Doctoral (Ph.D.) Thesis

Molecular Determinants of Myofibrillar Contractile Function in the Heart

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INTRODUCTION

Chronic heart failure (CHF), accompanied by unacceptably high morbidity and mortality, is a prominent public health problem in the developed societies. Despite the novel therapeutic tools and recently reassessed approaches in the management of patients with CHF the prognosis remains poor. These rigorous facts unambiguously emphasize the need for investigations in order to get further insight into the pathomechanism of CHF. New recognitions may enable us to improve present therapeutic strategies and to develop novel ones.

An increase in the end-diastolic volume results in enhanced contractility of the myocardium. The cellular mechanisms involved in the regulation of the Frank-Starling law are, however, still uncertain. The role of several components has been presumed, e.g. length dependent increase in the calcium affinity of troponin C and decreased lattice spacing thus increased probability of the actin-myosin interaction following stretch. Additionally, alterations in the passive tension may also have both direct and indirect effects on the actin-myosin interaction. Cardiac function is dramatically altered at the organ level in CHF. Presumed changes of the Frank-Starling mechanism as well as those of myofilament Ca\(^{2+}\)-sensitivity in CHF are still controversial. Similarly, alterations in the content, distribution and phosphorylation of the contractile proteins are also ambiguous. For instance, a decrease in myosin light chain 2 (MLC2) content and various changes of its phosphorylation state were observed in CHF. MLC2 phosphorylation is known to be associated with increased Ca\(^{2+}\)-sensitivity hence its phosphorylation state in CHF is of particular importance in terms of contractile force determination. It is also to note that former studies proved regional differences in the electrical and passive mechanical properties of myocytes. We decided therefore to examine the stretch-dependent contractile properties in control and failing hearts taking into account the localisation of the myofilaments across the myocardial wall.
The thick filament component cardiac myosin binding protein C (cMyBP-C) is expressed exclusively in the heart. It contains specific phosphorylation sites and binding sites for actin, titin, and myosin. Its multiple interactions with other sarcomeric proteins predict structural and regulatory roles for the protein. Indeed, mutations in the human gene of the cMyBP-C could be associated with familial hypertrophic cardiomyopathy. Despite, the precise role of cMyBP-C in cardiac contractility regulation and length-dependent activation is still controversial. In previous studies hampered MyBP-C function resulted in increased, unchanged or decreased Ca\(^{2+}\) responsiveness. Protein kinase A (PKA) phosphorylates several contractile proteins including cMyBP-C and the phosphorylation results in decreased myofilament Ca\(^{2+}\) sensitivity. The PKA-phosphorylation of cMyBP-C was shown to modify both the protein’s interaction with actin and myosin and the activity of the myosin ATPase. Replacement of native cTnI by the non PKA-phosphorylatable slow skeletal isoform of TnI (ssTnI) was shown to eliminate the effect of PKA on Ca\(^{2+}\) sensitivity. Based on this observation an exclusive role of cTnI in the mediation of PKA effects on active myofilament contractility with the exclusion of cMyBPC was concluded. In contrast, the cMyBP-C knock out mouse model by Carrier and colleagues exhibited striking impairment of LV relaxation. This finding strongly suggests altered Ca\(^{2+}\) sensitization in the absence of cMyBP-C.

Clinical trials have proved the efficacy of the positive inotropic agent levosimendan in the treatment of heart failure. For cardiac mechanism of action several diverse possibilities (e.g. Ca\(^{2+}\) sensitization, PDE-inhibition, increase in [cAMP]) were suggested. Hence, comparison of inotropic, Ca\(^{2+}\) sensitising and PDE inhibitory potentials of levosimendan with those of the PDE inhibitor enoximone in a therapeutically relevant concentration range may further elucidate its mechanism of action. Furthermore, characterization of the levosimendan metabolite OR-1896 with a half-life of ~80 h may explain sustained hemodynamic effects following levosimendan administration.
Aims

Functional and structural parameters of myofilaments were analysed in order to characterize distinct pathophysiological and therapeutic aspects of chronic heart failure. Applying a wide range of physiological and biochemical methods our experimental research work focused on the following objectives:

I. The Frank-Starling law in heart failure
- To examine relationship between stretch-induced alterations in passive and active contractile properties regarding localisation within the myocardial wall.
- To identify possible underlying contractile protein changes and modifications.
- To compare myofilament stretch-response in control and failing rat heart.

II. Involvement of cMyBP-C in the regulation of myofilament contractility
- To characterize in vitro the myofibrillar contractile properties in a knock out mouse model lacking completely the cardiac isoform of MyBP-C.
- To analyse the impact of cMyBP-C on the stretch- and PKA-phosphorylation induced alterations in active and passive myofilament contractile properties.

III. Levosimendan and OR-1896 – mechanism of action in the myocardium
- To compare the inotropic, Ca$^{2+}$-sensitizing and PDE-inhibitory potentials of levosimendan and OR-1896 in guinea pig heart.
- To reveal the presumptive myocardial mechanism of action of these compounds at their clinically relevant plasma concentrations.

IV. Levosimendan and enoximone – Ca$^{2+}$-sensitization and PDE-inhibition
- To characterize in parallel both the contractile effects at the organ and myofilament level and the PDE inhibitory potentials of levosimendan and enoximone.
- In view of the observed functional properties to define possible differences in their mechanisms of inotropy within the myocardium.
MATERIALS and METHODS

Animals, animal models
Cardiac tissue from rats at 14 weeks following myocardial infarction was used as a model for ischemic heart failure. Effect of cMyBP-C on myofilament contractility was examined in a knock out mouse model with a complete lack of the cMyBP-C. Pharmacological studies were conducted in guinea pig hearts.

Isolated heart preparations
Langendorff-perfused hearts
Cannulation of the aorta allowed retrograde perfusion of the heart. A latex balloon introduced into the left ventricle and the isovolumetric left ventricular pressure signal was recorded. $+\frac{dP}{dt_{\text{max}}}$ was calculated at steady coronary flow and heart rate. Levosimendan and OR-1896 were added in cumulative manner to the modified Tyrode solution.

Working hearts
Anterograde perfusion of the heart was attained by pulmonary vein cannulation. At constant left atrial filling pressure and left ventricular afterload aortic and coronary flow rates, as well as the heart rate were monitored. For perfusion solution modified Krebs-Henseleit buffer (KHB) was used and tested drugs were added in cumulative manner.

Force measurements in myocyte-sized preparations
Myocyte isolation and mounting
Myocyte-sized permeabilized preparations were obtained by enzymatic isolation or mechanical disruption of frozen tissue sample followed by detergent treatment. The demembranated cardiomyocytes were then mounted either with silicone adhesive or optical glue to a highly sensitive force transducer under continuous microscopic video monitoring.

Solutions
Activating solutions containing Ca\(^{2+}\) in various concentrations were obtained by mixing relaxing and maximally activating solutions Ca\(^{2+}\) buffer solutions.

**Experimental protocol**

We used two different protocols to determine myofilament contractile properties. In the first case, passive tension was examined by gradually stretching the cell from slack length to 2.3 μm SL in relaxing solution. Isometric contractures were evoked by positioning the myocyte to the tip of a microcapillary and superfusing it at two different sarcomere lengths with activating buffer solutions containing Ca\(^{2+}\) in increasing concentrations. In the other case the myocyte was transferred from the relaxing solution to Ca\(^{2+}\)-containing solution. In this arrangement the amplitude of force was measured by a slack test protocol. To determine active force passive force component obtained in relaxing solution was subtracted from the peak isometric force developed at various [Ca\(^{2+}\)] levels.

Active force at submaximal levels of activation was normalized to the maximal activation. The active force-[Ca\(^{2+}\)] relationship was characterized by the Ca\(^{2+}\)-sensitivity of isometric force production (pCa\(_{50}\)). The change in pCa\(_{50}\) (ΔpCa\(_{50}\)) described numerically the stretch-induced increase or PKA-induced decrease in Ca\(^{2+}\)-sensitivity, respectively.

**Tested compounds, PKA**

Levosimendan and OR-1896 were kindly provided by Orion Pharma. Enoximone and the catalytic subunit of the PKA were purchased from Sigma.

**Biochemical assays**

**SDS-PAGE**

Titin isoforms and content were analysed with 2.5–7 % acrylamide gradient gels. For cTnI analysis total cardiac protein homogenates were separated by 15 % SDS-PAGE. For visualization Coomassie blue staining or specific antibodies and enhanced chemiluminescence were used.
Western immunoblot
In freshly dissected myocardial strips MLC and TnI composition was analysed with Western immunoblotting following separation on urea gel or SDS-PAGE, respectively. MLC2 isoforms were specifically detected with a cardiac MLC2 antibody. Total TnI content and the PKA phosphorylated form of cTnI was determined applying specific antibodies and ECL.

Real-Time Quantitative RT-PCR
RT-PCR was performed using specific primers for ssTnI and cTnI. Absolute mRNA levels were also calculated from standard curves generated by RT-PCR on serials dilutions of cloned ssTnI and cTnI.

Phosphodiesterase-activity assays
PDE III and PDE IV were isolated from guinea pig hearts, human platelets or human myeloid leukaemia promonocytic cell line (U-937). PDE inhibitory potential of levosimendan, OR-1896 and enoximone were measured by liquid scintillation detection of \[^{3}H\]5’AMP product following \[^{3}H\]cAMP cleavage.

Data analysis
The sigmoidal Ca\(^{2+}\)-force relations were fitted to a modified Hill equation:

\[
F = F_{\text{max}} \times \left( \frac{[\text{Ca}^{2+}]^{n_{\text{Hill}}}}{[\text{Ca}_{50}]^{n_{\text{Hill}}} + [\text{Ca}^{2+}]^{n_{\text{Hill}}}} \right)
\]

, where \(F\) is the steady-state force at a given [Ca\(^{2+}\)]; \(F_{\text{max}}\) is the steady Ca\(^{2+}\)-activated force at saturating [Ca\(^{2+}\)]; \([\text{Ca}_{50}]\) (or pCa\(_{50}\)) corresponds to the [Ca\(^{2+}\)] at which \(F = F_{\text{max}}/2\) (a measure of Ca\(^{2+}\)-sensitivity of the myofilaments) and \(n_{\text{Hill}}\) is the Hill constant, a measure for the steepness of force-pCa relation characterizing the cooperativity within the contractile machinery.

Values are given as mean\(\pm\)S.E.M. Statistical significance was accepted at \(P<0.05\). For calculation one-way or 2-way analysis of variance (ANOVA) followed by Dunnet’s two-tailed test, Student unpaired t-test, Wilcoxon signed-rank test and Holm-Sidak t-test were used according to the applicability.
TRANSMURAL STRETCH-DEPENDENT REGULATION OF CONTRACTILE PROPERTIES IN RAT HEART AND ITS ALTERATION AFTER MYOCARDIAL INFARCTION

RESULTS
Stretch-dependent contractile properties of isolated skinned myocytes from the subepicardial (EPI) and subendocardial (ENDO) layers were analysed and compared in sham and post myocardial infarction (PMI) rat hearts. Myofilament Ca$^{2+}$-sensitivity ($p\text{Ca}_{50}$) was characterized under isometric conditions at 1.9 µm and 2.3 µm SLs. At 1.9 µm SL no significant difference in Ca$^{2+}$-sensitivity between wall layers of sham and PMI hearts was detected (sham EPI: 5.60±0.03, sham ENDO: 5.60±0.01, PMI EPI: 5.59±0.03, PMI ENDO: 5.62±0.02). In contrast, stretching the sarcomeres to 2.3 µm SL resulted in larger calcium-sensitisation in sham ENDO cells than in sham EPI ones ($\Delta p\text{Ca}_{50}$: 0.24±0.02 and 0.11±0.01, respectively). Interestingly, stretch-induced Ca$^{2+}$-sensitisation was identical in the two corresponding myocardial layers of PMI hearts ($\Delta p\text{Ca}_{50}$: 0.14±0.01 and 0.14±0.01). Additionally, steady-state stiffness of the myofilaments was also characterized by stretching the cells from slack length to 2.3 µm SL in relaxing solution. Both in sham and PMI hearts ENDO cells proved to be stiffer than EPI cells ((in mN/mm$^2$) sham EPI: 4.3±0.2, sham ENDO: 8.3±0.4, PMI EPI: 4.8±0.5, PMI ENDO: 8.3±0.9). Detailed analysis of titin proved no isoform difference between the two layers. Instead, it revealed a gradient in the number of titin molecules per half-sarcomere (characterized by the titin/MHC ratio) within the ventricular wall. This ratio proved to be lower in the EPI layers of both sham and PMI animals (sham EPI: 0.22±0.02, sham ENDO: 0.31±0.02, PMI EPI: 0.26±0.02, PMI ENDO: 0.34±0.03). Phosphorylation states of various sarcomeric regulatory proteins were explored across the left ventricular free wall in sham and in PMI hearts. The molecules were examined before and after 1-min stretch (corresponding to ~1.9 µm SL and...
~2.3 µm SL, respectively). We observed no difference in the PKA phosphorylation level of troponin I among the examined groups. Furthermore, by means of urea gel separation four different forms of the myosin light chain 2 (the two ventricular isoforms, VLC2a and VLC2b and their phosphorylated forms, P-VLC2a and P-VLC2b) could be separated. VLC2a was less phosphorylated in the ENDO layer of PMI than in that of sham hearts and the degree of its phosphorylation was not affected by stretch in any of the samples. On the other hand, stretch resulted in a marked increase of the VLC2b phosphorylation in the ENDO-sham cells but not in ENDO-PMI ones.

**DISCUSSION**

For a better understanding of the molecular determinants that underlie the Frank-Starling relationship, this study aimed the characterization of the length-dependent contractile properties of cardiomyocytes from healthy and failing hearts, taking into account their localization within the left ventricular free wall. Stretch-induced Ca\(^{2+}\)-sensitisation (\(\Delta pC_{a_{50}}\)) and passive tension developed at 2.3 µm SL showed a remarkable relationship. In sham hearts a positive correlation between passive tension and the extent of stretch-induced Ca\(^{2+}\)-sensitisation could be established. In contrast, diminished stretch-sensitisation in the subendocardial layer of the PMI hearts blunted the positive correlation. Furthermore, phosphorylation states of various sarcomeric proteins were explored in order to explain the gradient in stretch sensitization across the left ventricular free wall in sham and its alteration in PMI hearts.

**Transmural contractile properties and passive tension**

In our work we demonstrated for the first time a gradient of passive and active mechanical properties across the left ventricular free wall which is considerably altered in the failing, remodelled heart. The increasing number of titin molecules per half-sarcomere (characterized by the titin/MHC ratio) correlated well with the gradient in myofilament stiffness observed along the myocardial wall. The
Transmural contractile properties

present work also confirms previous observation that Ca$^{2+}$-sensitivity of the contractile machinery is more correlated to stretch-induced modification in passive tension than to SL. In fact, titin has been proposed to be involved in the Frank-Starling mechanism at least by reducing the lattice spacing. The SL changes across the ventricular wall during a cardiac cycle are uncertain. It has been proposed, however, that during filling, the nonuniform transmural SL distribution observed in the unloaded state becomes more uniform with the subendocardial sarcomeres elongating more than the subepicardial ones. Also, higher circumferential strains and ejection fraction were observed in the endocardium. In line with these observations our results predict that heterogeneous cellular mechanical properties may contribute to the nonuniform deformation of the ventricular wall.

Stretch-induced Ca$^{2+}$ sensitization and the phosphorylation of the regulatory MLC2

According to recent studies PKA phosphorylation of cardiac troponin I decreases Ca$^{2+}$ sensitivity and is thought to enhance length-dependent activation of the myofilaments. The lack of difference in cTnI phosphorylation among the examined samples suggested no substantial role for cTnI in the regulation of stretch-induced Ca$^{2+}$-sensitization. Instead, structural changes in the thick filaments in response to stretch have been proposed for cardiac muscle. In addition, data suggest that stretch-dependent acivation of cardiac sarcomere cannot solely be explained by lattice spacing, implying involvement of other regulatory mechanisms. Hence, we postulated the existence of a passive tension-induced signalling pathway that could act on top of the length-induced change in interfilament lattice spacing.

Under our conditions a localization-dependent increase in VLC2b phosphorylation could be paralleled with the increased stretch-induced Ca$^{2+}$-sensitization. This result is in accordance with previous observations that myosin light chain kinase (MLCK) phosphorylates MLC2 and treatment of the
myofilaments with MLCK increases Ca^{2+} sensitivity. An increase in the population of myosin heads that could interact with actin was proposed for a feasible mechanism of action. MLC phosphorylation could facilitate the movement of the S1 myosin subfragment away from the thick-filament backbone toward the thin filament. Based on our previous experience that MLCK sensitizes myofilaments, we propose that stretch can result in MLC2 phosphorylation by a yet undefined mechanism.

Stretch-sensitization and chronic heart failure
The myocardial remodeling during the development of heart failure affected this regulatory pathway as manifested in the parallel diminution of both VLC2b phosphorylation and stretch-sensitization in the subendocardial layer of the failing myocardium. Interestingly, disappearance of the positive correlation between passive tension and the extent of stretch-induced Ca^{2+}-sensitization presumed functional alterations in the ENDO-PMI myofilaments. This proposal was supported by the observed decrease in myosin light chain 2 isoform VLC2b phosphorylation in the ENDO-PMI myofilaments. The relationship between the phosphorylation of the VLC2b and the extent of stretch-induced Ca^{2+}-sensitisation suggests an important role for VLC2b in the Frank-Starling mechanism. Additionally, it is presumable that the remodeling affects stretch-dependent regulation of myofilament contractility predominantly in the sub-endocardial layer of the myocardial wall.
ACTIVATION OF MYOFILAMENTS IN A HYPERTROPHIC MOUSE MODEL LACKING THE CARDIAC MYOSIN BINDING PROTEIN C: EFFECT OF LENGTH AND PKA PHOSPHORYLATION

RESULTS
First, we analyzed the passive mechanical properties of myocytes isolated from transgenic mice lacking cMyBP-C in young (~5 weeks, KO-5) and adult (~55 weeks, KO-55) mice exhibiting mild and severe hypertrophy, respectively. Passive tension at 2.3 µm SL did not differ between myocytes isolated from WT and KO mice at the same age. Passive properties were also analyzed following PKA stimulation. Basal phosphorylation level of cTnI and the extent of additional PKA phosphorylation were evaluated by Western blot and proved to be similar in WT and KO cells (27 and 30%, respectively). In agreement with previous observations, PKA treatment decreased the passive tension in both WT and KO myofilaments.

Then the SL-dependent active contractile properties of WT and KO left ventricular myocytes were compared. At both ages, KO myocytes showed higher Ca$^{2+}$ sensitivity at 1.9 µm SL than WT ones (P<0.01, values for pCa$_{50}$ were 5.79±0.03, 5.77±0.01, 5.87±0.02 and 5.85±0.01 in WT-5, WT-55, KO-5 and KO-55 mice, respectively. Increasing SL to 2.3 µm produced a leftward shift of the Ca$^{2+}$-tension relationship (ΔpCa$_{50}$). At 2.3 µm SL, there was no significant difference in the pCa$_{50}$ between WT and KO at either age. The length-dependent activation decreased in KO-5 and KO-55 myofilaments compared with WT. This effect was, however, more pronounced in adult (in pCa unit, WT-55= 0.23±0.01, KO-55=0.16±0.01) than in young mice (WT-5=0.18±0.02, KO-5= 0.14±0.01). Deficiency of cMyBP-C was also associated with reduced slopes of the Ca$^{2+}$-tension relationships at both SLs as indicated by the lower value of Hill coefficients in KO myofilaments, although significance in KO-5 mice at short (1.9 µm) SL was not reach (P=0.17). Maximal active
force values were not significantly different between the WT and KO groups at either SL or age. The Ca\(^{2+}\) sensitivity of myofilaments preincubated with PKA was determined at 1.9 and 2.3 µm SLs and was compared with previously obtained values in control cells. PKA treatment of WT myocytes induced a rightward shift of ~0.13 pCa unit of the tension–pCa curves at both SLs and ages indicating an overall desensitization of the myofilaments to Ca\(^{2+}\). The sensitivity of Ca\(^{2+}\) activation to length after PKA treatment remained unchanged (P<0.01) even when expressed as Ca\(^{2+}\) concentration (ΔEC\(_{50}\)). In KO myocytes, PKA phosphorylation reduced the pCa\(_{50}\) by ~0.03-0.04 pCa unit and had a significant effect only at 1.9 µm SL in KO-5 animals where pCa\(_{50}\) was reduced by 0.07 pCa unit (P=0.012). Maximum tension values were unaffected and the Hill coefficient in KO myofilaments increased to the same extent as in WT.

TnI expression in WT and KO myocytes was analyzed by Western blot with an antibody that recognizes both skeletal and cardiac TnIs. In KO myocardium, preferentially in young animals appeared a lower band of TnI with a molecular weight similar to that of the ssTnI isoform present in soleus muscle. In our conditions, ssTnI represented only 1 and 4% of total TnI in WT-5 and WT-55 mice, and 24% and 7% in KO-5 and KO-55 mice, respectively. The amount of mRNA for ssTnI and cTnI, determined by quantitative RT-PCR did not vary between WT and KO animals and was independent of the age. The crossing point cycle number (CP), used as an index of mRNA expression, did not vary in KO mice when expressed relative to WT at both ages. We observed that the CPs were 24.5±1.2, and 30.4 ±0.8 for cTnI and ssTnI, respectively, indicating that cTnI mRNA amount was approximately 125-fold higher than that of ssTnI mRNA. Furthermore, contractile parameters (the EC\(_{50}\) i.e. the Ca\(^{2+}\) concentration at which half-maximal force is generated and the extent of PKA-induced desensitization of myofilaments) were expressed as a function of the ssTnI content. Analysis of these relationships indicated no correlation between
contractile parameters following PKA treatment and the amount of ssTnI. Instead, the Ca\(^{2+}\) sensitivity of myofilaments at both SLs was rather dependent on the presence of cMyBP-C.

**DISCUSSION**

In the present study, we examined the contractile properties of cardiac myofilaments isolated from cMyBP-C KO and WT mice, at different ages and degrees of hypertrophy. We used physiologically relevant methods for changing the Ca\(^{2+}\) sensitivity of the contractile system: stretch and PKA phosphorylation.

The observed Ca\(^{2+}\) sensitization of myofilaments in cMyBP-C KO mice at short SL is in agreement with previous data obtained by partial removal of cMyBP-C and was similar at both ages, therefore independent of the degree of cardiac hypertrophy. As previously shown with phosphorylation of MLC2 in cardiac muscle, increase in Ca\(^{2+}\) sensitivity and decrease in cooperativity can be paralleled with increased flexibility and decreased order of myosin heads. The increase in Ca\(^{2+}\) sensitivity at 1.9 µm SL in KO mice is accompanied by a decrease in the slope of the tension-pCa curve reflecting a decrease in cooperativity in myosin head attachment and further supporting a decisive role for cMyBP-C in the regulation of myosin head order and movements. The cMyBP-C dependence of Ca\(^{2+}\) sensitivity is consistent with studies applying disruption of normal cMyBP-C function by the addition of S2 or C1C2 fragments, MyBP-C replacement by a truncated form and partial MyBP-C extraction. Nevertheless, our results are in contrast with those obtained in another KO model expressing a truncated cMyBP-C form. Observations presented here suggest that for the differences in Ca\(^{2+}\) sensitivity reported in the studies the different SLs used could, at least in part, be responsible.

cMyBP-C appears to modulate Ca\(^{2+}\) sensitivity through a mechanism regulated by stretch. In the absence of normal cMyBP-C, Ca\(^{2+}\) sensitivity is already enhanced and therefore, further increase in SL has substantially less effect. In
line with our results, previous studies reported the largest effects of cMyBP-C deficiency on $\text{Ca}^{2+}$ sensitization at the shortest SL. Thus, it is predictable that the effects of cMyBP-C manipulation is reduced or abolished with stretch. The present study further supports the view that cMyBP-C is be considered as a myofilament regulatory protein. cMyBP-C phosphorylation by itself decreases interfilament distance. In addition, since cMyBP-C can interact with both titin and myosin, it could transmit part of the strain developed by titin to the myosin heads, as observed in skeletal muscle.

The PKA effect on length-dependent activation reported by Konhilas et al. (2003) was unconfirmed in the present study. In our hands PKA phosphorylation had almost no effect on $\text{Ca}^{2+}$ sensitivity in KO myocytes at either SL, despite equal increases in cTnI phosphorylation of WT and KO. This is in line with the work by Yang et al. (2001) showing impaired response for isoproterenol in mice expressing a mutated cMyBP-C lacking both the titin and myosin binding sites at the C-terminus. The reason for this discrepancy is unclear but differences in the methods and basic level of PKA phosphorylation may play a role.

ssTnI is transiently expressed in the heart during development and in mouse ssTnI is the major isoform at early embryonic stage. In addition, ssTnI is re-expressed in various pathological models suggesting its protective effect against the $\beta$-adrenergic $\text{Ca}^{2+}$-desensitization. In the present study, a maintained expression or a re-expression of ssTnI was observed in young KO animals that disappeared with age. RT-PCR analysis, however, showed similar mRNA levels in KO and WT at both ages, assuming post-transcriptional regulation. Interestingly, PKA desensitization of myofilaments did not correlate with the level of ssTnI expression. It seems thus unlikely that ssTnI is responsible for the observed effects rather they can be linked to the lack of cMyBP-C. As alternative hypotheses for the negligible effect of ssTnI the inadequate quantity expressed or insufficient incorporation into the sarcomeres could be proposed.
THE EFFECTS OF LEVOSIMENDAN AND OR-1896 ON ISOLATED HEARTS, SKINNED MYOCYTES AND PHOSPHODIESTERASE ENZYMES OF THE GUINEA PIG

RESULTS

Positive inotropic effects of levosimendan and OR-1896 in paced Langendorff-perfused hearts with constant coronary flow

The positive peak of the first derivative of the left ventricular pressure signal (+ dP/dt\textsubscript{max}) was regarded as a measure of the contractile state of the myocardium. The drug-free mean baseline values of + dP/dt\textsubscript{max} were 1129±85 mm Hg s\textsuperscript{-1} for the levosimendan group and 1239±67 mm Hg s\textsuperscript{-1} for the OR-1896 group. This parameter revealed highly similar concentration dependences for levosimendan and OR-1896 (n=5 in each group). The maximal drug-induced increase in left ventricular + dP/dt\textsubscript{max} corresponded to relative \( E_{\text{MAX}} \) values of 26±4% and 25±3% over the drug-free baseline (P < 0.05), with a slightly lower (P < 0.05) EC\textsubscript{50} value (15±2 nM) for levosimendan than that for OR-1896 (25±1 nM).

Ca\textsuperscript{2+}-sensitizing properties of levosimendan and OR-1896 in isolated permeabilized myocyte-sized preparations

The maximal Ca\textsuperscript{2+}-activated force production (P\textsubscript{o}) of permeabilized myocyte-sized preparations was determined at pCa 4.75 under drug-free conditions. P\textsubscript{o} normalized to the cross-sectional area was 27±6 kN m\textsuperscript{-2} (n = 20). This value is comparable to that found elsewhere. Isometric force production was measured at maximal (pCa 4.75), and submaximal [Ca\textsuperscript{2+}] (pCa 5.8, pCa 6, pCa 6.2 and pCa 7) (n = 7–10 in each group), using a supramaximal concentration (10 \( \mu \)M) of levosimendan or OR-1896. Neither 10 \( \mu \)M levosimendan nor 10 \( \mu \)M OR-1896 affected either the maximal (P\textsubscript{o}, at pCa 4.75) or the lowest Ca\textsuperscript{2+}-activated force (at pCa 7) (P>0.05). The lack of effect at pCa 7 is in agreement with the
previous observations suggesting unaltered crossbridge dissociation at the diastolic \([Ca^{2+}]\). However, both levosimendan and OR-1896 increased the isometric force production at intermediate \([Ca^{2+}]\) (P < 0.05). \(Ca^{2+}\)-force relationships (obtained by fitting the Hill equation to the mean relative force values) in the absence or presence of 10 \(\mu\)M levosimendan or 10 \(\mu\)M OR-1896 revealed identical \(Ca^{2+}\)-sensitizing characteristics for the two compounds. \(pCa_{50}\) corresponded to \(pCa 5.88\pm0.01\) under control conditions, while it was increased (P < 0.05) identically by 10 \(\mu\)M levosimendan or 10 \(\mu\)M OR-1896 (to \(pCa 5.98\pm0.03\) or \(pCa 5.98\pm0.02\), respectively).

Concentration-dependent \(Ca^{2+}\)-sensitizing and phosphodiesterase-inhibitory functions of levosimendan and OR-1896

The concentration dependences of levosimendan- or OR-1896-induced \(Ca^{2+}\) sensitization were assessed during repeated activations at a single steady submaximal \([Ca^{2+}]\) applying the tested compound in increasing concentration. The drug-free isometric force was \(5.2\pm1\ kNm^{-2}\) (19\% of \(P_o\)) at \(pCa 6.2\) and the amplitude of the isometric \(Ca^{2+}\) contractures increased with the concentration of both levosimendan and OR-1896 (n=20). Force values were expressed in relative terms compared to drug-free baseline. The \(Ca^{2+}\)-sensitizing potential of levosimendan could be illustrated by an \(EC_{50}\) value of \(8\pm1\ nM\) and by a maximal increase in the isometric force production (\(E_{MAX}\)) of 51\%\%\%. In comparison, the \(EC_{50}\) for OR-1896 was \(36\pm7\ nM\), while the maximal force increase corresponded to a relative value of 52\%\%\%.

The phosphodiesterase (PDE) -inhibitory potentials of levosimendan and its metabolite were tested on purified PDE isoforms. Increasing concentrations of levosimendan or OR-1896 decreased the PDE III activity in a dose dependent manner. The half-maximal inhibition of PDE III was achieved at a concentration (\(IC_{50}\)) of \(2.5\ nM\) by levosimendan while in the case of OR-1896 the \(IC_{50}\) was \(94\ nM\). Similarly to the results with PDE III, increasing concentrations of levosimendan or OR-1896 also progressively decreased the PDE IV activity.
However, to attain half-maximal inhibition of PDE IV, significantly higher drug concentrations were required. The IC$_{50}$ value for levosimendan was 25 μM, while that for OR-1896 was 286 μM.

**DISCUSSION**

This study compares the concentration dependences of the positive inotropic effects of levosimendan and OR-1896 with those of their possible underlying mechanisms. Myocardial contractile state was assessed in Langendorff hearts at constant preload, heart rate and coronary flow. Under these conditions, myocardial contractility could be increased either by an increase in the Ca$^{2+}$-sensitivity of the contractile system and/or by an increase in the amplitude of the Ca$^{2+}$ transient due to PDE inhibition.

Using permeabilized myocyte-sized preparations and isolated PDE isoforms, the effects on Ca$^{2+}$-sensitivity and PDE inhibition could be dissected. Levosimendan and OR-1896 proved to be positive inotropes, with almost identical potentials in Langendorff hearts. Several earlier experimental efforts furnished evidence for the idea that levosimendan-induced positive inotropy does not require PDE inhibition and hence the activation of the β-adrenergic intracellular pathway. For instance, the protein kinase A (PKA) inhibitor KT 5720 did not decrease the levosimendan induced positive inotropy in Langendorff-perfused guinea pig hearts and levosimendan did not increase the L-type Ca$^{2+}$ current ($I_{Ca-L}$) in rat and rabbit myocytes at concentrations lower than 1 μM. Furthermore in enzymatically dissociated guinea pig myocytes levosimendan increased cell shortening without changes in [Ca$^{2+}$]$_i$ with an EC$_{50}$ value of 9 nM. This data matches well with our value (EC$_{50}$ = 8 nM) obtained in permeabilized myocytes. Hence, the increase in force of isolated cells with intact membranes was presumably due primarily, if not exclusively, to Ca$^{2+}$-sensitization.

Of the four different PDE isoenzymes (PDE I, II, III and IV) of the human myocardium, PDE III is frequently cited as a mediator of the positive inotropic
effect. This assumption is based on experiments with hypothetically PDE III-selective compounds (e.g. milrinone), a concept of intracellular compartmentation of the G protein-coupled signaling pathways and/or the apparent $K_m$ value of PDE III for cAMP. It has also been demonstrated that PDE inhibition decreases the predicted intracellular compartmentation of cAMP and this may allow effective interactions between the different PDE isoforms. Indeed, the experimental evidence in rat or rabbit myocytes suggested that a marked increase in L-type Ca$^{2+}$ current or positive inotropism through cAMP signaling could be achieved only if at least two PDE subtypes were inhibited simultaneously. In particular, dual inhibition of low $K_m$ cAMP PDEs (i.e. PDEs III and IV) was shown to be a prerequisite for the development of a PDE dependent elevation in the $[cAMP]_i$ and of positive inotropy. When only one PDE isoenzyme was inhibited, cAMP was presumably metabolized by other PDE isoenzymes, thereby preventing significant increase in cAMP content and consequently the activation of cAMP-dependent PKA. Conversely, PDE inhibitors (PDEIs) with poor PDE selectivity were more potent elevators of $[cAMP]_i$ levels in cardiomyocytes. The claim that PDE inhibition may not contribute to the positive inotropic effects of levosimendan and its metabolite is therefore supported by their PDE isoenzyme selectivity being higher than most other PDEIs. In our in vitro enzyme inhibition tests, the selectivity factors (PDE III over IV) for levosimendan and OR-1896 were 10000 and 3000, respectively. The corresponding value for milrinone has been found to be only 14. This makes it difficult to reproduce the selective PDE III inhibitory effect of levosimendan with other drugs in cardiac preparations. Additionally, we found a poor correlation between the concentration dependencies of OR-1896 induced positive inotropy and PDE III inhibition. Hence, despite its somewhat smaller PDE selectivity factor than that of levosimendan, Ca$^{2+}$-sensitization rather than PDE III inhibition explained the positive inotropic effect of OR-1896. In human atrial cardiomyocytes, it was suggested that PDE III inhibition was responsible
Effects of levosimendan and OR-1896 in the guinea pig heart

for the increase in L-type Ca$^{2+}$ current at lower than 1 μM of levosimendan concentrations. An increase in L-type Ca$^{2+}$ current via PDE inhibition would, however, also increase the amplitude of the intracellular Ca$^{2+}$ transient. In contrast, levosimendan did not increase [Ca$^{2+}$]$_i$ in ventricular muscle strips from end-stage failing human hearts. Further, the hemodynamic effects of levosimendan in chronically failing human hearts were inconsistent with those of sympathetic activation and of the sympathomimetic agent dobutamine. Hence, the levosimendan-induced increase in cardiac output was better explained by stronger systolic contractions due to a Ca$^{2+}$-sensitizing mechanism than by alterations in the sympathetic controlled heart rate and [Ca$^{2+}$]$_i$. These data are in accord with the idea that the PDE IV isoform is also present and functional in both healthy and failing human ventricular myocytes. The activity of PDE IV, or other PDE subtypes, may therefore possibly prevent intracellular cAMP accumulation following levosimendan application.

The results of our model investigation in cardiac preparations of healthy guinea pigs may explain in part the controversy regarding the mechanism of levosimendan-induced inotropy. We suggest that, despite the PDE III-inhibitory potential of levosimendan and OR-1896, these agents may leave the intracellular cAMP-dependent signaling unaltered because they do not inhibit PDE IV at their therapeutic plasma concentrations. However, the expression levels and intracellular localization may possibly influence the interactions between the different PDE subtypes. Accordingly, alterations in these parameters may give rise to a certain degree of variability in the consequences of selective PDE inhibition during health and disease.

In conclusion, our data support the hypothesis that levosimendan and OR-1896 both exert positive inotropy via a Ca$^{2+}$-sensitizing mechanism and not via simultaneous inhibition of the PDE III and IV isozymes in the myocardium at their maximal free plasma concentrations.
Comparison of levosimendan and enoximone in the guinea pig heart

TWO INOTROPES WITH DIFFERENT MECHANISMS OF ACTION: CONTRACTILE, PDE-INHIBITORY AND DIRECT MYOFIBRILLAR EFFECTS OF LEVOSIMENDAN AND ENOXIMONE.

RESULTS

Isolated working-hearts: inotropic effects of levosimendan and enoximone

The effects of levosimendan and enoximone on the cardiac pump function were studied in isolated guinea pig hearts perfused in the working-heart mode. The baseline parameters in the three experimental groups (control, n = 5; levosimendan, n = 7 and enoximone, n = 8) did not exhibit significant differences. Test compound were added to the perfusion buffer in a cumulative manner so as to obtain increasing drug concentrations. The effects of levosimendan or enoximone on the cardiac contractility were established in the range of drug concentrations between 1 nM and 1 μM. Drug effects were compared with drug-free control parameters obtained at identical solvent concentrations and time points. 10 nM and higher concentrations of levosimendan significantly increased aortic output and coronary flow (P < 0.05). Although enoximone did not influence the aortic output up to 1 μM, at concentrations of 50 nM and higher it increased the coronary flow. Levosimendan significantly augmented the cardiac output (CO) starting from a concentration of 10 nM onward. Over 50 nM, however, a saturation of the levosimendan-induced contractile effects could be observed. A significant increase in the CO in response to enoximone was observed at concentrations of 500 nM and higher (ie, 1 μM). The enoximone-induced cardiac response was not accompanied by a significant increase in heart rate. In contrast, levosimendan exerted a positive chronotropic effect at concentrations of 100 nM and higher. Assessment of left ventricular stroke volume showed substantially similar concentration dependence to that of CO.
Single permeabilized cardiomyocytes: effects of levosimendan and enoximone on Ca$^{2+}$ sensitivity of contractile filaments

Maximal Ca$^{2+}$-activated force was obtained at pCa 4.75 under drug-free conditions and normalized to the cross-sectional area ($P_o$). The value of $P_o$ (20.9±1.8 kN/m$^2$, $n = 23$) was similar to those described by others. The effects of levosimendan and enoximone on the Ca$^{2+}$-regulated force production were studied at a constant submaximal level of Ca$^{2+}$ activation (at pCa 6.2). Under drug-free conditions, the Ca$^{2+}$-induced isometric force at pCa 6.2 was 3.5±0.3 kN/m$^2$, 17.2±0.9% of $P_o$ ($n = 23$). After determination of the control isometric force at pCa 6.2, levosimendan ($n = 15$) or enoximone ($n = 8$) was added to the activating solution in increasing concentrations (in the ranges 0.1 nM–10 µM or 1 nM–10 µM, respectively). Concentrations of levosimendan 1 nM and higher significantly increased the peak isometric force. At 1 mM levosimendan, the amplitude of force was augmented by 55.5±6.8% over the drug-free baseline. The EC$_{50}$ value for the levosimendan concentration–isometric force relationship was 8.4 nM. No significant effect of enoximone on isometric force could be demonstrated in the studied concentration range.

Phosphodiesterase inhibition: effects of levosimendan on the activities of isoenzymes PDE III and PDE IV

Enzyme activity assays on purified PDE isoforms were used to characterize the PDE-inhibitory potentials of levosimendan and enoximone. PDE III and PDE IV were progressively inhibited by both levosimendan and enoximone in a dose-dependent manner. The drug concentration required for half-maximal inhibition of the catalytic activity (IC$_{50}$), however, indicated major differences between the two compounds. Levosimendan inhibited PDE III with an IC$_{50}$ of 1.4 nM and PDE IV with an IC$_{50}$ of 11 µM. The IC$_{50}$ values for PDE III and PDE IV inhibition by enoximone were 1.8 µM and 160 µM, respectively.
DISCUSSION
This study analyzed the pharmacological efficacies of two positive inotropic agents, levosimendan and enoximone. Isolated working heart experiments revealed maximal cardiotonic effect at concentration well below 1 μM for levosimendan and close to 1 μM for enoximone. In the concentration range between 1 nM and 1 μM the levosimendan-induced positive inotropy was more pronounced than that of enoximone. This difference can be explained by their different mechanisms of action: Ca$^{2+}$ sensitization in the case of levosimendan and a far less selective inhibition of isoenzymes PDE III and PDE IV in the case of enoximone. Besides, the vasodilatory potential of these agents affects the coronary microcirculation, with additional effects on the ventricular pump function. During the working heart measurements direct myocardial and indirect vascular mechanisms could be characterized separately. Consistent with their cardiotonic potentials, both compounds increased the CO and the stroke volume. The positive inotropic effect of levosimendan, however, included both increased aortic and coronary flows, whereas that of enoximone was reflected only through the increased coronary flow suggesting a more pronounced contribution of coronary vasodilation to the positive inotropy in the latter case.

Although PDE III is frequently cited as a mediator of the positive inotropic effect, other PDE isoenzymes (PDE I, II and IV) of the human myocardium may also contribute to regulation of the [cAMP]i. Several strong experimental evidences detailed in the previous chapter support the idea that inhibition of only a single subtype of PDE isoenzymes is insufficient to induce [cAMP]i elevation (e.g. decrease in the intracellular compartmentation of cAMP and subsequent interactions between the different PDE isoforms; lack of increase in $I_{Ca-L}$, positive inotropism and in [cAMP]i, etc.). Furthermore, dual inhibition of low-$K_m$ cAMP PDEs (i.e. PDE III and PDE IV) has been shown to be a prerequisite for the development of a PDE-dependent elevation in [cAMP]i and of positive inotropy. When only one PDE isoenzyme was inhibited, cAMP was presumably
metabolized by another PDE isoenzyme, thereby preventing an increase [cAMP]_i and the consequent activation of cAMP-dependent PKA. Conversely, PDEIs with poor PDE selectivity are more potent elevators of [cAMP]_i.

To elucidate the pharmacological background of the distinct positive inotropic profiles of levosimendan and enoximone, the concentration dependences of their putative intracellular effects, ie, PDE inhibition and Ca^{2+} sensitization, were examined. We failed to demonstrate major contractile effects on enoximone administration up to a concentration of 1 μM in isolated guinea pig hearts. In investigations involving enoximone at higher concentrations the induced contractile effects increased in a monotonous fashion up to 1 mM. Since PDE III inhibition becomes maximal at concentrations in the low-micromolar concentration range, augmentation of the positive inotropic effect of enoximone in a range of concentration well above this level cannot be explained by isolated PDE III inhibition. Alternatively, the combination of PDE III inhibition with some additional positive inotropic mechanism that is sensitive to enoximone concentrations higher than 1 μM should be considered. A direct myofibrillar effect of enoximone can be excluded because of the lack of influence on the isometric force production up to a concentration of 10 mM in permeabilized cardiac myocytes. Hence, the most probable candidate for the additional positive inotropic mechanism is the inhibition of another PDE isoenzyme. The PDE III and PDE IV inhibitory profiles of enoximone were poorly separated suggesting gradually increasing PDE IV inhibition in addition to PDE III blockade at concentrations close to and above its IC_{50} for PDE III. Furthermore, this assumption may well explain the monotonous increase in force with increasing enoximone concentrations until 1 mM through a graded intracellular cAMP accumulation as evidenced earlier in ventricular homogenates and slice preparations of the guinea pig.

The increase in isometric force in permeabilized myocytes demonstrated levosimendan to be a Ca^{2+} sensitizer with an EC_{50} of 8 nM, further supporting its
postulated interaction with the troponin C molecule in the contractile thin filament. The IC$_{50}$ of levosimendan for PDE III inhibition, however, was also found close to this concentration. The concentration dependences of levosimendan-induced Ca$^{2+}$ sensitization and PDE III inhibition therefore overlapped with the development of positive inotropy in isolated guinea pig hearts. This fact hampered the discrimination between the contributions of Ca$^{2+}$ sensitization and PDE inhibition to the positive inotropic effect of levosimendan. Interestingly, however, maximal contractile responses in isolated working hearts were attained at levosimendan concentrations at which PDE IV inhibition could not possibly occur. Moreover, in a previous study, higher than 0.1 mM levosimendan concentrations were required to elevate the [cAMP]. Accordingly, simultaneous inhibition of PDE III and PDE IV did not contribute to the positive inotropic response of levosimendan below this concentration.

The claim that PDE inhibition may not contribute to the positive inotropic effects of levosimendan, despite its PDE III inhibitory potential, is also supported by its PDE isoenzyme selectivity being higher than those of most other PDE inhibitors. In vitro enzyme inhibition tests in this and a previous study provided selectivity factors (PDE III over IV) for levosimendan close to 10,000. The corresponding value for enoximone varies between 4 and 60. The results of our enzyme activity assays revealed a selectivity factor of 90. Hence, in accord with others, our data are indicative of significantly less selective PDE inhibition following enoximone than following levosimendan application. Hence, our results support the hypothesis that levosimendan exerts positive inotropy via a Ca$^{2+}$-sensitizing mechanism, whereas enoximone does so via PDE inhibition with a limited PDE III versus PDE IV selectivity. Additionally, vascular effects, e.g. coronary vasodilation, are likely to have more pronounced role in the mechanism of positive inotropy induced by enoximone than in that induced by levosimendan.
CONCLUSIONS

This thesis summarizes the results of our experimental work on different, pathophysiological and therapeutic aspects of chronic heart failure. Cardiac contractility was characterized at the cellular level and underlying regulatory mechanisms were explored applying up-to-date biochemical methods.

1. We have shown transmural gradients of stiffness and stretch-dependent \(\text{Ca}^{2+}\) sensitization in the rat left ventricular myocardium. Increased stretch-sensitization in the sub-endocardium was associated with stretch-induced phosphorylation of VLC2b. In heart failure following myocardial infarction the gradient of stretch-dependent \(\text{Ca}^{2+}\) sensitization disappeared in parallel with a lack of VLC2b phosphorylation in the sub-endocardium.

2. In a knock out mouse model we demonstrated that the lack of cMyBP-C increased \(\text{Ca}^{2+}\) sensitivity at short SL, reduced the SL-dependent change and almost abolished the PKA-induced decrease in \(\text{Ca}^{2+}\) sensitivity. This implies a role for cMyBP-C in the regulation of cardiac contractility.

3. Comparison of positive inotropic, \(\text{Ca}^{2+}\)-sensitizing effects and PDE-inhibitory potentials of levosimendan and OR-1896 indicated that at their maximal free plasma concentrations both compounds exert positive inotropy via \(\text{Ca}^{2+}\) sensitization and not via simultaneous inhibition of PDE III and IV isozymes in the myocardium. Additionally, the PDE III versus PDE IV selectivity of levosimendan proved to be higher than that of most other PDE inhibitors.

4. Comparison of levosimendan and enoximone exposed distinct differences between the myocardial mechanisms of positive inotropy exerted by a \(\text{Ca}^{2+}\)-sensitizer with pronounced and a PDE inhibitor with limited PDE III versus PDE IV selectivity, respectively.
LIST of PUBLICATIONS

In extenso publications related to this thesis


2. Cazorla, O., Szilagyi, S., Vignier, N., Salazar, G., Krämer, E., Vassort, G., Carrier, L., Lacampagne, A. (2005) Activation of myofilaments in a hypertrophic mouse model lacking the cardiac myosin binding protein C: effect of length and PKA phosphorylation. († authors contributed equally to this work; submitted)


Other in extenso publication


Published abstracts


**Oral presentations**


Hungarian Society of Cardiologists, Balatonfüred.


Poster presentations


