Contractile Function of the Human Myocardium

Impact of Troponin Phosphorylation

Viola Kooij

ISBN: 978-90-8570-716-5

Cover: Schematic representation of the cardiac thin filament

Printed by: Wöhrmann Print Service

Acknowledgements:

Financial support by the Netherlands Heart Foundation and the J.E. Jurriaanse Stichting for the publication of this thesis is gratefully acknowledged. Additional financial support was kindly provided by the Dondersfonds.

VRIJE UNIVERSITEIT

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op dinsdag 15 februari 2011 om 13.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

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geboren te Rotterdam

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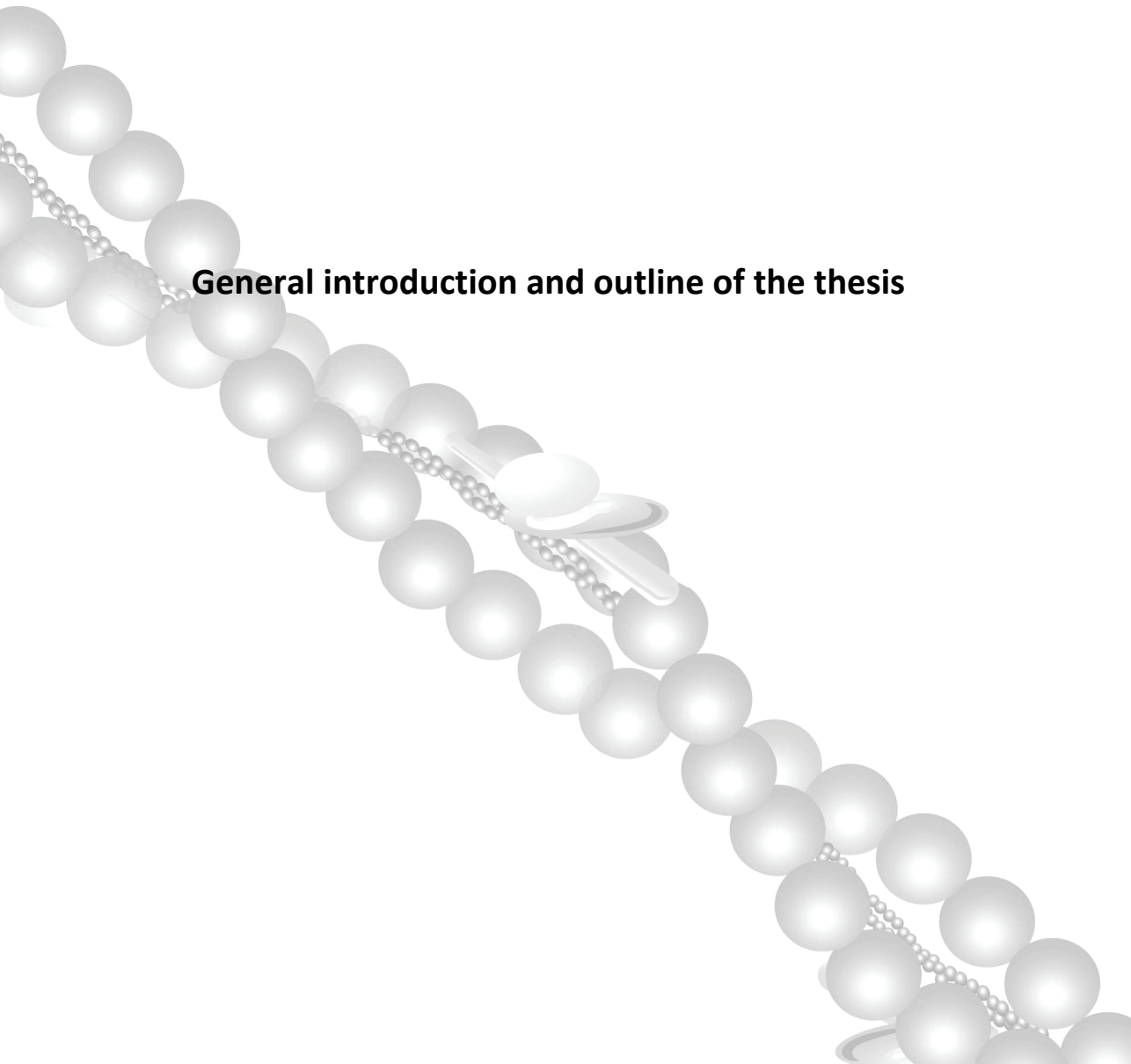
copromotor: dr. J. van der Velden

Contents

Chapter 1



General introduction and outline of the thesis

Heart failure

Heart failure (HF) is a condition in which the heart cannot pump enough blood to meet the demands of the body. It is the end stage of a number of cardiovascular diseases such as arterial hypertension, valvular heart disease, myocardial diseases and coronary heart diseases [1]. The failure of the heart triggers multiple 'counter mechanisms' throughout the body. The activation of intracellular signalling cascades in the heart and vasculature can alter cellular and organ morphology (remodelling) and thereby the function of the heart. These 'counter mechanisms' are initially able to compensate reduced cardiac performance, but they eventually become detrimental in the progression of heart disease [1,2].

Cardiac disease represents one of the main causes of death in the Netherlands. According to the Heart Foundation there are currently ~180.000 people diagnosed with heart failure in the Netherlands and every year this number of patients increases. The incidence of heart failure increases with age and the majority of the patients is older than 75 years. The rates of morbidity and mortality are very high and imposing enormous social and economical costs [2], therefore both prevention and treatment of cardiac disease is of great significance. For this reason, intensive research is a necessity to gain knowledge of the (patho)physiology of the heart, which will eventually help us in the development of new therapeutic strategies.

Cardiac contraction

The heart is a muscular organ enclosed in a fibrous sac, the pericardium. The walls of the heart, the myocardium, are mainly composed of cardiac muscle cells. A thin layer of endothelial cells lines the inner surface of the cardiac chambers. The heart can be seen as two separate pumps, the right part of the heart pumps blood through the lungs and the left part pumps blood to the other organs. Each half/part consists of an atrium and a ventricle separated by the atrioventricular (AV) valves, which permits blood to flow from atrium to ventricle, but not backwards [3-5]. The cardiac cycle includes all events associated with the flow of blood through the heart during one complete heartbeat. The cardiac cycle consists of a period of relaxation called diastole, during which the heart fills with blood, followed by a period of contraction called systole, when the pressure is high and the blood is ejected.

William Howell in 1884, Otto Frank in 1894 and Ernest Starling in 1918 demonstrated that the greater the volume of blood entering the heart during diastole, the more forcefully the heart contracts during systole. As a consequence, with other factors equal, stroke volume

increases as cardiac filling increases. This phenomenon is known as the Frank-Starling law of the heart and is based on the mechanical properties of myocardial muscle cells [6].

Structure of the sarcomeres: thin and thick filaments

Cardiac muscle, like skeletal muscle, is striated. When viewing under a microscope, a distinct pattern of light and dark bands perpendicular to the long axis is observed. The dark stripes crossing the cardiac muscle cell are called intercalated discs; cell membranes that separate individual cells. Furthermore, human cardiac muscle cells tend to have one or two nucleus and have, unlike most skeletal muscle, branching cells (Figure 1).

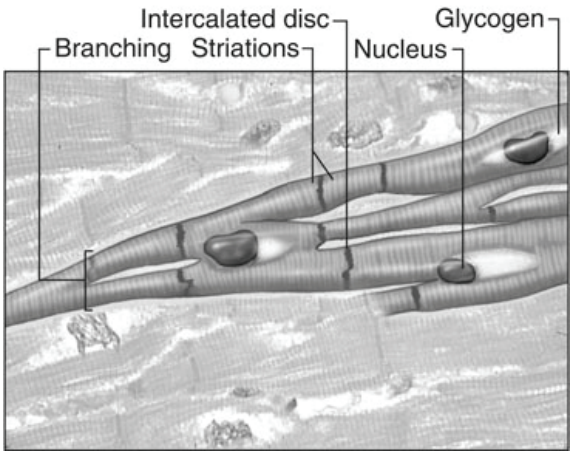


Figure 1. Cardiac muscle: a striated appearance. Figure modified from Widmaier et al. Vander's human physiology [3].

The striated pattern in the cardiac cells is a result of the arrangement of thick and thin filaments into myofibrils (Figure 2). The thick and thin filaments in each myofibril are arranged in a repeating pattern, the sarcomere. The striations are formed by alternating regions of greater and lesser optical density named A- and I-bands, respectively. The sarcomere is bounded by dense Z-discs at the centre of each I-band. The A-band is positioned around the lighter H-zone (Figure 2) [7,8].

Thick filament proteins

The sarcomeric thick filaments (Figure 3) are mainly composed of the motor protein myosin II. Myosin II is a polymer which is composed of two heavy chains (MHC) of ~200 kDa each, which are interconnected through a long twisted tail domain. In the heart, two different isoforms of MHC are present: α-myosin heavy chain (α-MHC) and β-myosin heavy chain (β-MHC). Human myosin is mainly composed of β-MHC with up to 5% α-MHC. The extending domains of the myosin heavy chain are called the two myosin 'heads'. Two myosin light chains (MLC-1 and MLC-2) of ~20 kDa are bound to the neck region of each head. The myosin 'head' is called a cross-bridge, since it bridges the gap between the thick myosin filaments and the thin actin filaments in muscle. The four light chains consist of two essential light chains (ELC, the MLC-1) and two regulatory light chains (RLC, the MLC-2) [9]. Each cross-bridge has ATPase activity, which is activated by interaction with actin.

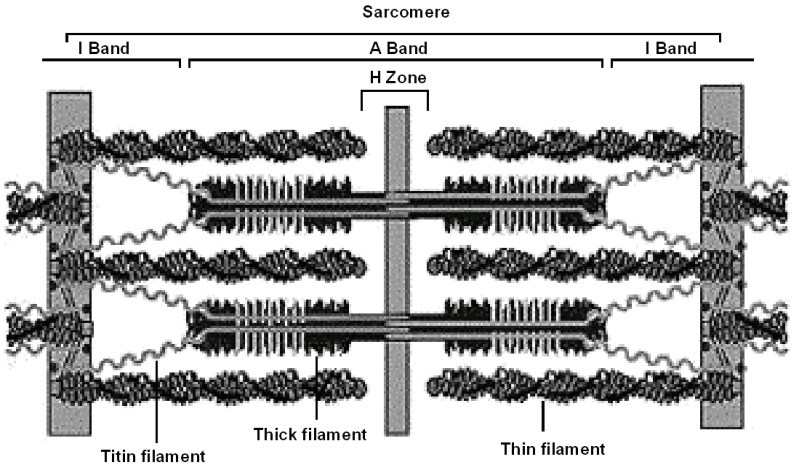


Figure 2. Cardiac sarcomere: schematic representation of the arrangement of the thick and thin filaments. Adapted from Gregorio et al. 2000 Trend in Cell Biology [7].

Force and filament movement depend on the energy released from ATP hydrolysis. In the presence of Ca^{2+}, the myosin cross-bridges bind to actin, which promotes the release of Pi and then ADP. During this process a structural transition in the cross-bridge, suggested to be a change of tilt or change of shape, is thought to produce a relative sliding force between thin actin and thick myosin filaments. Cross-bridges can be released from actin when they bind another ATP molecule [8].

Cardiac myosin binding protein C (cMyBP-C) is a sarcomeric protein associated with the thick filaments, located in the cross-bridge containing A-band of the sarcomere (Figure 2). Cardiac MyBP-C has been assigned a role in assembly and stability of the sarcomere as well as in the modulation of contraction [10]. It has been demonstrated that cMyBP-C knockout mice were viable but showed significant cardiac hypertrophy, myocyte disarray and fibrosis [11]. Two models have been proposed for the arrangement of cMyBP-C in the sarcomere. In the first 'collar' model it is suggested that cMyBP-C molecules form a ring around the thick filament [12]. The second model proposes that the C-terminal runs parallel to the myosin backbone, while the N-terminal domain interacts with neighbouring actin filaments [13,14]. The C-terminal region of cMyBP-C also contains binding sites for the giant protein titin.

Titin is the largest protein found in mammals with a molecular weight of ~3000-3700 kDa depending on isoform composition. At the N-terminus titin is anchored in the Z-disk and to the thin filament and at the C-terminus it is bound to the thick filament. The region of titin located in the I-band (Figure 2) is a complex molecular spring element, which consists of a PEVK domain (rich in proline (P), glutamate (E), valine (V) and lysine (K)), tandem Ig segments and variable N2B and N2A elements [15]. Titin is a major determinant of passive tension in cardiomyocytes. The passive tension results from extension of the I-band region of titin, which elongates as sarcomere length increases. The N2B element alone is present in the stiffer N2B isoform, while both the N2A and N2B elements make up the more compliant N2BA isoform. Both isoforms are co-expressed within the cardiac sarcomere and their ratio determines the passive stiffness of cardiomyocytes [16].

Thin filament proteins

The thin filaments (Figure 3) consist mostly of the ~45 kDa globular protein G-actin. G-actin monomers polymerise spontaneously to form the backbone of the thin filament, F-actin. F-actin is a double helix. The actin helix appears as two right-handed helices, which twine slowly around each other [8,17]. In striated muscle, tropomyosin (TM) is wrapped around the F-actin backbone as a α-helical coiled-coil dimer.

TM modulates the actin-myosin interactions and functions to stabilize the actin structure. It comprises 284 amino acid chains that spans seven actin monomers and contains seven quasi-repeating regions. The TM molecules are linked together through a head-to-tail association, which allows adjacent TM molecules to function as a cooperative unit. The Ca^{2+}-

binding protein complex, cardiac troponin (cTn), is bound to TM [18,19]. The thin filament is comprised of actin, TM and cTn in a 7:1:1 ratio.

Cardiac troponin is a heterotrimer, which consists of the subunits: troponin C (cTnC), the Ca^{2+} binding subunit; troponin I (cTnI) an inhibitor of the actin-myosin reaction that shuttles between tight binding to actin and tight binding to Ca^{2+}-cTnC; and troponin T (cTnT), which binds to TM. The interactions among the cTn subunits, TM and actin are Ca^{2+} sensitive and allow for Ca^{2+} induced conformational changes within the troponin complex, modification of the TM position on the actin filament and the initiation of contraction [9]. During the diastolic phase, the intracellular Ca^{2+} concentration is low and under these conditions, cTnI binds to actin-TM and inhibits the binding of myosin and thereby force generation. During systole, intracellular Ca^{2+} concentrations increase and Ca^{2+} will bind to cTnC. This induces conformational changes in cTnC, which are transmitted and ultimately cause a redistribution of cross-bridges towards the force generating state [20].

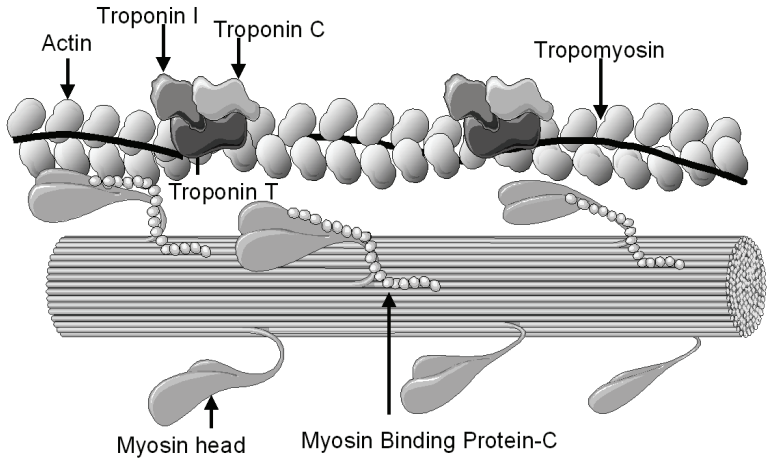


Figure 3. Thin and thick filaments. Schematic representation of the thick and thin filaments. The thick filaments are composed of myosin molecules with myosin binding protein C attached. The thin filaments are mainly composed of actin with tropomyosin and the troponin complex attached.

A three-state model of the thin filaments has been proposed by Geeves et al. (1984) to model the regulation of the interaction between actin, cTn, TM and myosin [21]. TM can occupy three distinct positions on actin. Under relaxing conditions, TM blocks the interaction between actin and myosin heads sterically, called the 'blocked' state. Herein, not even weak myosin binding is possible. Binding of Ca^{2+} to cTnC releases cTn from actin and allows movement of TM to another position on actin, thereby allowing the reaction of weakly bound cross-bridges. This

is called the 'closed' state only permitting weak myosin binding. With increased Ca^{2+} concentrations, the transition from weak to strong cross-bridges pushes TM further to the so-called 'open' state, allowing full force-generation by the cross-bridges [9,18,19].

Phosphorylation of myofilament proteins in the healthy and failing heart

Phosphorylation is a well-studied post-translational covalent modification known to play an important role in myofilament function. Differences are found in cTnI phosphorylation levels between tissues from end-stage failing and healthy human donor hearts, which indicates a role for altered cTnI phosphorylation in the pathophysiology of the heart [22-24]. The functional significance of cTnI phosphorylation under physiological and pathophysiological conditions is important and therefore a topic of extensive research.

Protein kinase A-mediated phosphorylation

β-adrenergic receptor stimulation causes an increase in cardiac output to meet the demands of the body, while under stress or during exercise. Upon β-adrenergic stimulation, there is a direct activation of the cAMP-dependent protein kinase A (PKA) in the cardiac muscle that phosphorylates several proteins. PKA has multiple intracellular targets within the cardiac cell; among them are the thin filament protein cTnI, the thick filament protein cMyBP-C and titin.

cTnI has two sites that are substrates for PKA, Ser23 and Ser24 (Figure 4) [25]. PKA-mediated phosphorylation of cTnI at sites Ser23/24 reduces myofilament Ca^{2+}-sensitivity [26]. This can account for the faster relaxation observed in the heart in response to increased catecholamine levels. Zhang et al. showed that both serines must be phosphorylated in order to observe the decrease in myofilament Ca^{2+}-sensitivity [27]. Also, phosphorylation of Ser23 occurs following phosphorylation of Ser24 [28]. In addition cMyBP-C phosphorylation might be involved in the alteration of the Ca^{2+}-sensitivity and more research is warranted on the matter to dissolve discrepancies in results. β-adrenergic signalling is reduced in heart failure due to downregulation and desensitisation of β-adrenoreceptors [29]. Furthermore, reduced phosphorylation levels of cTnI have been reported in end-stage failing human myocardium compared with non-failing donor hearts [22,23,30]. Cardiomyocytes from human end-stage failing hearts showed a significantly higher myofilament Ca^{2+}-sensitivity when compared to cardiomyocytes from healthy donor hearts. This sarcomeric alteration was normalized upon

treatment with exogenous PKA. This indicates that cTnI phosphorylation by PKA is a prominent determinant of sarcomeric (dys)function in both health and disease [23,31].

Three PKA-mediated phosphorylation sites on cMyBP-C, Ser265, Ser300 and Thr274 have been identified [32,33], while recently a fourth PKA-phosphorylation site has been identified in the M-domain of human cMyBP-C at Ser311 [34]. Reduced phosphorylation levels of cMyBP-C have been reported in end-stage failing human myocardium compared with non-failing donor hearts, however, the exact functional role of cMyBP-C phosphorylation remains elusive [23,31]. Harris et al. showed, in a mouse model, that the absence of cMyBP-C is sufficient to trigger cardiac hypertrophy, impaired systolic cardiac performance and depressed myocyte contractile properties [35]. Stelzer et al. showed that mouse lacking cMyBP-C exhibited a significant acceleration of cross-bridge kinetics, which was evident as an accelerated rate of force redevelopment [36]. Furthermore, they showed that PKA-treatment of wild type mouse myocardium also accelerated force redevelopment, similar as observed with ablation of cMyBP-C, indicating a distinct role for PKA-mediated phosphorylation of cMyBP-C in cross-bridge kinetics [37]. Recently, using recombinant cMyBP-C, it was demonstrated that phosphorylation of the regulatory motif, the M-domain, reduced binding to actin and eliminated actin cross-linking. The authors suggested that the N-terminus of cMyBP-C interacts with F-actin through multiple binding sites and that binding at one or more sites is reduced by phosphorylation [38].

Passive tension, caused by the extensibility of titin, can also be modulated by phosphorylation. β-adrenergic mediated phosphorylation of PKA leads to phosphorylation of the N2B-unique sequence, which reduces passive stiffness in rat and human cardiomyocytes [39,40]. Our group has reported a higher passive stiffness in diastolic heart failure patients compared to systolic heart failure patients, which could be corrected with PKA treatment [41]. In the same year, Krüger et al. reported that PKA phosphorylates titin in human myocardium and that PKA-mediated phosphorylation reduced passive stiffness of human cardiac muscle [39].

Protein kinase C-mediated phosphorylation

Protein kinase C (PKC) is able to modify thin filament function via phosphorylation of cTnI and cTnT. It has been more difficult to ascertain a role for PKC phosphorylation of these proteins than for PKA due to complexities in the PKC signalling pathways, including the presence of multiple isoforms, translocation and localization of each activated PKC isoform, and multiple end-targets [42]. There are approximately 12 different isozymes comprising the PKC family. They

are divided in two groups, the conventional PKC isoforms that are calcium activated (α, β_I, β_{II} and γ) and the calcium independent isozymes (ε, ϕ, η, δ, ζ, ι, ν and μ) [43,44].

PKC is able to phosphorylate cTnI at different sites: Ser23/24, Ser42/44, Thr143, Ser76 (or Thr77), and cTnT at Thr194, 203, 284 and Ser89 (Figure 4) [45-47]. An earlier report also showed Ser1 on cTnT as a phosphorylation site [48]. The exact functional consequences of cTnI and cTnT phosphorylation by PKC are largely unknown, since diverse PKC-mediated changes have been reported in the responsiveness of myofilaments to Ca^{2+} both in terms of sub-maximal and maximal force generation. Evidence suggests that phosphorylation at Ser43/45 on cTnI decreases maximum Ca^{2+}-activated tension in skinned fibres and maximal sliding velocity in in vitro motility assays [49-53]. However, these studies were performed in rodents. Our group reported an unchanged maximum Ca^{2+}-activated tension upon incubation with the catalytic subunit of PKC in human skinned cardiomyocytes [24]. Furthermore, PKC phosphorylation has been reported to decrease Ca^{2+}-sensitivity [51,54], while phosphorylation of Thr144 was found to sensitise cardiac myofilaments to Ca^{2+} [55].

Ser265, Ser300 and Ser1169 are the major PKC-mediated phosphorylation sites found in cMyBP-C [32,33]. A specific role for PKC-mediated phosphorylation of cMyBP-C on the contractility has yet to be determined.

Recently, Hildago et al. demonstrated with phosphorylation assays and mass spectrometry that the PEVK region of titin is a PKCα substrate. They identified Ser170 and Ser26 as specific PKCα phosphorylation sites. They also showed that PKCα increased the passive tension and that the passive tension further increased when cells were first dephosphorylated by protein phosphatase 1 (PP1) [56]. However, we did not observe an increase in passive force in human cardiomyocytes incubated with the catalytic subunit of PKC [24,57].

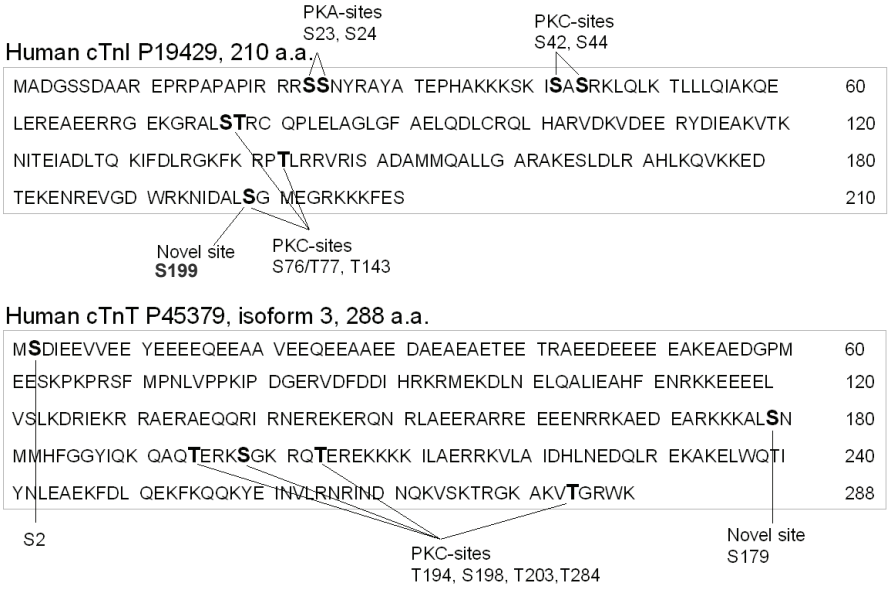


Figure 4. Amino acid sequence of cTnI and cTnT. Human cardiac cTnI and cTnT sequence containing the PKA and PKC phosphorylation sites. Cardiac TnT isoform 3 is the dominantly expressed isoform in human adult hearts [58]. The positions of the novel sites (identified in chapter 3) are also indicated. Amino acid sequence based on UniProt KB (cTnI P19429 and cTnT P45379).

Aim and methods

Discrepant observations have been reported in rodent studies on functional consequences in response to myofilament phosphorylation by PKC. Moreover, it is unknown if data from rodent studies can be extrapolated to human. The general aim of this thesis was to determine the influence of PKA- and PKC-mediated phosphorylation of the cTn complex on force development in human donor and end-stage failing cardiomyocytes. Moreover, we aimed to determine a specific role for cTn phosphorylation on contractile properties. Since both PKA and PKC have multiple myofilament proteins as targets for phosphorylation, we used a method in which human recombinant cTn complex is selectively exchanged with endogenous cTn complex in permeabilised cardiomyocytes. This exchange method enabled us to determine force characteristics with direct control of the occupancy of the various phosphorylation sites, without interference from other target proteins.

Exchange method

The cTn subunits (cTnT, cTnI and cTnC) were separately expressed in *Escherichia coli* cells and purified using ion-exchange chromatography. The purified subunits were reconstituted into full cTn complex by mixing the subunits in a 1:1:1 molar ratio. For a direct determination of the effects of cTnI phosphorylation by PKA on contractility in human cardiac preparations without alteration of phosphorylation of other contractile proteins, recombinant cTn complex was incubated with the catalytic subunit of PKA until saturated phosphorylation levels were reached. A similar method was used to study the effects of PKCα mediated phosphorylation of cTn. However, for this purpose recombinant cTn(DD) complex, phosphorylated with PKCα, was used. The recombinant cTn(DD) complex had the two PKA sites Ser23/24 on cTnI mutated into aspartic acid (D) to mimic phosphorylation and thereby ruled out cross-phosphorylation by PKCα.

The exchange is performed in human cardiac myocytes isolated from donor and end-stage failing hearts obtained during transplant surgery and stored in liquid nitrogen. Cardiac tissue is mechanically disrupted to obtain single cardiomyocytes. Triton X-100 treatment is used to permeabilise the cells, which makes it possible to control the intracellular medium and makes the exchange of cTn possible. The suspension of cells was immersed with an exchange solution containing a high concentration of cTn complex (0.5 or 1.0 mg/ml) and left overnight at 4°C. In this way, the recombinant cTn complex in the exchange solution replaces the endogenous complex (Figure 5).

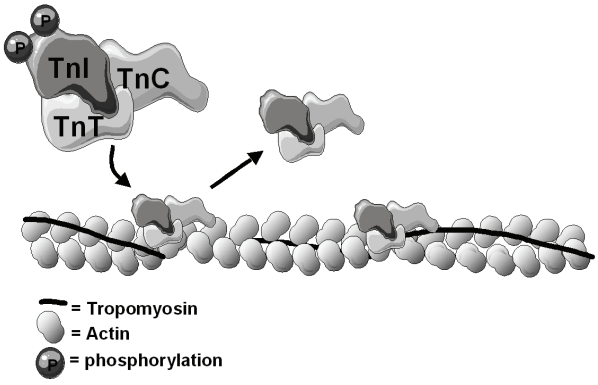


Figure 5. Exchange method. Schematic representation of phosphorylated cTn complex exchanged with endogenous cTn complex. A high concentration of recombinant cTn complex is added to the cardiomyocytes in order to replace the endogenous cTn complex.

Force measurements in single cardiomyocytes

After the exchange protocol, a single cardiomyocyte was glued with silicon adhesive between a force transducer and a motor in a droplet of relaxation solution. The glue was allowed to cure for 40 minutes. The force transducer as well as the motor was connected to joystick-controlled micromanipulators (Figure 6).

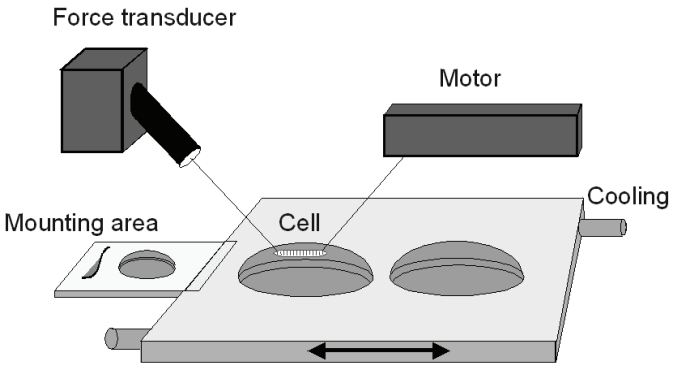


Figure 6. Schematic overview of the myocyte set-up. In the mounting area, a droplet of the suspension of cells and a thin line of silicon glue were applied. The tips of the force transducer and motor were transferred through the thin line of glue and a cardiomyocyte was glued between the tips. Adapted from Van der Velden et al. 1998 Cardiovasc. Res. [59].

Movement of the stage of the inverted microscope could transfer the cell to a well containing relaxation solution. Lateral movement was used to transfer the cardiomyocyte to the next well, which contained the activation solution. The relaxing and activating solutions were maintained at 15°C by means of water circulation (the inlet and outlet of this circulation were connected to a temperature controlled water bath).

Outline of the thesis

In **chapter 2**, we determined the direct effects of PKCα and PKCε phosphorylation of contractile proteins in skinned cardiomyocytes from human donor and end-stage failing heart tissue. Force measurements were conducted on single cardiomyocytes, which were incubated with PKCα or PKCε.

In **chapter 3**, we investigated the effects of targeted PKCα-mediated phosphorylation of cTn on the contractile properties of the myofilaments. PKCα-phosphorylated recombinant cTn complex was exchanged with endogenous cTn in cardiomyocytes from end-stage failing patients.

In **chapter 4** we focused on the effects of PKA-mediated phosphorylation of cTnI on Ca^{2+}-sensitivity of force development. Endogenous cTn from healthy donor cardiomyocytes was exchanged with unphosphorylated cTn complex, while failing cardiomyocytes were exchanged with PKA-phosphorylated cTn complex.

In **chapter 5** the contractile effects of PKA mediated phosphorylation and of pseudo-phosphorylation of cTnI in human cardiomyocytes were compared. The pseudo-phosphorylated cTn complex has the PKA sites Ser23 and Ser24 mutated in aspartic acid (D).

In **chapter 6** the main conclusions of the major findings of this thesis are given followed by future perspectives.

In **chapter 7** a summary is given of the main findings of this thesis in both English and Dutch.