S5A demonstrates the sarcomere length range of each experiment and thereby the generated work). It is important to note that these SLs were commonly achieved at low pacing frequencies (1 and 2 Hz). At higher pacing frequencies, the end-diastolic SL decreases slightly. End-systolic SLs were always between 1.6 and 1.7 μm SL.
Figure 4. The power generation of a single cardiomyocyte upon isoprenaline (ISO) treatment. (A) Force-length (FL) relation of a single cardiomyocyte was monitored in Tyrode for several stretches (i.e., increase in pre-load). (B) After the baseline (BL) measurements, the cell was exposed to 100 nmol/L of ISO and subsequently the force-length relation was repeated. End-diastolic force-length (EDFL) and end-systolic force-length (ESFL) value can be determined for both Tyrode as well as ISO-treated cardiomyocytes. (C) For every pre-load step, the generated power (i.e., the area of a single loop) is higher with ISO-treated condition. (D) Schematic comparison between work-loops and isometric contractions and the response to isoproterenol, that has an effect on both the EDFL and the ESFL. The loop marked with (1) is at baseline. (2) is the loop after ISO with the same pre- and afterload as (1). (3) is the loop where the afterload is adjusted to measure the maximum amount of work. On the right a sketch of the equivalent isometric contractions. With the isometric contraction the measured change is heavily weighted towards increases in systolic force development, with only a small change in diastolic force. This is due to the EDFL being much shallower than the ESFL. Using work-loops, the functional effects on both diastole and systole are given equal weight. The change upon an inotrope stimulus is further enhanced when looking at the maximum work that can be performed for a given pre-load. Now work-loops show an approximate four-fold increase in work vs. a 50-75% increase in isometrically developed force. The figure is based on the loops collected in Figure 4C.

Figure 5. The post-rest potentiation effect. Switching the frequency from 8 to 1Hz, resulted in an increase in force development (A), sarcomere shortening (B) and ~5-fold increase in single cell work generation within the first contractions (C).
4. DISCUSSION

Using a force transducer based on laser interferometry in combination with a micro machined probe we were able to measure the force development of a single cardiomyocyte with unprecedented sensitivity, signal quality and responsiveness. Its sensitivity bridges the gap between Atomic Force Microscopy, which excels in the picN range and the classic force transducers used for muscle physiology, which show effective sensitivity ends in the pN range. The ability to fully submerge the force transducer also gives it very good baseline stability. The combination of nN-sensitivity, stability and a 7kHz resonance frequency for the first time opens up the possibility to control the force development at the level of a single intact myocyte using true feed-back.

4.1 Work loops and power generation

We have used the ability to control force to mimic the cardiac cycle at the single myocyte level. Our method provides reproducible linear EDFL as well as ESFL relations. Changes in the inotropic state of the cardiomyocyte by increasing the pacing rate or β-adrenergic receptor stimulation changes the ESFL and EDFL in a predictable manner. We are also able to measure the changes in cardiomyocyte generated work in response to changes in pre- and after-load. In particular the ability to do repeated measures will be useful in studying pharmaceutical interventions.

The focus of the experiments in the present study was to establish the relation between levels of pre-load, after-load and pacing frequency with the external work the myocyte produces. The amount of external work is what ultimately determines the capacity of the heart to pump blood. The amount of work per loop increases with pacing frequency up to 4Hz. At 6 and 8Hz the work per loop decreases slightly, mostly due to an elevated EDL. The power generation peaks at 6Hz. This is consistent with studies on ultra-thin rat trabeculae that can be paced at high rates, where isometric force peaks at 6Hz. In cardiomyocytes the pacing frequency has a limited effect on power generation between 4 and 8Hz (Figure 3C), while the major determinant of power generation is the pre-load level (Figures 3B). Rat ventricular myocytes appear to be adapted to produce most power over the physiological range of heart rates. Output per cardiomyocyte is further increased by β-adrenergic receptor stimulation.

The effects of β-adrenergic stimulation have been studied extensively in linear preparations such as permeabilized myocytes, unloaded intact myocytes or muscle strips like trabeculae or papillary muscle. Experiments on permeabilized myocytes are suitable to measure the effects of PKA-mediated phosphorylation on calcium-sensitivity and passive force of the myofilaments. Unloaded, intact myocytes are ideal for studying the effects on excitation-contraction coupling because of the ease with which calcium kinetics can be measured in combination with myocyte shortening. The current work-loop experiments on intact cardiomyocytes illustrate the contribution of both changes in sarcomere properties and in calcium handling. The combined effects of myofilament calcium desensitization, reduced passive force and enhanced calcium re-uptake during β-adrenergic receptor stimulation led to a small decrease in the slope of the EDFL relation. The reduced slope of the EDFL relation, while the pre-load stays constant, results in increased stretch of the cardiomyocyte at end-diastole. This extra stretch further enhances the force development in systole by length-dependent activation, on top of the increased force development caused by higher end-systolic cytosolic calcium levels. The increase in stretch during diastole and greater force development in systole both augment the area encompassed by the work-loop. It leads to an approximate 4-fold increase in the work generated by the cardiomyocyte (Figure 4C) upon β-adrenergic receptor stimulation.

4.2 Limitations of the used methods

Measuring work loops in isolated myocytes by controlling the force in parts of the cycle gives results similar to those achieved in PV-loop measurements of the whole heart. This is illustrated by the suitability of existing PV-loop analysis software to analyze the data. But it would go too far to consider these force-length loops as one dimensional PV loops. For example we have no dynamic force control in systole to mimic the changing impedance in the ejection phase. Better models have been made in the past using trabeculae. The use of a real-time programmable machine, such as the FPGA, will allow future modifications of the algorithm that do take these complexities into account. The data shown here do however properly represent some of the key aspects of the whole heart measurements, most importantly a consistent end-diastolic and end-systolic pressure (i.e. force) - volume (i.e. length) relation. This had been shown before by Irbe et al., however those graphs had to be painstakingly constructed by manually adjusting the feed-forward parameters with each change in pre- and after-load. The current method greatly simplifies the process. The data for Figure 2C where three pre-load levels were tested for a full range of afterloads took less than 13 seconds to collect. As it is feedback and not feed-forward it is also sufficiently robust to cope with beat-to-beat changes in contractility. An example is shown in Figure 5C where pacing is abruptly changed from 8Hz to 1Hz, resulting in an acute 5-fold increase in the amount of work performed per stroke.

The work loops in Figure 3B and the power calculated in Figure 3C are based on the length change of the piezo translator as force x length change determines the external work performed by the myocyte. Looking at Figures 2B however, and this is exemplary for all our data, there is a lot of internal shortening of the sarcomeres. The sarcomeres display no distinct isometric activation and isometric relaxation phase. With the current gluing procedure this cannot be avoided and it probably can never be completely avoided, even with better attachment methods. In the past, using the carbon fiber technique that has less compliance in the attachment we experienced the same. The classic study by ter Keurs et al where length-dependent activation in rat
trabeculae is described, shows internal shortening where end-systolic SL does not increase to the trabecula is stretched beyond 1.95 μm end-diastolic SL. At that point the majority of length-dependent force increase has already taken place. It appears as if in rats the end-systolic SL is of limited relevance for the force development, which for a given inotropic state is dominated by the end-diastolic SL.

The upper limit of the end-diastolic SLs in our experiments was found to be approximately 2.1 μm, but usually ended between 1.95-2.00 μm SL after which further increases in pre-load did not further stretch the cells. The experiments here were done at relatively high extracellular calcium levels (~1.8 mmol/L). We hypothesize that the intracellular calcium levels were such that the diastolic force levels had an active component (as previously shown in King et al) that was further enhanced by length-dependent activation upon stretch, effectively leading to a very non-linear relation between pre-load and SL increase. We do think that we cover the majority of the physiological range with maximal end-diastolic SLs of up to 2.1 μm. X-ray studies on intact mouse hearts and measurements on skinned mouse hearts come up with a range of 1.9-2.1 and 1.8-2.1 μm respectively. Older studies on fixed rat hearts showed SLs of 2.0-2.1 μm at diastolic filling pressures. The physiological range likely increases with the size of the animal, but even in canine hearts fixed at end-diastolic pressure, the SLs were below 2.1 μm. Rat cardiomyocytes have a compliance similar to mouse myocytes and we thus expect a comparable physiological SL range in mice.

4.3 Advantages of work-loop measurements at the single cell level

Work-loops offer a specific advantage over isometric contractions which have been the norm over the past decades, and that is the sensitivity to changes in diastolic properties. As the slope of the EDFL is shallow, a small change in the slope will result in a significant change in end-diastolic sarcomere length, while other parameters remain equal. As our data show, a 0.1 um increase in end-diastolic sarcomere length results in a ~100% change of the maximum work that can be produced at that pre-load. This is illustrated in the schematic drawing of Figure 4D. The figure also shows the contrast with equivalent isometric contractions, where a small change in the slope will result in a small change in the measured end-diastolic force that, although important, may sometimes not even be noticed. It could be argued that the bias towards systolic changes with studies using isometric contractions has led to a research bias towards treatments that affect systolic function. We therefore think that the ability to measure work-loops at the single cell level adds an important tool to study the functional consequences of disease and treatment options in animal models, notably with respect to diastolic dysfunction.

This paper describes for the first time a method that can be used practically in testing the effect of drugs or disease models that affect diastolic function by measuring its effect on the work that the myocyte can perform.

Funding

D.I. acknowledges the support of the European Research Council (grant agreement n. 615170) and of the Stichting voor Fundamenteel Onderzoek der Materie (FOM). J.v.d.V. is financially supported by the Netherlands organization for scientific research (NWO, VIDI grant 91711344) and CVON-consortium grant (ARENA). We also acknowledge support from the 7th Framework Program of the European Union (“BIG-HEART”, grant agreement 241577) and from ICIN-Netherlands Heart Institute.

Conflict of interest

M. Helmes is a shareholder in Ionoptix Ltd.
REFERENCES

30. Raman S, Kelley MA, Janssen PM. MIMICKING THE CARDIAC CYCLE IN INTACT CARDIOMYOCYTES
31. Chung CS, Granzier HL. Contribution of titin and extracellular matrix to passive pressure and measurement of sarcomere length in the mouse left ventricle. J Mol Cell Cardiol. 2011;50:731-739


ONLINE DATA

The experimental approach

The difference between the set-point and actual force is measured and a proportional correction signal is sent to the piezo translator. Two parameters affect the correction: the iteration frequency of the work-loop algorithm and the multiplier of the proportional correction. We set the iteration frequency close to the update frequency of the force transducer (20 kHz).

We then increased the multiplier of the proportional correction till we saw oscillations in the force trace due to the feed-back loop. At that point we halved the multiplier and that gave us reasonably good feed-back control without oscillatory artifacts. When tuned for experiments at room temperature we achieved an almost perfectly square work loop, a true force clamp, but at 37°C there was some initial overshoot of pre- and after load set-points.

At the end of systole the load clamp has to be released so that the myocyte can relax isometrically. We wanted to achieve this similarly to the termination of systole in the heart, which is the closing of the aortic valve when the ventricular pressure drops below the arterial pressure. In the ‘ejection’ phase of the after-load control, the piezo moves to shorten the myocyte in order to keep the force level equal. We found the best way to terminate this phase was to find out when the force dropped below the after-load level. This happens when the piezo translator reverses direction and starts to stretch the myocyte in order to maintain the after-load level. We achieved this by tracking the minimum position of the piezo translator during the ejection phase. As soon as the piezo translator position was at a user-defined threshold above the minimum, the systolic phase was deemed complete and the load clamp was terminated. We tried a similar approach for the diastolic phase. But as changes in late diastole are slow, any disturbance would make the algorithm terminate that phase too early. It was therefore more reliable to terminate the diastolic force clamp (the ‘pre-load’ clamp) at the next pacing mark.

As can be seen in Figure S3, the feedback was under-damped, resulting in an overshoot of the after-load and undershoot of the pre-load. Increasing the loop speed or the multiplication constant (Kp) of the proportional correction led to better clamping at the fast parts of the transient, but often resulted in force oscillations in phases where force changes were relatively slow. As under-damped feed-back had no great consequences for the data collected, we opted to under-dampen the response to avoid oscillation in the feed-back loop.

Cell isolation

Adult wild-type male and female Wistar rats (N=7) weighing ~300 grams were anesthetized with isoflurane. Subsequently hearts were quickly removed and rinsed in cold isolation-Tyrode (Tyro) solution (composition: 130 mmol/L NaCl, 5.4 mmol/L KCl, 3 mmol/L NaPyruvate, 25 mmol/L HEPES, 0.5 mmol/L MgCl2, 0.33 mmol/L NaH2PO4, 22 mmol/L glucose) containing 0.2
mmol/L EGTA (Tyro-EGTA) and pH 7.4. The heart was then cannulated through the aorta and perfused via the Langendorff apparatus for 2 min with Tyro-EGTA at 37ºC. Thereafter, the heart was perfused with enzyme-Tyro solution consisting of Tyro solution, 1.2 mg/mL collagenase (Type II, 265 U/mg; Worthington Biochemical, NJ, USA) and 50 μmol/L CaCl2 for a period of 7 min. The right ventricle and atria were removed, and the left ventricle was cut into small pieces and repetitively pipetted with a plastic Pasteur pipette for 3 min in stopping buffer solution-1 (SB-1; composition: Tyro solution, 0.6 mg/mL collagenase, 100 μmol/L CaCl2, and 10 mg/mL bovine serum albumin (BSA)). The cell suspension was filtered through a 350 μm nylon mesh into a 50 mL Falcon tube and centrifuged for 1 min at 27xg (20-23ºC). The pellet containing cardiomyocytes was resuspended in stopping buffer solution-2 (SB-2; composition: Tyro solution, 250 μmol/L CaCl2, and 10 mg/mL BSA) and incubated for 10 min in a water bath at 37ºC allowing cells to settle. The supernatant was discarded and cardiomyocytes were resuspended in stopping buffer solution-3 (SB-3; containing Tyro solution, 500 μmol/L CaCl2 and 10 mg/mL BSA), incubated for 10 min at 37ºC and subsequently resuspended for storage in Tyrode consisting of 1 mmol/L CaCl2, 133.5 mmol/L NaCl, 5.0 mmol/L KCl, 1.2 mmol/L NaH2PO4, 1.2 mmol/L MgCl2, 10 mmol/L HEPES and 11.1 mmol/L glucose. Similar Tyrode solution was made, but with 1.8 mmol/L calcium, which was used for intact cardiomyocyte experiments. Cardiomyocytes were used within the first 6h after isolation. All experiments were performed under approval of the Animal Care and Use Committee of the VU University Medical Center (Amsterdam) conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

**ONLINE FIGURES**

**Figure S1.** The experimental chamber. (A) aluminum insert used for heating the bath; (B) thermistor to measure the temperature of the insert; (C) platinum stimulation electrodes; (D) coverslip at the bottom from which the cells are picked up; (E) resistance wires wrapped around the insert for heating; (F) leads connecting the resistance wires to the to temperature controller.

**Figure S2.** Force-length relation at room temperature. (A) At room temperature (~20 ºC) we achieved square loops. (B) At the given range of after-load, we are able to detect the maximum work development of a single cardiomyocyte.
Figure S3. Screen shots from a work-loop collection experiment. (A) top; force tracing, middle; motor movement (all unfiltered data), bottom; displaying the 4 control phases of the work-loop algorithm. Diastole is the longest period, terminated by the next pacing pulse (tick marks at the bottom of each graph) (B) Workloops plotted from the traces in a. The small bulge in the top left corner of the loop is the threshold artefact, where the motor reversal marks the end of systole. The myocyte was paced at 2 Hz.

Figure S4. Example of the protocol at one pacing frequency (4Hz/240BPM). (A) Pre-programmed changes in after-load and pre-load, with below the resulting force and length tracings. (B) Plotting force vs. length of the same traces gives work-loops. High afterloads lead to narrow loops, producing little mechanical work. Lower after-loads lead to an increased amount of mechanical work. Of note: the data show hysteresis when afterload is reduced and then increased again.

Figure S5. Generated work and sarcomere length (SL) relation. (A) For each cell the maximum amount of work at three pre-load levels is plotted. Although there clearly is a positive correlation, the variation is high. The largest source of variation are difference in cross sectional area. The second source of variation is sarcomere length inhomogeneity. As we follow one particular region we are subject to sampling this inhomogeneity. A lot of this variation can avoided by plotting the relative change in work vs. change in sarcomere length (B). Here we find that for each 0.1 micron change in sarcomere length, the maximum amount of work doubles (106%).
Figure S6. Repeatability of the experiments. (A) At the top shows a screenshot of the raw data of a complete experiment, where the cardiomyocyte is paced at various frequencies and the after-load and pre-load are varied to establish the relation between end diastolic sarcomere length (EDSL) and the maximum amount of external work the cardiomyocyte can produce (for a less condensed view of the protocol, see figure S4). (B-I) In a number of cells (n=8), the range of pacing frequencies was followed by a return to the 4 Hz pacing frequency for which the pre-load and afterload vs. work relation was established again. These cells allow us to look at the repeatability of the measurements. Figure B through I show the maximum work vs. EDSL relation between the first and second run at 4 Hz (cell 1 corresponds to the raw data in figure A). As can be seen, the data are usually reproducible, with a tendency towards a small decrease in slope, which may be indicative of a minor run-down.

Figure S7. The comparison between the first and second run. (A) The slopes of work-end diastolic sarcomere length (EDSL) relation at first and second run. A paired t-test on these data confirms that there is no significant change between the first and second run. (B) These data were then put in a Bland-Altman plot which is used to indicate whether two measurements are in agreement. The repeated measures here are well within the confidence interval.

Video S1. Collecting cardiomyocyte force-length loops at varying pre- and after-loads. Real-time data collection of force and length (background), with in the foreground the xy-plot of force vs length, resulting in work-loops. The protocol followed was the same as in online-figure S4A, but of a different cardiomyocyte, paced at 2 Hz (A), 4 Hz (B) and 8 Hz (C).

Video S2. The post-rest potentiation effect of a single cardiomyocyte. This phenomenon was performed by abruptly switching the pacing frequency from 8 Hz to 1 Hz, resulting in a ~5 fold increase in cardiomyocyte work.
Pre-activation of cardiomyocytes determines contractile force; role of titin and calcium

Aref Najafi
Martijn van de Locht
Maike Schuizt
Ilse Bollen
Max Goebel
Coen AC Ottenheijm
Jolanda van der Velden
Michiel Helmes
Diederik WD Kuster

In preparation.
Titin functions as a molecular spring and cardiomyocytes are able, through splicing, to control the length of titin. We hypothesized that together with diastolic [$Ca^{2+}$]titin-based stretch pre-activates cardiomyocytes during diastole and is a major determinant of force production in the subsequent systolic phase. Through this mechanism titin would play an important role in active force development and length-dependent activation.

Mutations in the splicing factor RNA binding motif protein 20 (RBM20) result in expression of large, highly compliant titin isoforms. We measured single cardiomyocyte work loops that mimic the cardiac cycle in wild-type (WT) and heterozygous (HET) RBM20 deficient rats. In addition, we studied the role of diastolic [$Ca^{2+}$] in membrane-permeabilized WT and HET cardiomyocytes.

Isolated intact cardiomyocytes isolated from HET left ventricles were unable to produce normal levels of work (55% of WT) at low pacing frequencies, but this difference disappeared at high pacing frequencies. Length-dependent activation (force/SL relationship) was blunted in HET cardiomyocytes, but force/end-diastolic force relationship was not different between HET and WT cardiomyocytes. To delineate the effects of diastolic [$Ca^{2+}$] and titin pre-activation on force generation, measurements were performed in detergent-permeabilized cardiomyocytes. In these cells cardiac twitches were simulated by transiently exposing cardiomyocytes to 2 µM [$Ca^{2+}$]. Increasing diastolic [$Ca^{2+}$] from 1 to 80 nM increased force development twofold in WT. Higher diastolic [$Ca^{2+}$] were needed in HET. These findings are consistent with our hypothesis that pre-activation increases active force development. Highly compliant titin allows cells to function at higher diastolic [$Ca^{2+}$].

1. INTRODUCTION

During exercise and increased stress, cardiac work is adjusted to meet the increased demands of the body. Work of the heart is modulated by changing contractile force as well as relaxation capacity. Cardiac contraction and relaxation depend on preload (Frank-Starling mechanism, i.e. length-dependent activation), inotropic state (sympathetic drive) and afterload (peripheral resistance). The giant protein titin is a central player in cardiac muscle relaxation and contributes to impaired relaxation in cardiac disease (LeWinter & Granzier, 2013; Linke & Hamdani, 2014).

Titin's role in regulating passive tension is well-established. In the heart, titin is spliced into two isoforms, the stiff N2B isoform and the longer and more compliant N2BA isoform. Changes in titin isoform composition and phosphorylation occur during development of cardiac disease, which contribute to altered cardiac performance (LeWinter & Granzier, 2013; Linke & Hamdani, 2014). Titin is not only a passive spring element involved in regulating muscle stiffness, but can also influence cardiac contraction. Studies that elucidated the role of titin in cardiac contractility have mainly been performed in murine models. In the Greaser laboratory however a rat model was identified with very large titin isoforms (Greaser et al., 2008). Later it was established that a mutation in the splicing factor RBM20 caused these ‘giant’ titin proteins (Guo et al., 2012). In addition, mutations in RBM20 have been identified as a cause for dilated cardiomyopathy in patients, which were associated with expression of highly compliant ‘giant’ titin isoforms (Brauch et al., 2009; Guo et al., 2012; Beqqali et al., 2016). While large compliant titin isoforms reduce passive tension (Greaser et al., 2008; Patel et al., 2012; Methawasin et al., 2014) and thus may improve cardiac relaxation (Methawasin et al., 2014), expression of giant titin isoforms have been associated with reductions in maximal force (Patel et al., 2012; Mateja et al., 2013; Methawasin et al., 2014) and length-dependent activation (Patel et al., 2012; Methawasin et al., 2014). Moreover, reduced RBM20 expression, either via a mutation or knock-out, leads to ventricular dilation (Brauch et al., 2009; Guo et al., 2012) and decreased survival in rodents (Brauch et al., 2009). Interestingly, the effects seem to be dose-dependent, as heterozygotes have intermediate effects on titin length, passive force, slack sarcomere length and length-dependent activation (Methawasin et al., 2014). Having a large compliant titin isoform might also confer some benefits, as exercise capacity was increased in heterozygote RBM20 mutant mice (Methawasin et al., 2014).

We hypothesized that titin-based stress together with diastolic $Ca^{2+}$ pre-activates the cardiomyocyte during diastole and that this is a major determinant of force production in a subsequent contraction. To test this, we measured work in intact cardiomyocytes isolated from RBM20+/− (HET) and wild-type (WT) rats at different pacing frequencies. By using membrane-permeabilized cardiomyocytes, we defined the effect of increasing diastolic calcium levels on myofilament force production.
2. METHODS

2.1 Cell isolation
All animal experiments were performed under approval of the Animal Care and Use Committee of the VU University Medical Center (Amsterdam) conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Adult rat cardiomyocytes were isolated from WT (N=7) and RBM20 HET (N=7) male and female rats, weighing ~300 grams as described before (Helmes et al., 2016). The isolated cardiomyocyte measurements were performed in a tyrode solution (composition: 130 mmol/L NaCl, 5.4 mmol/L KCl, 3 mmol/L NaPyruvate, 25 mmol/L HEPES, 0.5 mmol/L MgCl₂, 0.33 mmol/L NaH₂PO₄, 22 mmol/L glucose) containing 1.8 mmol/L Ca²⁺ at 37°C.

2.2 Titin isoform composition analysis
Titin isoform composition was analyzed in rat left ventricular (LV) homogenates using a vertical Hoefer SE600 gel system (Hoefer Inc., USA) with a 1% agarose gel (1% w/v Sea Kem Gold agarose, BioWhittaker Cell Biology Products, USA), 30% v/v glycerol, 50 mmol/L Tris-base, 0.384 M glycine, and 0.1% w/v SDS, as previously described (Warren et al., 2003; Bollen et al., 2017b). Samples were normalized against myosin heavy chain content and loaded in triplicates.

2.3 Protein analysis
Cardiac troponin I (cTnI) phosphorylation and phospholamban (PLN) expression level and phosphorylation levels were determined in isolated cardiomyocytes (n=6 WT, n=5 HET) as described before (Najafi et al., 2016). In short, unphosphorylated cTnI was separated from mono- and bis-phosphorylated forms by Phostag SDS-PAGE followed by Western Blot. cTnI was visualized upon incubation with cTnI antibody (MA1-22700, ThermoFisher Scientific). PLN expression and phosphorylation was studied using site specific antibodies (Total PLN, antibody A010-14, P-Ser16 PLN: antibody A010-12AP, Badrilla). Total PLN levels were normalized to alpha-actinin and PLN-Ser16 phosphorylation was normalized to total PLN levels.

2.4 Intact cardiomyocyte work loop measurements
Single intact cardiomyocytes were attached to the cantilever of the force transducer and to the piezo translator as described previously (Helmes et al., 2016). Briefly, the tips (35 micron glass fiber) of the force transducer and piezo were coated with an aluminum silicate suspension (Iono-tix pre-coat). The pre-coat was air-dried after which they were dipped in MyoTak (Iono-tix). Finally, the cardiomyocyte was attached to the pre-coated and MyoTak coated glass fiber tips of the force transducer and piezo.

To mimic the cardiac pressure-volume (PV) relationship at the cellular level with an analogous force-length (FL) relationship, we implemented a feedback control system that, by modulating the cardiomyocyte length, can bound the force generated by the cardiomyocyte between a predefined preload and afterload. Preload and afterload is nomenclature in whole heart measurements and indicate the volume at end-diastole, and the aortic pressure against which the blood is ejected during systole, respectively. Here it refers to the target force levels of the feedback control during the diastolic and systolic phase of the myocyte contraction, respectively. The work loop protocol was performed as described previously (Helmes et al., 2016).

2.5 Pre-activation protocol of membrane-permeabilized cardiomyocytes
From a small piece of LV, cardiomyocytes were isolated as described previously (van der Velden et al., 1998). The membrane-permeabilized cardiomyocytes were glued by using shellac (wax-free Sigma Aldrich, 78471; 0.07 mg/ml 70% ethanol). The single membrane-permeabilized cardiomyocyte was glued between an piezo motor and force probe to determine the level of force upon activation with calcium. The experimental protocol that was performed, contained five sets of activations (mimicking time-limited contractions). Each set had a different diastolic [Ca²⁺] (1 nM, 80 nM, 160 nM, 250 nM and 400 nM Ca²⁺), while keeping the activating [Ca²⁺] constant (2 µM Ca²⁺) (see Fig 1). The use of a theta-glass perfusion system (VC-77CSP, Warner Instruments) allowed fast switching between solutions. After three cycles of activation and relaxation with 2 µM Ca²⁺ and 1 nM Ca²⁺, the diastolic [Ca²⁺] was increased from 1 nM to 80 nM, whereas the activating [Ca²⁺] remained the same (i.e. 2 µM). We repeated this procedure also for diastolic calcium solutions with 160 nM, 250 nM and 400 nM Ca²⁺, while the activation solution contained 2 µM Ca²⁺. The forces were normalized by averaging the force of the first three cardiomyocyte activations (i.e. 1 nM as diastolic and 2 µM Ca²⁺ as activation solution) and set at 1 at time 1 second from the switch of the pipet from diastolic to the activating calcium. We investigated if increasing diastolic calcium might result in a higher active force at 2 µM Ca²⁺ relative to the first activation (i.e. 1 nM Ca²⁺).

2.6 Data analysis
Data analysis and statistics were performed using Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean ± SEM of all single cardiomyocytes per rat group. Data was tested for normality by Kolmogorov-Smirnov normality test. When data was normally distributed and in the case of testing one variable in more than two groups, the groups were compared using one-way analysis of variance (ANOVA); in the case of two or more variables, the data were compared using a two-way ANOVA. If a significant value in two-way ANOVA was detected, a Holm-Sidak multiple comparison post hoc test was performed to identify significance within multiple groups. Significance was accepted when p<0.05. N indicates the number of animals; n indicates the number of cardiomyocytes measured.
PRE-ACTIVATION OF CARDIOMYOCYTES WITH CA2+

CHAPTER 6

3. RESULTS

3.1 Longer titin, but no changes in cTnI and PLN phosphorylation in HET compared to WT

Titin isoform expression was determined in the freshly isolated cardiomyocytes. As previously reported, longer titin isoforms were found in the HET compared to WT cardiomyocytes (Fig 2A). Because phosphorylation of cTnI and PLN are important regulators of cardiac contractility, we studied their phosphorylation levels. Using Phostag analysis to assess cTnI phosphorylation, we found no difference in cTnI phosphorylation between the two groups (Fig 2B). PLN expression and phosphorylation were measured by immunoblot analysis and no difference in PLN expression levels (Fig 2C) and phosphorylation (Fig 2D) was observed.

3.2 Reduced work output in RBM20 cardiomyocytes only at low pacing frequencies

To explain the previously reported higher exercise capacity in HET rats (Methawasin et al., 2014), we performed work loop experiments in isolated adult intact cardiomyocytes at different pacing frequencies.
frequencies (1-8 Hz). By stretching the cells to four different preloads and constantly changing the afterload, we could explore the parameter space of pre-load – afterload and work and could establish the pre- and afterload combination that produced maximal work (for details, see Helmes et al. 2016). This was performed at each pacing frequency for both WT and HET (Fig 3A & B). At low pacing frequencies the maximal work was significantly lower in HET compared to WT cells (Fig 3C). WT cells produced their maximal work at lower SL than HET. When increasing the pacing frequency to 8 Hz, the maximal work was no longer different between the groups, although the SL at which this occurred was still longer in HET (Fig 3D). When looking at the range of frequencies from 1 to 8 Hz, the maximum work output per contraction decreased in WT cardiomyocytes, while in HET, maximum work per contraction increased with each increase in pacing frequency (p interaction <0.05).

3.3 Systolic force development is dependent on end-diastolic force
For each contraction the end-diastolic force (EDF), end-diastolic sarcomere length and force development (end-systolic force – EDF) were measured. Because cardiomyocytes are stretched to different preloads during the work loop protocol, we could determine the EDF/EDSL ratio, and how much an increase in SL and in EDF influences developed force on the subsequent contraction. As expected, the EDF/SL relation was significantly lower in HET than in WT cardiomyocytes at the different pacing frequencies (Fig 4A). Previous articles reported blunted length-dependent activation (LDA) in this model, (Patel et al., 2012; Methawasin et al., 2014). Here we found that the developed force/SL relation was also severely blunted (Fig 4B). Thus with an equivalent increase in SL, the increase in force production is much smaller in HET compared to WT. However, the developed force/EDF relationship was not significantly different between WT and HET cardiomyocytes (Fig 4C). This means that a similar increase in EDF leads to a similar increase in force production in WT and HET. These data indicate that, regardless of titin isoform, the stretch-mediated end-diastolic force is the major determinant of the subsequent systolic force development.

3.4 Pre-activation of permeabilized cardiomyocyte increases force development
Based on the previous result (3.3) it is to be expected that increasing end-diastolic force by increasing diastolic [Ca\(^{2+}\)] will increase force development. To be able to control diastolic and systolic [Ca\(^{2+}\)] and test this hypothesis, we measured force development by detergent-permeabilized, mechanically isolated cardiomyocytes. Transient force development as occurs in intact cardiomyocytes was mimicked using a fast-solution switching setup. With this setup we exposed myocytes to activation calcium (2 µM Ca\(^{2+}\)) for 1 second (Fig 5A). To pre-activate the permeabilized cardiomyocyte, the diastolic [Ca\(^{2+}\)] was increased stepwise. Increasing diastolic [Ca\(^{2+}\)] from 1nM to 80nM increased the subsequent force production two-fold in WT cardiomyocytes (Fig 5B). The maximal effect of pre-activation was reached at 80 nM as a further increase in diastolic calcium reduced force development, mainly by increasing diastolic force.
PRE-ACTIVATION OF CARDIOMYOCYTES WITH CA2+

Figure 4. End-diastolic force not SL determines length-dependent activation. During the work loop protocol cells are stretched to different preload (end-diastolic force (EDF)), while SL is recorded. EDF to SL relationship shows that HET cardiomyocytes require much less force to be stretched, which holds true at different pacing frequencies (N=6, n=18 WT; N=6, n=17 HET) (A). As developed force is measured during the subsequent contraction, developed force to end-diastolic SL relationship can be measured. This relationship is a measure for length-dependent activation. This is severely blunted in HET cardiomyocytes (N=6, n=17 WT; N= 6 n=9 HET) (B). However, if EDF is plotted against subsequent developed force, only a small non-significant decrease is seen in HET cardiomyocytes compared with WT (N=6, n=21 WT; N=6, n=10 HET) (C).

Figure 5. Pre-activation of HET cardiomyocytes occurs at higher diastolic Ca2+. Tracing of a pre-activation protocol. Permeabilized cardiomyocyte was constantly perfused with different diastolic calcium solutions and then exposed to 1 second perfusions of activating Ca2+ (2 µM) (A). Activation was repeated 3 times and the average was used. Note that at high diastolic calcium concentrations, diastolic force is already being produced. Averaged data for active force development in WT and HET cardiomyocytes pre-activated by different diastolic calcium concentrations. WT N=5, n=11; HET: N=5, n=9.

4. DISCUSSION

The role titin plays in regulating passive stiffness has been extensively studied (Linke et al., 1994; Granzier & Irving, 1995). Cardiac muscle cells are able to decrease passive stiffness by favoring production of the longer more compliant N2BA isoform over the stiff N2B isoform (Cazorla et al., 2000; Freiburg et al., 2000). Levels of N2BA and N2B in the heart are regulated by splicing factors (Labeit & Kolmerer, 1995; Freiburg et al., 2000; Bang et al., 2001). Another mechanism to decrease passive stiffness is increasing PKA/PKG/Erk2 phosphorylation or decreasing PKC phosphorylation (Hamdani et al., 2017). The in vivo consequences of decreasing titin stiffness have been extensively studied since the seminal finding from Greaser et al. who identified a rat model that expressed a large highly compliant isoform (Greaser et al., 2008). It was later identified that mutations in the splicing factor RBM20 caused these compliant titin molecules (Guo et al., 2012) and that mutations in the RBM20 gene can cause dilated cardiomyopathy (Brauch et al., 2009; Guo et al., 2012). These compliant titin molecules lead to a severe reduction in passive force in cardiomyocytes in rats (Greaser et al., 2008; Patel et al., 2012), mice (Methawasin et al., 2014) and humans (Beqqali et al., 2016). The effects on active force development however are less clear. Maximal force production in permeabilized cardiomyocytes was reduced in RBM20 knock-out (KO) rats (Patel et al., 2012) and mice (Methawasin et al., 2014), but not in HET mice (Methawasin et al., 2014). Echocardiography derived fractional shortening was not changed in HET and KO rats (Guo et al., 2012), although both of them showed dilation and reduced survival. In mice, only homozygous loss of RBM20 resulted in reduced fractional shortening, but both HET and KO mice showed reduced in vivo contractility (measured as end-systolic elastance) (Methawasin et al., 2014). In myofibril experiments it was shown that kinetics of contraction (k act and k tr) of skeletal muscle are reduced in KO rats (Mateja et al., 2013). Furthermore, reductions in length-dependent activation were seen in rats (Patel et al., 2012), mice (Methawasin et al., 2014) and human (Beqqali et al., 2016). While mutations in RBM20 resulted in reduced contractility and dilated cardiomyopathy, RBM20 HET mice showed increased exercise capacity (Methawasin et al., 2014; Bull et al., 2016). Furthermore, in an experimental hypertensive model in mice, increasing titin compliance, through inducible functional knockout of RBM20, corrects subsequent diastolic dysfunction and increases exercise capacity (Methawasin et al., 2016).

Here we show that force development of cardiomyocytes is largely determined by the end-diastolic force. The end-diastolic force is a combination of passive and active force and the (Fig 5B). In HET cardiomyocytes, a different pre-activation response was seen. Almost no pre-activation takes place at low diastolic [Ca2+] (Fig 5B). In these cells, the maximal effect on pre-activation was seen at 400nM Ca2+.
interplay between them determines the force development. As the HET cardiomyocytes have a more compliant titin, they produce less work than WT cardiomyocytes at low pacing frequencies, where end diastolic [Ca\(^{2+}\)] is also low. When increasing pacing frequency to ≥ 6Hz the work produced per contraction is reduced in WT cells while HET cells produce more work compared to low pacing frequency. We show that HET cardiomyocytes are able to increase active force development when end-diastolic force is increased and when diastolic [Ca\(^{2+}\)] is high. These results give insight into the paradoxical findings that having long compliant titin results in contractile deficits at rest, but increased exercise capacity.

The effect of increasing titin stiffness on cardiac function has been assessed by removing part of the titin gene, resulting in shorter and thus stiffer titin proteins. Mice with stiff titin isoforms showed increased end-diastolic pressure volume relationships (Bull et al., 2016; Hinze et al., 2016) caused by increased passive stiffness of cardiomyocytes (Bull et al., 2016) and myofibrils (Elhamine et al., 2014). Stiffer titin isoforms lead to increased kinetics of contraction in myofibril experiments (Elhamine et al., 2014). In contrast to what is observed in mice with giant titin isoforms, these mice have a reduced exercise capacity (Slater et al., 2017).

In the current study we show that rat cardiomyocytes with compliant titin produce less work at low pacing frequencies comparable with the reduced contractility found in this model (Methawasin et al., 2014). But the difference in work output is no longer present at higher pacing frequencies, which could contribute to the increased exercise capacity seen in mice with more compliant titin (Methawasin et al., 2014; Bull et al., 2016). Increasing pacing frequency leads to an increase in diastolic Ca\(^{2+}\)-levels (Frampton et al., 1991; Layland & Kentish, 1999; Gattoni et al., 2016). Here we have shown that HET permeabilized cardiomyocytes produce more active force if they are exposed to high diastolic calcium levels (Fig 5B). WT cardiomyocytes show this pre-activation effect only at small increases of diastolic calcium and this effect gets smaller at higher diastolic calcium. Our measurements indicate that high diastolic calcium can overcome the reduced contractility of HET cardiomyocytes. The latter observation can explain the contradicting findings of reduced basal contractility and increased exercise capacity in RBM20 deficient models.

Pre-activation through increased end-diastolic force (or passive force in skinned cardiomyocytes) has been proposed previously for skeletal muscle (Granzier & Wang, 1993) and cardiac muscle (Cazorla et al., 2001). Multiple studies have shown that the level of passive force determines the amount of length-dependent activation (Granzier & Wang, 1993; Cazorla et al., 2001; Terui et al., 2008) and that titin plays an important role in this regulation (Terui et al., 2008). Consistent with previous data (Methawasin et al., 2014), developed force was less dependent on SL in HET than in WT cardiomyocytes (Fig 4B). However, the relationship between end-diastolic force and developed force was not different between WT and HET cells (Fig 4C). This indicates that it is not SL per se that determines LDA, but rather the level of end-diastolic force that is generated.

How the level of end-diastolic force determines the developed force of the subsequent contraction (pre-activation) is not exactly understood. From this and other studies it is clear that titin plays an important role. It has been suggested that stretching increases the ordering of myosin heads on the thin filament (Farman et al., 2011) or through titin based ordering of troponin C (Ait-Mou et al., 2016). Also, a more direct role for titin in active force development, through unfolding and refolding of its IgG domains, has been proposed (Rivas-Pardo et al., 2016). The findings in the current paper are consistent with both mechanisms.

Increases in titin compliance are not only caused by RBM20 mutations. They are a common feature in cardiac disease. Increased levels of the more compliant N2BA isoform (usually expressed as the N2BA:N2B ratio) are seen in most human disease samples. Patients with end-stage heart failure due to ischemic heart disease (Neagoe et al., 2002), dilated cardiomyopathy (Makarenko et al., 2004; Nagueh et al., 2004; Bollen et al., 2017a), heart failure with preserved ejection fraction (Borbely et al., 2009), peripartum cardiomyopathy (Bollen et al., 2017a) and hypertrophic cardiomyopathy (Nijenkamp, 2018) all show increased N2BA:N2B ratios. This increase in compliant titin isoform expression is usually considered to serve as compensation for the increased fibrosis seen in these disease states. Intriguingly, it could also be a way to cope with the high levels of diastolic calcium seen in end-stage disease.
REFERENCES


CHAPTER 6

PRE-ACTIVATION OF CARDIOMYOCYTES WITH CA2+


CHAPTER 7

Summary, general discussion and future perspectives
SUMMARY AND DISCUSSION

This aim of this thesis was to define mechanisms that modify cardiomyopathy pathophysiology. The most common cardiomyopathy, hypertrophic cardiomyopathy (HCM) is caused by mutations in genes encoding contractile proteins in the heart. But this causative mechanism is more complex than simply: gene mutation -> phenotype. For instance, the age of onset of disease varies a lot, even in patients with the same mutation. Some mutation carriers do not develop a phenotype at all. Clearly, there is more to HCM than only a mutation. This thesis focused on secondary disease triggers. In part I we focused on β-adrenergic receptor (β-AR) signalling in HCM (chapters 2-4). This pathway is part of the fight-or-flight response of the body and therefore one of the most important regulators of cardiac function. Dysregulation of this pathway is often seen in cardiac disease and we defined its role in HCM. We also studied the effect of exercise in a HCM model (chapter 4). Here we also studied the role of sex on HCM pathophysiology.

In the second part we developed a novel method to study cardiomyocyte function (chapter 5). This method allows us to mimic the pressure-volume loop of the whole heart, at the single cell level. This method was used to study the effect of a RBM20 mutation, associated with dilated cardiomyopathy (DCM). In rodents, this mutation leads to DCM with impaired systolic function, while exercise capacity is increased. We studied active and passive force development in this model to explain these aberrant findings.

Exercise and hypertrophic cardiomyopathy

Exercise is almost always considered as something highly beneficial. However in the latest guidelines from the European Society of Cardiology (ESC) and the American Heart Association (AHA), HCM patients are “recommended to avoid competitive sports” (ESC) or “should not participate in competitive sports” (AHA). These recommendations were made based on earlier findings that HCM was the most common cause of sudden cardiac death (SCD) in young athletes. More recent studies have contradicted these findings. Moreover, Saberi et al. presented a prospective population study in Australia and New Zealand where HCM accounted for only 4% of SCD cases, with only 6 cases of SCD occurring during or immediately after exercise. This observation might challenge the widely held view that HCM is the leading cause of SCD in young athletes, and whether competitive sports are truly increasing the risk of arrhythmia and SCD in HCM. The question remains whether exercise restriction is necessary to reduce the risk for SCD in athletes with HCM. On the basis of the Australia/New Zealand patient cohort study, the author proposed a new guideline for HCM patients, i.e. promoting light and moderate exercise in the clinical care of the patient, while for vigorous and high-intensity exercise a more extensive decision-making model is warranted. It is also important to note that the race of the patient could play a role in disease outcome. A recent study in Afro-Americans suggests that “black” patients with HCM are underrecognized and they are underreferred for effective therapy such as myectomy and implanting cardiovascular defibrillators. Clinical trials are under way which will establish if exercise is harmful or may even be beneficial in HCM.

We studied in chapter 4 the role of exercise in a mouse model of HCM. The HCM mice showed an increase in LV weight upon a 8-week exercise protocol compared with the sedentary mice. The myofilament Ca²⁺ sensitivity increased with exercise, which corresponded with lower cardiac troponin I (cTnI) phosphorylation in these animals. Finally, in HCM mice, exercise resulted in higher maximal generated tension of the sarcomere. These findings suggest that besides the adaptation of the heart, voluntary exercise might be a beneficial factor for the force generating capacity of the sarcomere. However it should also be considered that hyperactivation of the sarcomere might also affect the resting phase of the myocyte and thereby lead to a less efficient filling of the heart. Last and not least is the fact the male and female HCM mice reacted differently upon 8-weeks wheel running; this phenomenon will be discussed below.

β-adrenergic receptor signalling in HCM

During exercise heart rate and stroke volume (and thereby cardiac output) are increased due the sympathetic nervous system activation. (Nor)adrenaline is released and activates cardiac muscle cells by binding to the β-adrenergic receptor (β-AR). The increased contractility and relaxation of the heart following β-AR stimulation is mediated by protein kinase A (PKA) phosphorylation of different downstream targets. In different cardiac disease states, such as heart failure, dysregulation of this signalling pathway takes place.

In Chapter 2 we reviewed the role of β-AR signalling in cardiac disease in general. In Chapter 3 we focussed on its role in HCM. We studied basal β-AR signalling in an HCM-associated mouse model and found lower phosphorylation of the contractile protein cardiac troponin I, but normal phosphorylation levels of Ca²⁺-handling protein phospholamban (PLN). On a functional level, we found in membrane-permeabilized cells from the homozygous (KI) Mybpc3-targeted knock-in mice, a significantly higher myofilament Ca²⁺-sensitivity and passive tension. These two parameters were normalized to WT level with exogenous PKA pre-treatment. The restored myofilament Ca²⁺-sensitivity in KI to WT level after PKA treatment may indicate that the difference in Ca²⁺-sensitivity between KI and WT was mainly due to hypophosphorylation of the sarcomeric proteins rather than the sarcomeric mutation itself. This phenomenon was also observed in PKA-treated cardiomyocytes obtained from HCM patients who underwent myectomy surgery as well as a transgenic HCM mouse model. In line with the Ca²⁺-sensitivity pattern, PKA pre-treatment of permeabilized single cardiomyocytes reduced the high passive tension in KI cardiomyocytes, which indicates a lower PKA-mediated phosphorylation of titin in KI mice. To establish if the β-AR signaling route was affected in HCM cardiomyocytes, we studied the response to the β-AR agonist isoprenaline. β-AR stimulation in intact cardiomyocytes...
revealed a blunted increase in myofilament response in KI, while the Ca²⁺ handling response was maintained. This disparity was explained by an attenuated increase in cTnI phosphorylation in KI compared to WT, while the increase in PLN phosphorylation was maintained. The blunted myofilament response upon isoproterenol is in line with previous data in HCM-associated cMyBP-C null mice. It seems that the selective β-AR signalling to one compartment (sarcoplasmic reticulum, SR) over another (myofilaments) could be a compensatory mechanism to maintain cardiomyocyte force development, despite lower β-AR signalling. This would lead to a system where systolic function is maintained, but diastolic function is impaired. This is the phenotype that is seen at an early stage in HCM patients.

Sex as disease modifier in HCM
In various clinical studies sex-related differences in HCM disease onset and severity have been reported. A study by Olivotto and co-workers in a HCM patient population, reported that females were underrepresented, suggesting females are less likely to develop symptoms. Moreover, at disease onset females were on average 9 years older than males. This phenomenon was also found in a recent study in patients with overt HCM that underwent a myectomy. The latter study revealed that women demonstrate a higher degree of diastolic dysfunction than men at the time of myectomy. Also ‘heart failure’-associated titin isoform switch, to a more compliant isoform, was more pronounced in female than in male HCM patients. In contrast to the previous study, late in the 90s, it was reported that females undergo less cardiac remodelling. The study showed that interventricular septum thickness is lower in females compared to males. This phenomenon is in line with our LV mass data in female HET mice at sedentary state. A recent study in patients carrying MYBPC3 mutation showed larger atrial and ventricular dimensions and lower fractional shortening (FS) in males than in females. This is in contrast to our findings in chapter 4 in MYBPC3 mutant mice where FS was not different between male and female mice. We did find that LV weight and the size of cardiac lumen was lower in females compared to male mice, which is in line with the study by Page et al. in MYBPC3 mutation carriers. In a relatively small clinical study in male and female MYBPC3 mutation carriers, females showed a delayed onset of left ventricular hypertrophy compared with males. Similarly, Schulz Menger et al. found a lower remodeling index in HCM women than in HCM men. The remodeling index was based on the relation between LV weight and LV volumes. So, women may have some mechanisms, such as genetic (modifier genes on sex chromosomes) and hormonal factors, preventing the development of hypertrophy and resulting in later onset of disease in female patients, which may be related to under-representation of women with HCM.

In Chapter 4, an interesting finding in female HET mice was the high myofilament force generation compared with the male counterparts. However, the cellular passive stiffness, a measure of relaxation property of the cell was higher in females than in male mice, which is in line with the recent study in HCM patients, in which women showed more severe diastolic dysfunction than men. In addition to relaxation, it has been widely reported that also fibrosis might affect the relaxation property of the heart. Noteworthy, perturbed relaxation at cellular level together with fibrosis deposition (in females) as reported by Nijenkamp et al., might be a sufficient source for development of cardiac arrhythmias in female HCM individuals. However, the opposite was demonstrated by Maron and colleagues. SCD was significantly more likely to occur in male than in female athletes, although the mechanism is still unclear. A recent cross sectional study with HCM patients with cardiac phenotype (HCM LVH) and mutation carriers with no phenotype (genotype+ LVH), reported that vigorous exercise was correlated with increased LV mass only in genotype+ LVH+ patients, which were more frequently females (64%) upon exercise, the heart of the male and female HET mice adapt differently to the voluntary exercise protocol. Female HET showed an increase in Ca²⁺-sensitivity, lower cTnI phosphorylation and a significant increase in LV mass (i.e. hypertrophy). It seems that physiological stimuli elicit a sexually dimorphic cardiac response in HET MYBPC3-targeted knock-in mice (figure 1).

Developing new tools to study cardiomyocyte function
To fully understand primary cardiomyopathies we need to establish the functional defects occurring in cardiomyocytes. To expand our toolbox we validated in Chapter 5 a new method to investigate contractile function, specifically the force-sarcomere length relation of a single intact cardiomyocyte. These cardiomyocyte work-loop measurements mimic cardiac pressure-volume (PV) loops, that have been widely used in cardiovascular research. The ability to measure work-loops in intact cardiomyocytes fills the experimental void that is currently present. Current experimental protocols for assessing cardiomyocyte function, either use membrane-permeabilized cardiomyocytes or (un)loaded intact cardiomyocytes. Membrane-permeabilized cardiomyocytes are prepared by using a detergent that removes the lipid bilayer membrane, as well as intracellular organelles, but leaves intact the myofilaments. The permeabilized cells are glued between a force transducer and a length motor. With this technique, exogenous Ca²⁺ has to be provided to initiate contraction (as the SR is also inactivated by the detergent), thus providing insight on the myofilament function of the cardiomyocyte. Sarcomere length is held constant during contraction (i.e. isometric contraction). From this protocol, maximal force generation of cells can be established, Ca²⁺ sensitivity of contraction, passive stiffness, cross-bridge cycling kinetics, cooperativity and length-dependent activation. These measurements are used in chapters 3 and 4. Advantages of this technique are that they can be performed on cells isolated from frozen material (such as patient biopsies) and that you directly assess myofilament function. A drawback is that this experiment does not exactly match a cardiac contraction in timing and Ca²⁺-mediated contraction and relaxation. Also in the cardiac cycle, the cells are only isometric for a brief period, with shortening (during
Measurement of contractile function in intact cardiomyocytes are also possible. Most studies of intact cardiomyocyte function use unloaded measurements. In unloaded studies, single cells can shorten during contraction as they are not attached to other cells or a force transducer. The level and kinetics of shortening and re-lengthening are measured. Furthermore, by loading the cells with a Ca\(^{2+}\)-sensitive fluorescent indicator, the Ca\(^{2+}\) transient can be assessed. These measurements were performed in Chapters 3. Unloaded measurements do not fully mimic the situation in the intact heart, as no force is measured.

Attachment of intact cardiomyocytes to a force transducer was enabled by work of the Lederer laboratory. They developed MyoTak, a glue that does not perturb the lipid bilayer of the cell. This allows intact cardiomyocytes to be attached to a force transducer and length motor. Force being produced during a contraction (initiated by field stimulation) can be measured. Furthermore, by movement of the length motor cells can be stretched so that length dependent activation can be measured. A disadvantage of loaded cardiomyocyte experiments is that they are technically challenging, because of the gluing and low forces that are produced (5-10 times lower than in permeabilized cardiomyocytes). Additionally, these measurements are isometric and therefore do not fully represent the different phases of the cardiac cycle.

To faithfully reproduce the cardiac cycle in a single intact cardiomyocyte, a work-loop protocol was established in Chapter 5. To enable these experiments, a number of challenges had to be overcome. Most notably the low forces produced by an intact cardiomyocyte and a feedback mechanism to allow switching between isometric (the isovolumetric phases at the organ level) and shortening/lengthening (ejection/filling) phases of the work-loop. To accomplish this a new force transducer with high sensitivity and responsiveness was needed. We validated the use of a novel force transducer based on interferometry which has very high sensitivity. By controlling preload (by stretching the cell to a certain force is reached before starting contraction) and afterload (the force to which a cell isometrically contracts, before it is allowed to shorten), cardiomyocyte work-loops were measured. These force-length work-loops are not one to one comparable to one dimensional PV loops. With the current setup we are not able to control the dynamic force in systole to mimic the changes in impedance during the ejection phase. By using more advanced real-time programmable apparatus, which will allow future modifications of the algorithm that do take these complexities into account.

Dilated cardiomyopathy mutation lead to contractile deficits

After the validation of this new technique we determined the force-length relation in a cardiomyopathy model. We used a rat model that carried a RBM20 mutation. RBM20 mutations lead to the expression of high compliant titin and dilated cardiomyopathy. In addition, these studies revealed that RBM20 deficiency also results in fibrosis and reduced length-dependent activation. Interestingly, an enhanced exercise capacity and a reduction of diastolic stress have been reported in RMB20 animals. The maximal running distance, a measure of exercise capacity, was significantly increased in heterozygous RBM20 deficient mice. In Chapter 6 we measured the work development (which is measured as the area within the work-loop) in heterozygous RBM20 deficient rats at multiple pacing frequencies. We found that compliant titin leads to reduced work development at low frequency, but not at higher pacing frequency.

We identified that having a highly compliant titin leads to reduced end-diastolic force and that this end-diastolic force is an important determinant for the amount of active force that can be developed during the next contraction. Titin-based stiffness is one way of increasing end-diastolic force, but another mechanism would be increasing diastolic Ca\(^{2+}\) concentration (which also occurs at high pacing frequencies). We found, using permeabilized cardiomyocyte experiments, that cardiomyocytes from RBM20 mutant animals require a larger increase of diastolic calcium to increase their systolic force development than WT cells.

Although the RBM20 model is an extreme model of increasing titin compliance, changes in titin isoform expression are also seen in HCM and other cardiac diseases. It is an intriguing thought that the increased expression of compliant titin isoforms that is seen in heart failure, could be an adaptive mechanism in response to increased diastolic Ca\(^{2+}\) levels seen in end-stage cardiac disease. Normally, increased diastolic Ca\(^{2+}\) would lead to increased passive force, and therefore decreased filling of the heart. But by increasing titin compliance, passive force at the myofilament level is reduced somewhat, while active force is maintained. On the long-term this increased diastolic Ca\(^{2+}\) would lead to activation of Ca\(^{2+}\)-based pathological remodelling and would therefore still be detrimental. But it might function as a short-term fix for maintaining passive and active force.

According to the cross-bridge theory (i.e. the interaction between the thick and thin filament), there are two ways to increase cross-bridge forces: (1) by increasing the average force per cross-bridge, or (2) by increasing the proportion of attached cross-bridges (or a combination of the two). The increase in pacing frequency may induce a higher intracellular [Ca\(^{2+}\)] of the myocytes, which may interact with the compliant titin and thereby influence the active force development during systole. Many studies demonstrated the interaction between Ca\(^{2+}\) and the titin spring region, which could influence the muscle stiffness and force production. In the presence of Ca\(^{2+}\), the configuration of the extensible I-band of titin changes. Moreover, it has been reported that the stiffness of the I-band region increases when Ca\(^{2+}\) interacts with the PEVK and Ig domain of titin. The Ca\(^{2+}\)-titin interaction imposes a passive strain on the
SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

THICK FILAMENT PROTEINS, WHICH MAY REDUCE THE LATTICE SPACE AND ALTER THE ARRANGEMENT OF THE CROSS-BRIDGES.

Besides the change in myosin and cross-bridge recruitment, Ca\(^{2+}\)-dependent interaction between (a more stiff) titin and the thin filament have been observed in the late 90s. Titin binding to tropomyosin at the I-band modulates the stiffness of tropomyosin, leading to Ca\(^{2+}\)-cooperative activation (C-state transition) of additional near-neighborig functional units (i.e. 7 actin, 1 troponin and tropomyosin complex). At optimal SL, the even higher strain on tropomyosin (via greater titin tension on the thin filament), activates more functional units and more binding sites for myosin heads to interact.

All in all, mutations in cardiomyopathies exert direct and indirect effects on contractile function of cardiomyocytes. Together with secondary disease modifiers they lead to cardiac dysfunction. Understanding both the direct and indirect effects should enable the identification of therapeutic targets that specifically target cardiomyopathies.

FUTURE PERSPECTIVES

In this thesis we studied the direct and indirect effects of mutations in cardiomyopathy. In the first part we described that β-AR signaling is selectively disturbed in HCM secondary to the disease causing mutation. Preliminary data from HCM patient and non-failing donor samples revealed the same discrepancy in phosphorylation pattern as we found in our HCM mouse model. More research is needed to explore the exact mechanism behind the selectivity in β-AR signaling, e.g. different expression of the A-kinase anchoring proteins in HCM, changes in localization of phosphatases. More understanding of this pathway could also open a window for therapeutic opportunities to treat diastolic dysfunction in HCM. Currently, β-blockers (or dispyramide) represent a cornerstone of the treatment of overt HCM. They are (both) used as first-line therapy for obstructive and non-obstructive HCM. The main goal of β-blockers is to protect the heart from the chronic catecholamine stimulation of β1- β2-ARs, which results in “re-sensitization” of the β-AR signalling pathway. Besides the β-blockers, more research and understanding is necessary of other novel, but preclinical therapeutic treatments, such as adenylyl cyclase-5 inhibitors, lowering G protein-coupled receptor kinases expression and targeting PKA-A-kinase anchoring proteins interaction, which may be of relevance to treat HCM in the future.

Alteration in β-AR signaling or other secondary disease modifier is frequently investigated with one primary disease modifier, for example a gene mutation. It would be interesting to elucidate the role of multiple primary disease modifiers, together with a secondary disease modifier,
such as multiple gene mutations with exercise. In HCM, the presence of multiple pathogenic sarcomere mutations has been correlated with earlier onset and greater disease severity. In 3 families, multiple sarcomere mutations were associated with increased risk of sudden death, even in the absence of conventional risk factors. Future research should also take into account the disease progression of a small number of patients with double or triple sarcomere mutation, which (also) can be exposed to many disease modifiers.

There are multiple candidate (gene) modifiers outside of the sarcomere. These factors are for example environmental factors, gene modifiers and microRNAs. Genetic polymorphism variants that may affect HCM penetrance and expression have been predicted. An example of a gene modifier are variants in the calmodulin gene. A study by Carrier and colleagues reported that any calmodulin variant could affect Ca2+ handling and contractile properties of the heart in HCM. The authors showed that a polymorphism in the promoter region of calmodulin III was more prevalent in clinically affected gene carriers than in phenotype negative family members. Besides the gene modifiers, microRNAs have been proposed in HCM disease pathology. Interestingly, patients with MYBPC3 mutations have a specific miRNA expression profile that differs from non-failing donors. For example miR-451 seems to regulate cardiac hypertrophy and cardiac autophagy. The down-regulation of miR-451 in HCM may contribute to the development of hypertrophy and may be a potential therapeutic target for this disease. We investigate the in chapter 4 the effect of two disease modifiers (i.e. sex and exercise) in HET KI mice. Thus, it seems that there is a certain modulation of the disease penetrance with these factors, however more research is needed to elucidate the contribution of each disease modifier in mutation carriers or manifest HCM individuals with single or double mutations.

Testing this phenomenon is possibly doable in the laboratory by using engineered heart tissue (EHT) from patient. To main goals of the EHT technique are to produce in vitro tissues for cardiac repair; to advance in vitro models of heart function; and by mere fascination of observing a heart muscle beating in the dish. It is a promising method to investigate the 3D EHT in artificial hearts. The therapeutic experiments with heart cells recently became easier with the presence of multiple disease modifiers, but also to test therapeutic responses in these modifier in mutation carriers or manifest HCM individuals with single or double mutations. The therapeutic experiments with heart cells recently became easier with the new high throughput system from CytoCypher. This setup enables researchers to measure cardiomyocyte function in the presence of a drug with a high number of cells is a short period of time with a high accuracy and confidence.

REFERENCES

6. Sophie Wells MEJR, MD; Viraj Bhattacharjee, MS; Martin S. Maron, MD; Barry J. Maron, MD. Association Between Race and Clinical Profile of Patients Referred for Hypertrophic Cardiomyopathy. Circulation. 2018;137.


Hypertrofische cardiomyopathie (HCM) is een erfelijke hartspierziekte, waarbij de hartspier in de loop van tientallen jaren lokaal verdikt. De verdikte hartspier ontspant niet goed meer en verliest zijn elasticiteit. Het verdikte hartweefsel kan het uitpompen van bloed de aorta in gedeeltelijk blokkeren. HCM wordt veroorzaakt door mutaties in het DNA; meer specifiek de stukken DNA die coderen voor eiwitten betrokken bij contractie van het hart. De kleinste functionele eenheid van spieren zijn sarcomeren. De sarcomeren bestaan uit dikke- en dunne filamentewitten. De meeste mutaties in Nederland en wereldwijd zijn gevonden in de dikke-filamentewitten, namelijk in myosine zware keten (MHC) en myosine-gebonden eiwit C (cMyBP-C). De meeste HCM-gerelateerde mutaties resulteren in aminozuur substituties. Het mutante eiwit wordt opgenomen in de sarcomeer. Een eenheid van HCM het nadeel. De hartcellen van vrouwelijke HCM muizen bleken na inspanning gevoeller te zijn voor calcium, wat gepaard ging met een afname in cTnI fosforylatie en een toename in linkere sarcomeren. De post-translationele modificator waar wij naar gekeken hebben is de cMyBP-C; deze leiden meestal tot verminderde eiwit productie, waardoor er onvoldoende eiwit aanwezig is voor een normale sarcomeer functie.

Ongeveer 1:200 Nederlanders heeft HCM. HCM leidt in meer dan de helft van de gevallen tot klachten. De klachten kunnen variëren van: kortademigheid bij inspanning, vermoeidheid, hartkloppingen, hartritmestoornissen en pijn op de borst. Patiënten worden behandeld met bètablokkers of calciumantagonisten, geneesmiddelen die de samentrekking van het hart verminderen. Als patiënt ondanks het gebruik van de medicatie klachten overhouden, wordt een gedeelde van de verdikte hartspier chirurgisch verwijderd om zo de blokkade te verhelpen. Naast de mutatie zelf zijn er ook andere factoren die het ziekteproces kunnen beïnvloeden. In deel 1 van dit proefschrift heb ik eerst het effect van een post-translationele modificatie onderzocht en bepaald of dit eventueel kan bijdragen aan de functie van de sarcomeren. De post-translationele modificator waar wij naar gekeken hebben is de β-adrenerge signaaltransductieketen. Dit is een proces dat normaliter wordt aangezet bij inspanning. Maar dit proces is mogelijk overmatig stimuleerd in HCM en kan uiteindelijk verstoord raken in het ziekteproces. Uit eerdere patiënt studies is naar voren gekomen dat deze verstoring kan leiden tot verminderde fosforlyatie van de sarcomeer-eiwitten. De verandering in eiwit fosforylatie kan vervolgens de structuur en daarmee de functie van het eiwit verstoren. Voor ons onderzoek is er gebruik gemaakt van een HCM-geassocieerd muismodel, die de situatie van HCM patiënten goed nabootst. Uit deze muis werden enkele hartspiercellen geïsoleerd die vervolgens werden blootgesteld aan een β-adrenerge stimulator. Eiwitanalyse van deze cellen laten zien dat stimuleren van deze signaaltransductieketen een selectief fosforylatiepatroon veroorzaakt. Dit fosforlyatiepatroon is meer in het voordeel van de calciumhuiswond eiwit phospholamban dan de sarcomeer-eiwit troponine I (cTnI).

In deel 2 van dit proefschrift heb ik een nieuwe techniek ontwikkeld en gevalideerd. Met die techniek kunnen wij door middel van een gevoelige en hoge responsiviteit krachtopnemer de kracht van de RBM20 mutatie in RBM20 (RBM20Δ). Het maximale ontwikkelde werk van een intacte hartspiercel was lager in RBM20Δ cellen t.o.v. de gezonde ratcellen; echter op hoge frequenties (bijvoorbeeld 6 en 8 Hz) nam het werk van de HET myocyten toe, terwijl dat van gezonde cellen af nam. Diastolische calcium concentratie zou als modulator een rol spelen in de toename van het werk op hoge frequenties. Daarom hebben we hartcellen aan steeds hogere diastolische calciumconcentraties blootgesteld. Dat heeft geleid tot een steeds toenemende (relatieve) kracht van de RBM20Δ cellen. Daaruit kan geconcludeerd worden dat, diastolisch calcium in combinatie met compliante titine de kracht ontwikkeling van de hartspiercellen (positief) kan beïnvloeden.

In het tweede deel van dit onderdeel heb ik onderzoek gedaan naar het effect van sekse en inspanning op HCM. Eerder is aangetoond dat vrouwelijke mutatiedragers op latere leeftijd HCM ontwikkelen dan mannelijke mutatiedragers. Zodra de vrouwen echter eenmaal aan HCM lijden zijn zij symptomatischer dan mannelijke HCM patiënten. Daarentegen is bekend dat spontane hartdood in combinatie met inspanning significant vakerbij mannelijke dan bij vrouwelijke atleten plaatsvindt. Ik heb in hoofdstuk 4 van dit proefschrift onderzoek verricht naar het effect van geslacht en inspanning op het ziektebeeld van HCM muizen. Tijdens de sedentaire toestand waren de vrouwelijke muizen in het voordeel door meer kracht te ontwikkelen dan de mannelijke lotgenoten. Tijdens inspanning waren de vrouwen echter in het nadeel. De hartcellen van vrouwelijke HCM muizen bleken na inspanning gevoeller te zijn voor calcium, wat gepaard ging met een afname in cTnI fosforylatie en een toename in linkere sarcomeren. De post-translationele modificator waar wij naar gekeken hebben is de cMyBP-C; deze leiden meestal tot verminderde eiwit productie, waardoor er onvoldoende eiwit aanwezig is voor een normale sarcomeer functie.
APPENDIX

Curriculum vitae
List of publications
Acknowledgments/Dankwoord
Aref Najafi is geboren in Herat, Afghanistan. Op zijn 12de vluchtte Aref met zijn ouders naar Nederland vanwege de onveilige situatie in Afghanistan. Na een jaar kreeg de familie Najafi verblijfsvergunning en kon de familie zich gaan vestigen in Capelle aan den Ijssel. Aref mocht naar het IJsselcollege en begon met MAVO, niveau C om zijn taal verder te ontwikkelen. Na een jaar kon hij naar een niveau hoger (niveau D) en vervolgens besloot hij zijn HAVO te halen. Dat lukte hem en 2 jaar later had hij zijn HAVO diploma op zak en begon hij aan de opleiding biologie en medisch laboratoriumonderzoek (BML) op de hogeschool Rotterdam. De opleiding duurde 4 jaar en tijdens zijn opleiding liep Aref verschillende stages; 1) op het RIVM en 2) de afstudeerstage verrichtte hij op het experimentele Cardiologie van het Erasmus MC (EMC), onder de supervisie van Prof. Duncker, Dr. Merkus en Dr. De Beer. Vanaf dat moment begon zijn liefde voor het hart – en vaatonderzoek. Aref werd na de BML opleiding toegelaten op de Master Cardiovascular Research op het VU medisch Centrum (VUmc). De master duurde 2 jaar en gedurende de opleiding (en voor de 2de stage) mocht Aref nog een keer stage oplopen op het experimentele cardiologie van het EMC. De eerste stage van de master was Aref werkzaam op de afdeling Fysiologie van het VUmc, onder de supervisie van prof. Jolanda van der Velden en Nicky Boontje. Na zijn master begon Aref zijn promotieonderzoek op de afdeling fysiologie onder begeleiding van prof. Van der Velden en Dr. Diederik Kuster. De focus van het promotieonderzoek lag in hoe en in welke maten de modulatoren, zoals seks, calcium en beta-adernerge activatie de ziekte hypertrofe cardiomyopathie beïnvloeden. Tijdens zijn promotietraject verrichtte Aref onderwijstaken voor de afdeling fysiologie en samenwerking met Dr. Pieter Koolwijk. Aref was in zijn laatste jaar van het promotietraject de (vice)coördinator van de cursus humane anatomie en fysiologie. Ook was Aref voor gedurende 6 maanden de Coördinator van 3de jaars internationale bachelor geneeskunde studenten in het kader van Joint program for European medical studies (JPEMS). Verder heeft Aref voor 1 dag in de week, gedurende 6 maanden lesgegeven aan het Zadkine college voor de opleiding verpleegkunde, onder begeleiding van mevrouw Hannie Bouwdesteijn. Tot slot, mocht Aref in augustus 2017 beginnen als docent voor de opleiding BML, waar hij zelf negen jaar geleden op afstudeerde. Nu mag hij de toekomstige laboranten samen met de collega's opleiden. Hier eindigt zijn verhaal (of loopbaan) niet. Aref helpt de afgelopen 5 jaar graag vluchtelingen tijdens de transitie van een asielzoeker centrum naar een woning (in een gemeente). Zodra een vluchting naar een woning moet, probeert Aref de vluchtelingen wegwijs te maken in de gemeente en helpen met allerlei administratieve zaken die zij te maken krijgen in de woning. Zo probeert hij een zachte lading voor de vluchting te bewerkstelligen.


APPENDIX


ACKNOWLEDGEMENTS/DANKWOORD

“Be grateful for whoever comes, because each has been sent as a guide from beyond.”
— Rumi

Tot slot mijn dank aan iedereen die gedurende mijn promotietraject heeft geholpen. Want zonder de professionele bijdrage van collega’s en vrienden kon ik dit proefschrift niet samenstellen.

Als eerst wil ik Jolanda bedanken. Toen ik je voor het eerst ontmoette, ergens in 2010 voor mijn eerste master stage dacht ik: wat een vrolijke dame. Vanaf dat moment vond ik je geweldig! Ik wil je bedanken voor je vertrouwen en mijn deelname in je onderzoeksgroep. Ik heb veel van je geleerd, vooral het manuscript dat bij JMCC werd geweigerd, zat ik helemaal doorheen en jij hebt mij geholpen om door te zetten en uiteindelijk werd het in CVR aangenomen. Ondanks je drukke agenda kon ik altijd bij je terecht. Bedankt voor het bieden van mogelijkheden om mij verder te ontwikkelen op de afdeling, bijvoorbeeld mijn deelname aan de BKO, het JPEMS programma en de toestemming om 1 dag in de week les te geven op het Zadkine college. Tijdens mijn promotieonderzoek werd je professor en daarbij ook nog eens afdelingshoofd. Ik heb enorme bewondering voor je visie en motivatie binnen de afdeling Fysiologie en ben er trots op dat ik jou als promotor mocht hebben.

Diederik, jij was een sterke begeleider die altijd tijd probeerde te maken om werk-gerelateerde zaken te bespreken, zelfs toen je in Chicago was. Het eerst manuscript was nogal een drama, ik schreef niet goed en jij hebt mij goed en kundig geholpen met het schrijven van de eerste versie. Onze wegen hebben eerder gekruist, namelijk tijdens de experimentele cardiology periode bij Dirk Duncker. Tijdens mijn master kwam jij naar Amsterdam en daarna werd jij mijn co-promotor en wellicht in de toekomst komen we elkaar weer tegen. Ik heb heel veel van je geleerd en ik ben zeer blij om jou als begeleider mocht hebben. Ik ben geweldig Kuster!

Aimee, ik je bedanken voor alle ondersteuning op alle vlakken op de afdeling. Soms blijft je waarde op de afdeling op de achtergrond door alle hectiek van het labwerk, maar je bent zeer waardevol voor de afdeling. Bedankt voor alles.

Beste Chris of Christano (soms Zris) mij roomi van het MF gebouw. We hebben een fijne periode gehad en we hebben elkaar soms goed uitgeschilden, just for fun (sounds weird!). Ik wil nog steeds je moeder ontmoeten; hopelijk komt die dag dat je moeder een keer voor mij kookt. Super bedankt gozer voor de gezellige periode.
Vashco, “mijn bruur”. I would like to thank you for support during my PhD. For scientific questions as labwork and even the cover of my thesis, I could always ask you for help and you never said no. Thanks for everything vasco. You was a key player during my PhD. You are amazing. Thank you mijn bruur.


Ik wil bij deze de collega’s van o.a. de skeletspiergroep bedanken voor deze geweldige periode. Mijn dank aan Coen, Josine, Marloues, Martijn, Michi, Stefan, Rowan, Mark Eva, Bianca, Denise, Larissa, marit, Deli, Xu, Emmy, Pleuni, Silvia, Anoek Silvia, Vaishali en Barbara.

Michiel, Vanaf de eerste dag van mijn promotieonderzoek werd ik aan jou gekoppeld. Jij bent een intelligente man die zeer betekenisvol was voor mijn promotieonderzoek. Ik mocht vanaf het begin met de Ionoptix opstellingen aan de slag en bedankt voor de ondersteuning daarbij en je input bij de manuscripten.

Max, jij was een belangrijke schakel in mijn onderzoeken. We hebben hele mooie momenten meegemaakt in het MF gebouw en daarna in O2. Ook veel stressvolle momenten. En de agressie die daarbij ontstaat is beangstigend, vooral met die baard van je. Maxie super bedankt voor alles; jij bent geweldig. Ik wil meteen ook Ruud bedanken voor zijn inzet in mijn onderzoeken.

Elza, wat ben ik blij om jou als collega op de HR te mogen hebben. Ik heb heel fijn met je in de wetenschap samengewerkt (resultaat: 1 manuscript) en deze samenwerking kunnen we voortzetten in het onderwijs. Vincent, eerst mijn stagebegeleider en nu een geweldige collega. Ik kan (nog steeds) heel veel van je leren. Bedankt voor alle support.

Pieter, Ton en Rob wil ik bedanken voor alle ondersteuning tijdens mijn onderwijstaken en BKO voor de afdeling. Ik heb er heel veel plezier aan gehad. Bedankt heren.

Ook de collega’s van de vaatgroep, Peter, Jurjen, Joana, Manon en andere collega’s die ik op dit moment niet op kom, wil ik bedanken voor de fijne periode op de afdeling fysiologie.

De collega’s van de elektronica Duncan, Ger, Peter en Andreas wil ik bedanken voor alle hulp en expertise met de myocytopstellingen. Ik heb heel veel van de opstellingen geleerd en aantal aspecten blijven mij nog een raadsel.

Ik wil tot slot mijn collega’s van de Hogeschool Rotterdam bedanken. Margriet, bedankt voor je vertrouwen, advies en begeleiding op de HR. Karin, ik kan heel veel van je leren. Bedankt voor je wijze adviezen. Ook wil ik alle (andere) roomies: Astrid, Heleen, Esther, Meihe, Rinske en Monique bedanken voor alle hulp en ondersteuning om mij wegwijs te maken in mijn eerste jaar op de HR. Ook ander collega’s van de HR: Mike, Barbara, Jos, Ralph Esther Appeldoorn, Corrie, Cora, Joep, Maarten, Jan, Eddy, Paula, Danny, Peggy, Esra, Adriaan, Ronald en Pinar bedankt voor alle momenten.

I would like to thank our colleagues from Hamburg, Lucie Carrier itself en her research group for support and for the development of the mybpc3 mice.

Ik wil mijn familie, vooral mijn ouders Asef en Fatemeh bedanken voor de ondersteuning en liefde. Ook mijn dank aan Freezant en je onvoorwaardelijke liefde, Mahboubeh en Omid voor jullie vertrouwen en support. Niet te vergeten, Rayan mijn liefste baba. Ik hou van jullie allemaal!