Summary

Post-translational protein modifications are important determinants of cardiac performance. One of the most important post-translational modifications known to exert a regulatory role in myofilament function is phosphorylation. Alterations are known to occur in the phosphorylation status of contractile proteins in patients with heart failure. They can be detrimental or beneficial for the progression of the contractile dysfunction of heart failure. Therefore, it is of great importance to reveal the alterations in the phosphorylation status of contractile proteins in human heart failure and their influence on the functional properties of cardiac tissue.

In this thesis, the effects of PKA-and PKC-mediated phosphorylation of contractile proteins on force development were studied in healthy and failing human cardiac tissue.

Chapter 1

In Chapter 1, a general overview is given concerning: heart failure; cardiac contraction; structures of the sarcomeres; and phosphorylation of the myofilament proteins in the healthy and failing heart. Furthermore, an introduction to the methods used in these studies is given and the aim of this thesis is defined.

Chapter 2

In this chapter, single cardiomyocytes isolated from end-stage failing hearts and healthy donor tissue were incubated with PKCα and PKCε. Hereby, we investigated the role of PKCα and PKCε mediated phosphorylation on contractility in human failing and healthy cardiomyocytes. Protein analysis showed that incubation with both PKC isoforms increased the phosphorylation of cTnI and cMyBP-C in failing cardiomyocytes. Furthermore, after dephosphorylation of cTnT by AP in donor tissue, it became apparent that cTnT is also a substrate for PKCα. Both PKC isoforms caused a decrease in Ca^{2+}-sensitivity in failing cardiomyocytes. The Ca^{2+}-sensitivity of donor cardiomyocytes was not affected, presumably caused by an overall high basal phosphorylation level. Both PKCα and PKCε showed no effects on maximal force and only PKCα resulted in a modest but significant reduction in passive force. To investigate the reduction in passive force, the phosphorylation level of the filament protein titin was analysed. However, the results showed no significant increase in phosphorylation of titin upon incubation with PKCα in either donor or failing cardiomyocytes.

Chapter 3

Triggered by the findings of Chapter 2, we determined whether the decreased Ca^{2+}-sensitivity after PKCα incubation was due to phosphorylation of only troponin. To explore the direct effects of cTn phosphorylation by PKCα, an elegant cTn exchange method was used. The advantage of this technique is that it allows to study the direct effects of PKCα phosphorylated cTn complex on contractility in human cardiac myocytes without altering other contractile proteins. In this cTn complex, the protein kinase A (PKA) sites Ser23/24 on cTnI are mutated into aspartic acids (cTnDD) to rule out *in vitro* cross-phosphorylation of these sites by PKCα. cTn(DD) complex was pre-treated with PKCα (cTn(DD + PKCα)) and partially exchanged (~70%) with endogenous cTn complex in end-stage failing cardiomyocytes. Results showed that exchange with cTn(DD + PKCα) increased the Ca^{2+} sensitivity compared to the cTn(DD) group. Interestingly, the Ca^{2+}-sensitivity was decreased by subsequent incubation of the exchanged cardiomyocytes with PKCα. Furthermore, a depression of the maximal force generating capacity of the cardiomyocytes was observed in the cells exchanged with cTn(DD + PKCα). Western blot analysis of cTn incubated with PKCα revealed phosphorylation at known PKC sites on cTnI, Ser42 and Thr143. Furthermore, two novel phosphorylation sites were identified using mass spectrometry: Ser199 on cTnI and Ser179 on cTnT. These sites may contribute to the complex effects of PKCα-mediated phosphorylation observed in human myocardium.

Chapter 4

In Chapter 4, we determined whether a PKA-induced decrease in Ca^{2+}-sensitivity was solely due to cTnI phosphorylation or depends on the phosphorylation status of other sarcomeric proteins. Using the same exchange method as used in Chapter 3, endogenous cTn in donor tissue (with a high baseline cTnI phosphorylation level) was exchanged (up to 66%) with unphosphorylated cTn complex (cTn). PKA incubated cTn (cTn(PKA)) complex was exchanged into failing cardiomyocytes in which cTnI was largely dephosphorylated. Phos-tag (a method to distinguish phosphorylation species of a protein) gel analysis showed an increase of un-phosphorylated cTn in donor cells exchanged with cTn complex, while failing cells exchanged with cTn(PKA) demonstrated an increase in bis-phosphorylated cTn complex. A very similar distribution of un-, mono- and bis-phosphorylated cTnI in donor control and in failing tissue exchanged with PKA-phosphorylated cTn complex was observed.

Donor cardiomyocytes showed an increase in Ca^{2+}-sensitivity upon exchange with unphosphorylated cTn complex relative to the donor control value. Exchange of cTn(PKA) in failing cardiomyocytes showed no effect on the Ca^{2+}-sensitivity relative to the failing control. Subsequent incubation of the cardiomyocytes with PKA resulted in a significant decrease in Ca^{2+}-sensitivity in both donor and failing tissue. These results indicate that the phosphorylation background of other myofilament proteins influences the impact of PKA phosphorylation of cTnI on Ca^{2+}-sensitivity. We demonstrate that the other myofibrillar targets of PKA, notably cMyBP-C and/or titin, might play an important role in modulating the effect of cTnI phosphorylation on Ca^{2+}-sensitivity in human myocardium.

Chapter 5

Since pseudo-phosphorylation is often used to study the functional implications of cTnI phosphorylation, we determined whether pseudo-phosphorylation of Ser23/24 mimics the physiological effects. In this chapter recombinant cTn complex is used in which Ser 23 and 24 on cTn are replaced by aspartic acids (D). Endogenous cTn complex in failing cardiomyocytes is partially (~70%) replaced by recombinant cTn(PKA) and cTn(DD) complex. Force measurements were conducted to compare the effects on the contractile properties of the cardiomyocytes. A significantly reduced Ca^{2+}-sensitivity was observed in the cardiomyocytes exchanged with cTn(DD), however Ca^{2+}-sensitivity remained unaltered after exchange with cTn(PKA). This dissimilar effect could be due to some dephosphorylation of the contractile proteins of the cardiomyocytes in the cTn(PKA) group, or due to the influence of the phosphorylation background that is abolished in the cTn(DD) group. Dephosphorylation (~19%) of the cTn(PKA) complex could result in a discrepancy between the bis-phosphorylation levels of cTn(PKA) and cTn(DD), since cTn(DD) is not able to dephosphorylate. To tackle this problem, we conducted exchange experiments where ~45% exogenous cTn(DD) complex is incorporated in the failing cardiomyocytes. This would lead to a lower level of cTnI bis-phosphorylation (predicted value 51.2%) compared to cells exchanged with ~70% cTn(PKA) (predicted value 59.4%). A small but significant reduction in Ca^{2+}-sensitivity of force was observed with ~45% exchange of cTn(DD) complex, which was not present in failing cardiomyocytes exchanged with cTn(PKA) complex. These results support the idea that the influence of the phosphorylation background is only present in the cTn(PKA) group and might be abolished in the cTn(DD) group.