

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**The importance of myofilament Ca<sup>2+</sup>-sensitivity in heart  
failure**

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Head of the **Examination Committee:** Prof. Dr. György Balla, MHAS  
Members of the Examination Committee: Dr. István Baczkó, Ph.D.  
Dr. Béla Juhász, Ph.D.

The Examination takes place at the Department of Pediatrics, Medical and Health Science  
Center, University of Debrecen  
April 24, 2012. 11:00

Head of the **Defense Committee:** Prof. Dr. György Balla, MHAS  
Reviewers: Dr. Violetta Kékesi, C.Sc.  
Dr. János Magyar, D.Sc.

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Dr. Béla Juhász, Ph.D.

The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine,  
Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen  
April 24, 2012. 13:00

# 1. Introduction and Background

## 1.1. Definition and clinical aspects of heart failure

Heart failure (HF) is a complex clinical syndrome, that can be caused by any structural or functional cardiac abnormality, which deteriorates the systolic and/or diastolic function of the left ventricle (LV).

Nowadays HF is a leading cause of death all over the world. The prevalence of the disease is especially high in the industrialized countries. Despite the significant improvement in HF treatment in the last decade, its mortality is comparable or even exceeds that of malignancies. This emphasizes the fact, that the search for new therapeutical approaches of HF is imperious. Thus, both multicenter, randomized, prospective clinical trials and basic reasearch of the disease's molecular pathophysiology are essential in the struggle against HF.

Chronic HF leads to an abnormal remodeling of the myocardium through disadvantageous hemodynamical parameters, myocardial ischemia, direct myocardial damage and a number of other factors as well. These detrimental effects are associated with a deteriorating cardiac function and altered heart geometry. As a result, the ejection fraction and the cardiac output will be reduced, which induces an enhanced activity of the neurohumoral system: catecholamines from the adrenal glands will induce positive ino- and chronotropic effects, while the renin-angiotensin-aldosterone system will favour water retention and vasoconstriction to maintain normal blood pressure and cardiac output.

Beside lifestyle changes, today's conventional HF therapy includes also the application of certain drugs, already in the early stage of the disease. However, in a certain proportion of patients the maximal tolerable doses of these pharmacons are still unable to reduce the symptoms and complaints, thus their quality of life and prognosis remains poor.

In part of the chronic HF patients not responding to conventional pharmaceutical therapy, the so called  $\text{Ca}^{2+}$ -sensitizing treatment can be helpful. In contrast to cardiac glycosides and catecholamines,  $\text{Ca}^{2+}$ -sensitizers do not enhance cardiac performance through increasing intracellular  $\text{Ca}^{2+}$ -concentration ( $[\text{Ca}^{2+}]_i$ ), but through acting directly on the contractile proteins. This feature of these drugs enables reducing undesirable effects of  $\text{Ca}^{2+}$ -overload, as  $\text{Ca}^{2+}$ -sensitizers are able to enhance contractility without increasing oxygen consumption or inducing severe arrythmias.

Regarding the high morbidity and mortality rates of HF, understanding the molecular pathophysiology of the disease in detail is essential.

## 1.2. Molecular background of heart failure

The molecular background of myocardial remodeling results mainly from altered expression, phosphorylation status, structural- and functional modifications of contractile proteins, as well as regulatory proteins of intracellular  $\text{Ca}^{2+}$ -homeostasis of the cardiomyocytes. The so far collected data in the literature shed light to many aspects of the molecular changes during HF, however there remained several questions and contradictions awaiting to be clarified. One possible way of clarification is to develop such animal models, which mimick the human pathology in a large degree, thereby enabling the tests of potentially new therapeutic approaches.

Myocardial protein alterations occurring in the molecular background of myocardial remodeling during the development of chronic HF affect the myocardial structure and are responsible for disturbances in cellular functions ranging from intracellular signaling through cardiomyocyte contractility to metabolism. In this context, a number of studies have implicated the involvement of myofibrillar proteins in the progressive decay of the myocardial function. In the failing human heart, a down-graded  $\beta$ -adrenergic responsiveness was found to be associated with an increased  $\text{Ca}^{2+}$ -sensitivity of force production through a diminished level of protein kinase A (PKA)-mediated troponin I (TnI) and myosin binding protein C (MyBP-C) phosphorylation, although these relationships were not fully confirmed in all models of HF. Some of the discrepancies arise from the complexity of the human pathology, the difficulty of finding reliable human control materials, and possible species-dependent differences between signaling pathways in large and small mammals. It follows that investigations in appropriate animal models of HF are of great importance, as they will possibly elucidate HF-specific protein alterations with direct relevance for the human pathology.

## 1.3. $\text{Ca}^{2+}$ -dependency of contractile force

Determining the  $\text{Ca}^{2+}$ -dependency of contractile force generated by the myofibrillar structures of the heart is difficult, as the myoplasmatic  $\text{Ca}^{2+}$ -concentration ( $[\text{Ca}^{2+}]$ ) changes continuously and very rapidly during the cardiac cycle. However, examining the  $\text{Ca}^{2+}$ -dependency of contractile force is possible in permeabilized (in other words "skinned") cardiac preparations, whose myoplasmatic  $[\text{Ca}^{2+}]$  can be regulated, and the generated force can be parallelly measured at controlled sarcomere lengths (SLs). Plotting the force values as a function of  $[\text{Ca}^{2+}]$  results in a typical sigmoid curve. The  $[\text{Ca}^{2+}]$  is usually expressed in  $-\log$  units (pCa).

The sigmoid curve can be fitted with a Hill-equation, which contains the most important quantitative parameters of the  $\text{Ca}^{2+}$ -dependency of the force generated by the contractile system. The  $[\text{Ca}^{2+}]$  required for the "half-maximal" force to develop is  $[\text{Ca}_{50}]$  (or  $p\text{Ca}_{50}$ ), which characterizes the  $\text{Ca}^{2+}$ -sensitivity of the contractile apparatus in itself.

The increase in  $p\text{Ca}_{50}$  (decrease in  $[\text{Ca}_{50}]$ ) results in the leftward shift of the  $\text{Ca}^{2+}$ -force relationship (increase in  $\text{Ca}^{2+}$ -sensitivity, e.g. upon increasing the SL or applying  $\text{Ca}^{2+}$ -sensitizer drugs), the decrease in  $p\text{Ca}_{50}$  (increase in  $[\text{Ca}_{50}]$ ) has opposite effects (decrease in  $\text{Ca}^{2+}$ -sensitivity, e.g. upon  $\beta$ -adrenergic stimulation). The Hill-coefficient ( $n_{\text{Hill}}$ ) is characteristic for the steepness of the sigmoid curve, and also for the degree of cooperativity within the contractile system.

#### **1.4. $\beta$ -adrenergic signaling and cardiomyocyte contractility in the normal and failing heart**

Enhancing the contractility of the cardiomyocytes is achieved through  $\beta$ -adrenergic stimulation of the sympathetic nervous system.  $\beta$ -adrenergic receptor stimulation induces PKA-mediated phosphorylation of various membrane- and intracellular proteins (e.g. L-type  $\text{Ca}^{2+}$ -channels, ryanodine receptors, phospholamban) resulting in enhanced sarcoplasmic reticular  $\text{Ca}^{2+}$ -uptake. Consequently, sarcoplasmic  $\text{Ca}^{2+}$ -loading, systolic cytosolic  $\text{Ca}^{2+}$ -concentrations and therefore, contractile force will increase. At the myofilament level,  $\beta$ -adrenergic activation induces phosphorylation of various myofibrillar proteins as well, which will alter several determinants of cardiomyocyte contractility (maximal force generating capacity,  $\text{Ca}^{2+}$ -sensitivity, passive tension). PKA-mediated phosphorylation of the thin filament protein, TnI and the thick filament protein, MyBP-C decreases myofilament  $\text{Ca}^{2+}$ -sensitivity, thereby contributing to an acceleration of cardiac relaxation in diastole (positive lusitropy). In addition, several studies revealed a role for PKA-mediated phosphorylation of titin in regulating the passive force of cardiomyocytes.

In healthy human myocardium the enhanced contractility, reduced  $\text{Ca}^{2+}$ -sensitivity and the consequent positive ino- and lusitropic responses upon  $\beta$ -adrenergic stimulation are beneficial for the cardiac performance. During HF, neurohumoral stimulation increases to compensate for reduced cardiac pump function. Although neurohumoral activation aims at maintaining cardiac contractile force, eventually it becomes detrimental as it leads to the imbalance between kinases and phosphatases regulating contractile function, and it initiates cardiac remodelling and phosphorylation changes of regulatory proteins, which impair cardiomyocyte function. Eventually the  $\beta$ -adrenergic signalling pathway becomes desensitized upon chronic

stimulation, which will limit myocardial response to the increased demand. Down-regulation of  $\beta$ -receptors induces PKA-mediated hypophosphorylation of proteins regulating  $\text{Ca}^{2+}$ -homeostasis and contractility, and the latter will lead to increased myofilamentary  $\text{Ca}^{2+}$ -sensitivity and cardiomyocyte passive tension.

Several previous animal studies came to consistent conclusions and suggested that coordinated changes may occur in at least three different parameters of cardiomyocyte mechanics:  $\text{Ca}^{2+}$ -sensitivity, force generation and passive stiffness. Similarly to human HF, an increase in myofilament  $\text{Ca}^{2+}$ -sensitivity was found at the infarcted area of post-infarction porcine hearts, which could be normalized by *in vitro* PKA treatment of cardiomyocytes. On the contrary, in post-infarction and chronic pressure overload rat models of HF reduced  $\text{Ca}^{2+}$ -sensitivity and increased TnI phosphorylation was confirmed compared to controls. These alterations were explained by enhanced PKC $\alpha$  activity and signaling. Maximal  $\text{Ca}^{2+}$ -activated force ( $F_o$ ) was found to be significantly lower not only in the previous post-ischemic porcine and rat models, but also in cardiomyocytes from the remodelled myocardium of a similar mouse model. Finally, changes in the passive tension ( $F_{passive}$ ) seem to be also relevant, and thereby to contribute to cardiac mechanical dysfunction in certain conditions. However,  $F_{passive}$  did not significantly differ between failing and non-failing hearts in a post-ischemic mouse and a pig model.

### **1.5. Regional alterations in pacing-induced heart failure**

A well-established large animal model of HF, induced by continuous cardiac pacing at a frequency three- to fourfold higher than the spontaneous heart rate, is mostly applied to dogs, pigs, sheep and monkeys. One characteristic of this model is the presence of regional differences in functional and molecular remodeling. For example, in a canine tachy-pacing HF model, regional differences were noted in the extent of action potential prolongation and intracellular  $\text{Ca}^{2+}$ -homeostasis abnormalities. Moreover, cardiac resynchronization therapy in these hearts furnished non-uniform responses. In a porcine model of HF, sustained pacing-induced dyssynchronous left ventricular (LV) activation caused more pronounced decreases in LV systolic thickening and circumferential shortening in the anterior/anterolateral region (paced site) as compared with the inferoseptal region (opposite site). This observation was unrelated to a uniform increase in glucose uptake in the corresponding regions. However, these changes did correlate with an asymmetrical myocardial expression of natriuretic peptides.

In the present study, we set out to investigate whether alterations in PKA-dependent myofilament phosphorylation are associated with the *in vivo* found regional contractile dysfunction in the porcine model of pacing-induced HF.

### **1.6. Role of Ca<sup>2+</sup>-sensitizers in heart failure**

Troponins of cardiac thin myofilaments are central in the regulation of the contractile process. Given its Ca<sup>2+</sup>-sensor and Ca<sup>2+</sup>-binding properties, cardiac troponin C (TnC) - one of the 3 troponin subunits - acts as a Ca<sup>2+</sup>-operated molecular switch, turning myocardial force production on and off during cardiac systoles and diastoles.

Ca<sup>2+</sup>-sensitizers used in chronic HF present a class of cardiotoxic agents that are capable of producing a positive inotropic effect by acting on the cardiac excitation–contraction coupling process at the level of the myofilaments by modifying Ca<sup>2+</sup>-binding to TnC, thin filament regulatory sites, and/or directly the cross-bridge cycling.

Their mechanism of action is not associated with an increase in the intracellular [Ca<sup>2+</sup>] concentration at therapeutic doses, and thus, does not induce Ca<sup>2+</sup>-related deleterious effects such as lethal arrhythmias.

From all the Ca<sup>2+</sup>-sensitizing drugs levosimendan is one of the most thoroughly investigated. Besides its positive inotropic effect it influences the cardiovascular system beneficially, in part through its active metabolite OR-1896 (vasodilation, cardioprotection, phosphodiesterase-inhibition). It induces Ca<sup>2+</sup>-sensitization by modulating Ca<sup>2+</sup>-binding to TnC. Essentially it stabilizes the protein's Ca<sup>2+</sup>-bound state without affecting maximal Ca<sup>2+</sup>-activated force of cardiomyocytes.

An interesting observation was that direct myofilamentary effects can develop in the case of such compounds, which were originally designed to bind myofilament independent molecular targets. SR33805 is a fantofarone derivative characterized as a potent Ca<sup>2+</sup>-inhibitor, which can hypothetically cause Ca<sup>2+</sup>-sensitizing effects as well. In a previous study SR33805 was found to reduce the amplitude of Ca<sup>2+</sup>-transient, however, at the same time it was able to increase shortening of intact ventricular cardiomyocytes in healthy rats. Despite being a potent Ca<sup>2+</sup>-inhibitor, the drug possessed a favourably weak negative inotropic effect in electrically stimulated rabbit-isolated cardiac muscle preparations. The application of SR33805 at a low concentration induced a significant increase in maximal Ca<sup>2+</sup>-activated force and decrease in shortening time, while the application of a larger dose clearly induced Ca<sup>2+</sup>-sensitizing effects, particularly at a larger SL.

Previous preclinical investigations could not clarify, whether the  $\text{Ca}^{2+}$ -sensitizing effect of SR33805 can be exerted on the failing myocardium as well. Furthermore, it is not yet decided, whether the  $\text{Ca}^{2+}$ -sensitization can be associated to alterations in phosphorylation status of regulatory proteins of myocardial contractility.

## **2. Aims**

Based on the aforementioned findings, the main aims of our research were:

1. To investigate hypothetical dysregulation of myofilamentary contractile protein phosphorylation and related abnormalities of  $\text{Ca}^{2+}$ -sensitivity of isometric force production in isolated cardiomyocytes in a porcine model of pacing-induced HF.
2. To elucidate the possible  $\text{Ca}^{2+}$ -sensitizing effects of SR33805, a known  $\text{Ca}^{2+}$ -antagonist drug, on the remodeled myocardium of a post-ischemic rat model.

## **3. Materials and Methods**

### **3.1. Experimental animal models**

#### *3.1.1. Porcine model of pacing-induced heart failure*

Ten male, sexually mature minipigs (35–40 kg) were chronically instrumented in a partner laboratory of Pisa, Italy. HF was induced by pacing the LV anterior wall at 180 beats/min for 3 weeks. Pigs were considered to develop severe HF when the LV end-diastolic pressure was  $\geq 20$  mmHg and the ejection fraction was  $< 40\%$ . Untreated animals were used as healthy controls. Hemodynamic measurements, MRI and PET examinations were performed in controls and in HF animals and identified regional wall motion abnormalities. The minipigs were then sacrificed and their hearts were removed, dissected and immediately frozen in liquid nitrogen. The anterolateral wall (paced site) and inferoseptal region (opposite site) of the LVs were separated (for the sake of a consistent nomenclature - the indicated regions of the control animals were also referred as paced or opposite sites, although these hearts were not paced at all).

#### *3.1.2. Post-infarction rat model*

Male Wistar rats (5 weeks old) were subjected to permanent coronary ligation in the proximal segment of the left anterior descending artery (LAD) to produce myocardial infarction (MI) in



a partner laboratory of Montpellier, France. MI rats were compared with sham-operated animals without ligature. Animals were investigated 18 weeks after MI. *In vivo* echocardiography and tissue Doppler imaging were performed to assess cardiac contractility before and after an intraperitoneal bolus of SR33805 (20 mg/kg). Then the animals were sacrificed, their hearts were removed and used for mechanical and biochemical measurements.

### **3.2. Immunohistochemistry**

After embedding in cryomatrix resin, porcine heart samples were sectioned in a cryostat at -20 °C to obtain 10- $\mu$ m-thick slices. All subsequent steps were performed at room temperature. A monoclonal mouse anti-TnI primary antibody was used to analyze TnI independently of its phosphorylation status, and a polyclonal rabbit anti-TnI (phospho S22 + S23) was employed to assess PKA mediated TnI phosphorylation (P-TnI). Slides were incubated overnight at +4 °C, followed by a 30-min incubation at room temperature with anti-mouse-Cy3 (red) and anti-rabbit-Cy2 (green) antibodies in order to visualize cardiac TnI and its PKA specific phosphorylated form. Microscopic images of tissue cuts were recorded with a NIKON Eclipse 80i Fluorescein microscope and an Olympus FluoView 1000 Confocal microscope.

### **3.3. TnI back-phosphorylation**

A back-phosphorylation assay was performed to assess the phosphorylation level of TnI in protein homogenates of permeabilized cardiomyocytes. Permeabilized cardiomyocytes were obtained via mechanical isolation and Triton-permeabilization of cardiac tissue samples. The reaction mixtures contained the isolated cardiomyocyte fraction, <sup>32</sup>P-labeled ATP (final specific activity: 1500 cpm/pmol) and PKA. The reaction was performed at room temperature for 15 min. At the end, sodium dodecyl sulfate (SDS) sample buffer was added, and the samples were boiled for 5 min. Samples separated by polyacrylamide gel electrophoresis (PAGE) were transferred onto nitrocellulose membranes. Phosphate incorporation into TnI was detected by autoradiography. Band intensities (reflecting phosphate incorporation) were determined by densitometry using Image J software.

### **3.4. Western immunoblot**

Porcine and rat LV tissue samples were homogenized in radioimmune precipitation assay and urea buffer. After precise adjustment of protein concentrations the homogenates were boiled for 10 min, separated by PAGE, then transferred to nitrocellulose membranes. Membranes containing porcine samples were probed with antibodies against TnI and the signal was detected with a peroxidase-conjugated anti-rabbit IgG-specific antibody. Amount of TnI in rat myocardial samples was also detected by anti-TnI antibody, while its PKA-mediated phosphorylated form was probed with phospho-specific antibody. Regulatory myosin light chain (MLC2) isoforms in rat myocardium were specifically detected with a cardiac MLC2 antibody. The bands were visualized by enhanced chemiluminescence and evaluated with Image J software.

### **3.5. PKA activity measurements**

Frozen LV tissue samples of rat hearts were defrosted and subsequently disrupted in modified isolation solution. The cytosol was separated by centrifugation. Endogenous PKA activity was then tested at room temperature in a reaction mixture containing the cytosol fraction, <sup>32</sup>P-labeled ATP (final specific activity: 2500 cpm/pmol) and a reaction mixture containing both PKA-inhibitor H-89 or SR33805. Reactions were initiated by adding the cytosol fraction, then 10 µl of reaction mixture was dropped onto phosphocellulose paper at 0 and 15 min. The radioactivity on the papers was measured by scintillation counting. Effects of SR33805 on recombinant PKA activity was also tested. PKA activities were expressed as % of maximal activity (activity in the absence of inhibitor) over baseline (activity in the absence of kinase).

### **3.6. Flow cytometry**

LV tissue samples of porcine hearts (~0.04 g wet weight) were mechanically disrupted and permeabilized. Thereafter, the isolated cardiomyocytes were dual-labeled with antibodies. Monoclonal mouse anti-TnI antibody, and a phosphorylation-sensitive polyclonal rabbit anti-TnI antibody (phospho S22 + S23) were used as primary antibodies, followed by a 30-min incubation on ice with anti-mouse-Cy3 and anti-rabbit-Cy5 antibodies to label cardiac TnI and P-TnI. The cell suspensions were gently sonicated, and then filtered through a 50-µm-wide cell filter. Flow cytometric experiments were carried out on a FACSArray flow cytometer. Fluorescence intensities for single particles were determined in 6 animals per group. 6,000-10,000 particles were measured per animal.

### 3.7. Force measurements in permeabilized cardiomyocyte preparations

For measuring the mechanical function of cardiomyocytes from porcine and rat hearts, identical methods were employed. The force at saturating  $[Ca^{2+}]$  ( $F_o$ ), the passive force component ( $F_{passive}$ ), the  $Ca^{2+}$ -sensitivity of isometric force production ( $pCa_{50}$ ), a parameter characterizing the steepness of the  $Ca^{2+}$ -force relationship ( $nHill$ ), and the rate constant of force redevelopment at saturating  $Ca^{2+}$ -levels ( $k_{tr,max}$  - specific for the maximal turnover rate of the actin-myosin cross-bridge cycle) were used as parameters of the myofibrillar function. Moreover, these mechanical data were determined at SLs of 1.9 and 2.3  $\mu m$  to test for possible alterations in length-dependent  $Ca^{2+}$ -sensitization, i.e. in the molecular background of the Frank-Starling mechanism. The pCa, i.e.  $-\log[Ca^{2+}]$ , values of the relaxing solution and the activating solution used during the force measurements were 10 and 4.75, respectively.

A single permeabilized cardiomyocyte was attached to a mechanical measuring system at 15 °C. After precise SL adjustment to 2.3  $\mu m$ , isometric  $Ca^{2+}$ -contractures were evoked during repeated activation-relaxation cycles, by moving the myocyte from  $Ca^{2+}$ -free relaxing solution to activating solutions of gradually increasing  $[Ca^{2+}]$ . Isometric force values were normalized for maximal  $Ca^{2+}$ -activated active force, and  $Ca^{2+}$ -force relations were plotted and fitted with a modified Hill function. During single  $Ca^{2+}$ -contractures, once the peak force was reached, the rate constant of force redevelopment ( $k_{tr,max}$ ) was determined by a quick release-restretch maneuver in the activating solution. As a result of this intervention, the force dropped from peak level to zero, allowing determination of the total force level ( $F_{total}$ ), and then started to redevelop. The cardiomyocyte was next returned to the relaxing solution, where a shortening to 80% of the original preparation length was performed to assess  $F_{passive}$ . The active isometric force ( $F_o$ ) was calculated by subtracting the passive force from the total isometric force.  $Ca^{2+}$ -force relationships of porcine cardiomyocytes were determined before and after *in vitro* PKA exposures. To assess the effects of PKA, cardiomyocytes were incubated for 20 minutes in PKA-containing solution. Mechanical parameters of rat cardiomyocytes were determined before and after *in vitro* SR33805 treatment (10  $\mu M$ , 10 min, room temperature).

### 3.8. Data analysis, statistics

$Ca^{2+}$ -force relations were fitted to a modified Hill equation:

$$F = F_o [Ca^{2+}]^{nHill} / (Ca_{50}^{nHill} + [Ca^{2+}]^{nHill})$$

where  $F$  is the steady-state force at a given  $[Ca^{2+}]$ , while  $F_o$ ,  $nHill$  and  $Ca_{50}$  (or  $pCa_{50}$ ) denote the maximal  $Ca^{2+}$ -activated force at saturating  $[Ca^{2+}]$ , and the slope and midpoint of the sigmoidal relationship, respectively.

The force redevelopment after the release-restretch maneuver was fitted to a single exponential function in order to estimate the rate constant of force redevelopment ( $k_{tr,max}$ ) at the maximal  $[Ca^{2+}]$  level:

$$F(t) = F_i + F_a(1 - e^{-k_{tr,max}t})$$

where  $F(t)$  is the force at any time ( $t$ ) after the restretch at the maximal  $[Ca^{2+}]$ , and  $F_i$  and  $F_a$  denote the initial force after the restretch and the amplitude of  $Ca^{2+}$ -activated force redevelopment, respectively. Each experimental preparation was fitted individually, the fitted parameters were pooled, and the mean values are reported.

Statistical analyses of the back-phosphorylation assay and mechanical measurements were performed by ANOVA followed by a Bonferroni post test for multiple comparisons between groups. Relationships between two continuous variables were assessed with linear regression analysis. Differences due to *in vitro* treatments during mechanical measurements were analyzed using a paired Student's t-test. Statistical significance was accepted at  $P < 0.05$ . For analysis of the thousands of single data points obtained from the flow cytometric measurements, maximum likelihood mixed effects multilevel modeling with interaction between groups and sites were used. Estimation of the pacing effect was based on mean log relative signal intensities  $[\log(P-TnI/TnI)]$  for the level of phosphorylation, and on interquartile ranges (IQRs) for the heterogeneity of phosphorylation. Logarithmic transformation of the data improved the normality of the distribution of the variables.

## 4. Results

### 4.1. Alterations of troponin I phosphorylation in the porcine model of pacing-induced heart failure

To test homogeneity in PKA mediated myofilament protein phosphorylation, the level of TnI phosphorylation was visualized during immunohistochemical studies on myocardial tissue samples in the anterior/anterolateral (pacing site) and in the inferoseptal regions (opposite site) in HF and control hearts. In general, labeling of tissue slices with an antibody non-sensitive for TnI phosphorylation resulted in a homogeneous staining pattern in all groups. Similarly, labeling control samples or those from the HF opposite site for P-TnI showed also homogeneous staining patterns. In contrast, we observed variable P-TnI intensities in the HF

paced region. Areas with given staining intensities were formed by clusters of cardiomyocytes with lower P-TnI signal levels at the paced site of HF hearts.

The mean levels of myofilament protein phosphorylation were tested in a back-phosphorylation assay. The mean phospho-specific TnI signal intensity of protein homogenates of permeabilized cardiomyocytes from the paced sites of the HF animals ( $58 \pm 14.49$  AU) was significantly higher than that in the control pigs ( $5.41 \pm 1.34$  AU;  $P < 0.05$ ), which was suggestive of a PKA-dependent phosphorylation deficit of TnI at the HF pacing site. The difference in the overall phosphorylation level of TnI between the paced and the opposite sites of the HF pigs did not reach significance ( $P > 0.05$ ) (HF opposite site:  $40.3 \pm 9.86$  AU).

Flow cytometry measurements allowed the quantitative evaluation of regional differences in the level of P-TnI in suspensions of a vast number of permeabilized cardiomyocytes. Distribution histograms of logarithmically transformed relative signal intensities [ $\log(\text{P-TnI}/\text{TnI})$ ] were expressed for the two regions of interest in the HF and control groups. The distribution histogram of the pacing site in the controls demonstrated close to Gaussian distribution, in contrast with the histogram of the HF pacing site, which appeared to be asymmetrical due to an increase in cardiomyocyte count at relatively low  $\log(\text{P-TnI}/\text{TnI})$  ratios. In agreement with the results of our back-phosphorylation assay, the mean value of  $\log(\text{P-TnI}/\text{TnI})$  at the HF pacing site was significantly lower than that at the same site in the control animals ( $-0.41$  vs.  $-0.28$  for the HF pacing site and the control pacing site, respectively;  $P = 0.0071$ ), and hence both of these assays pointed to a relative reduction in P-TnI in the paced region of failing hearts. Moreover, the IQR in this group was significantly higher than that at the pacing site of the controls or at the opposite site of failing hearts ( $0.53$  vs.  $0.36$  or  $0.42$ , for the HF pacing, control pacing and HF opposite sites, respectively;  $P = 0.0093$  vs. control pacing site;  $P = 0.0047$  vs. HF opposite site). The widening of the IQR at the HF pacing site reflected the increased scatter in the levels of relative TnI phosphorylation at the pacing site among the cardiomyocytes of failing hearts.

We illustrated mean levels of  $\log(\text{P-TnI}/\text{TnI})$  intensities with 95% confidence intervals for each animal as well. In general, the  $\log(\text{P-TnI}/\text{TnI})$  values for the control hearts were scattered in a narrower range than those for the failing hearts. Further, in the control animals the means of  $\log(\text{P-TnI}/\text{TnI})$  were randomly higher or lower for the pacing or opposite sites, and the differences between these two sites were rather small. In contrast, in HF the means for the  $\log(\text{P-TnI}/\text{TnI})$  values at the pacing sites were lower than those at the opposite sites.

Moreover, the differences between the two sites of individual failing hearts were about twice the mean difference detected within the control group.

A hypothetical difference in cardiomyocyte mechanics was also studied by means of direct force measurements on single permeabilized cardiomyocytes isolated from HF and control animals. Mean  $pCa_{50}$  values of control hearts at the pacing site did not differ significantly from those of the opposite site in control and HF hearts before PKA treatments ( $5.77\pm 0.01$ ,  $5.75\pm 0.01$  and  $5.73\pm 0.03$ , respectively). However, mean  $pCa_{50}$  (and hence  $Ca^{2+}$ -sensitivity of force production) was significantly higher at the pacing site of HF hearts ( $5.86\pm 0.03$ ) than at all the other sites of HF and control hearts. Moreover, PKA exposures reduced  $pCa_{50}$  by the largest degree in cardiomyocytes of the pacing site of HF animals, thereby eliminating the differences in the  $Ca^{2+}$ -sensitivities of force production among the four groups of cardiomyocytes. In addition, the distributions of  $pCa_{50}$  at the pacing sites of the failing and the control hearts resembled the characteristics of those obtained in our flow cytometric assays, and were therefore also in agreement with the biochemical and histochemical findings. Furthermore, *in vitro* PKA treatments evoked a wider range in  $Ca^{2+}$ -sensitivity reductions at the pacing site than at all other sites.  $F_{passive}$  at the HF pacing site was significantly higher than at the HF opposite site, however, no significant differences in  $F_o$  and in maximal actin-myosin cross-bridge cycle rates ( $k_{tr,max}$ ) were found between the different experimental groups.

#### **4.2. Effects of SR33805 on $Ca^{2+}$ -sensitivity and protein kinase A activity of cardiomyocytes in post-infarction rat model**

Electric stimulation of intact, unstretched, isolated cardiomyocytes loaded with  $Ca^{2+}$ -fluorescence indicator dye is a technique, which enables parallel measurements of changes in cell shortenings and in  $Ca^{2+}$ -transients. Mechanical measurements in isolated, permeabilized cardiomyocyte preparations allows the analysis of  $Ca^{2+}$ -responses of the myofilaments independently from  $Ca^{2+}$ -release of the sarcoplasmic reticulum. Application of 10  $\mu$ M of SR33805 restored cell shortening in intact cardiomyocytes from infarcted rats (MI) without affecting the amplitude of the  $Ca^{2+}$ -transient. Direct force measurements in cardiomyocytes isolated from healthy control and MI rat hearts were performed to characterize the function of the contractile machinery, and to assess effects of *in vitro* SR33805 on contractile parameters of cardiomyocytes from infarcted rats (MI-SR). Myofilament  $Ca^{2+}$ -sensitivity at short SL was not affected by MI, however it was significantly increased after *in vitro* SR33805 treatment (by  $\sim 0.07$  pCa units). Stretching the cells to 2.3  $\mu$ m SL induced a leftward shift of tension in

both control and MI groups as indicated by the higher  $pCa_{50}$ . However, the shift of the force– $pCa$  curve after stretch was lower in MI cells, resulting in a lower  $pCa_{50}$ . Myofilament  $Ca^{2+}$ -sensitivity at 2.3  $\mu m$  SL was improved after SR33805 incubation of MI cells (by  $\sim 0.18$   $pCa$  units). The difference between  $pCa_{50}$  at long and short SLs ( $\Delta pCa_{50}$  - used as an index of the length-dependent activation of the contractile machinery) was completely restored following the incubation of MI cells with SR33805 due to the stronger effect of the drug at long SL. Other contractile parameters ( $F_{passive}$ ,  $F_o$ ) measured at both SLs were similar between all groups.

To verify whether the SR33805 effect was associated with a modification in the phosphorylation level of regulatory proteins, TnI and MLC2 phosphorylation levels were measured in stretched, permeabilized muscle strips by Western blot analysis. MI reduced the phosphorylation level of MLC2 by  $\sim 33\%$ . SR33805 - although moderately - induced a significant increase in MLC2 phosphorylation in the MI tissues. The level of phosphorylation, however, remained still 22% lower than in control cells. For TnI a specific antibody was used, which recognizes the phosphorylated sites on Ser23/24 (the major cAMP-dependent PKA sites). TnI phosphorylation was increased in the MI myofilaments. Incubation of samples in SR33805 significantly reduced the TnI phosphorylation when compared with MI ( $\sim 30\%$ ) and control ( $\sim 20\%$ ) cells.

The reduction of TnI phosphorylation level observed upon SR33805 treatment suggested either an increase or a decrease of phosphatase and PKA activity, respectively. To this aim, the effect of SR33805 on the PKA activity was measured with a radioactive activity assay. A recombinant PKA was first used to test a possible direct interaction of the drug with the kinase. The activity of recombinant PKA was decreased by 10  $\mu M$  SR33805 to a similar extent than that by H-89, a standard PKA inhibitor. This result suggested that SR33805 can directly inhibit PKA *in vitro*. This inhibitory effect of SR33805 was also tested on the endogenous PKA activity of control rat hearts and similar results were found.

In summary, SR33805 targeted the phosphorylation of Ser23/24 in TnI by inhibiting the PKA activity and resulting in an increase in myofilament  $Ca^{2+}$ -sensitivity.

## 5. Discussion

### 5.1. Cellular inhomogeneity in the background of regional contractile dysfunction upon pacing-induced heart failure

Our experiments revealed a non-uniform down-regulation of myocardial PKA-dependent TnI phosphorylation in a porcine model of pacing-induced HF. Interestingly, the cell-to-cell variability in the level of P-TnI was larger in the vicinity of the pacing electrode than in non-paced regions of the LV. Consequently, we propose that the inhomogeneity in PKA-dependent contractile protein hypophosphorylation may contribute to the regional mechanical dysfunction of the remodeled myocardium in this model of HF.

Dyssynchrony in ventricular contractile responses is considered to be a major contributor of the mechanical dysfunction in the remodeled LV, and hence electrical resynchronization offers benefit for HF patients with a significant QRS prolongation. Regional myocardial differences have repeatedly been noted in various models of HF induced by electrical stimulation. However, it is currently not entirely clear which signaling processes are primarily involved in regional mechanical alterations and how they are linked with potential changes in the  $\text{Ca}^{2+}$ -responsiveness of the myofilaments and/or with the  $\text{Ca}^{2+}$ -handling of cardiomyocytes.

In view of the central position of the  $\beta$ -adrenergic cascade in the regulation of myocardial contractility, several previous investigations addressed its hypothetical involvement in the regional characteristics of pacing-induced HF. Indeed, the efferent sympathetic function and  $\beta$ -adrenergic responsiveness appeared to be reduced in association with the development of pacing-induced HF in dogs with region-specific alterations in function, although the biochemical and cellular parameters of  $\beta$ -adrenergic signaling did not clearly exhibit regional characteristics. At the myofilamentary level, a decrease in basal  $\beta$ -adrenergic signaling would predict an increase in the  $\text{Ca}^{2+}$ -sensitivity of myofibrillar force production, a change often correlated with the coordinated hypophosphorylation of TnI and MyBP-C.

During our experiments we focused not only on regional, but also on intraregional aspects of a hypothetically blunted PKA-mediated TnI phosphorylation, along with the development of pacing-induced HF in pigs. MRI imaging in the experimental animals detected regional contractile dysfunction, which could be characterized with a more pronounced decrease in LV systolic thickening and circumferential shortening in the paced, anterior region as compared with the inferoseptal, opposite region (~7% vs. ~31% and ~-5% vs. ~-7% at the paced and opposite sites, respectively). We hypothesized that tissue-level alterations in TnI



phosphorylation driven by the  $\beta$ -adrenergic system might be associated with regional differences in the mechanical function of the remodeled myocardium.

Our back-phosphorylation assays revealed significantly decreased TnI phosphorylation at the pacing site of the HF animals as compared with the controls, consistently with a hypothetical down-regulation of the  $\beta$ -adrenergic system at the pacing site of the HF animals and hence implying an increase in the  $\text{Ca}^{2+}$ -sensitivity of myofibrillar force production ( $pCa_{50}$ ). Accordingly, the mean  $pCa_{50}$  values were significantly higher at the HF pacing site than at the opposite site or both sides in controls, and this difference could be eliminated by *in vitro* PKA treatments. In addition, immunohistochemistry, flow cytometry and direct force measurements confirmed a relatively high cell-to-cell variability in the relative P-TnI levels at the pacing site of the HF animals.

Overall, our observations suggested that alterations in the myofilament function of the cardiomyocytes were mirrored by  $pCa_{50}$  at the pacing site of the HF animals, a change that can be correlated to PKA-dependent TnI and MyBP-C phosphorylation. Therefore, we propose that dysregulation of PKA-dependent contractile protein phosphorylation may contribute to the regional contractile dysfunction observed in the porcine model of pacing-induced HF. Additionally, the increase in  $F_{passive}$  at the pacing site due to PKA-dependent hypophosphorylation of other myofilament proteins (e.g. titin) could participate in the increase in LV end-diastolic pressure in these hearts (from  $\sim 6$  Hgmm to  $\sim 20$  Hgmm). Nevertheless, our present investigations did not clarify to which extent regional alterations in myofilament protein phosphorylation were involved in functional hemodynamic alterations (e.g. reduction in the ejection fraction from  $\sim 76\%$  to  $\sim 35\%$ ).

To the best of our knowledge, this is the first analysis of tissue-level inhomogeneities in cardiomyocyte TnI phosphorylation through a combination of a number of sensitive methods (protein biochemistry, histochemistry, direct force measurements and cell cytometry) during the development of non-ischemic HF. Our data revealed that the level of TnI phosphorylation spans a wider range in the vicinity of the pacing electrode than elsewhere, and suggested that it can be distinctly different in clusters of cardiomyocytes during pacing-induced HF. At present, we cannot provide an explanation for this distribution pattern of P-TnI staining intensity; we speculate that it may be related to the physical characteristics of the stimulating electrical current on the myocardium.

Overall, the rapid pacing of the LV induced the remodeling of porcine hearts and led to HF, which could be characterized by an overall decrease in PKA-mediated TnI phosphorylation close to the site of electrical stimulation. The level of PKA-dependent

phosphorylation of TnI exhibited a larger cell-to-cell variation in the paced region of the LV than in more remote areas or in controls. This intraregional inhomogeneity in this  $\beta$ -adrenergic-dependent variable affects the  $\text{Ca}^{2+}$ -sensitivity of myofibrillar force production, and may thereby contribute to dyssynchronous and hence impaired LV contractions in the porcine model of pacing-induced HF.

## **5.2. Beneficial effects of SR33805 on the contractile system of the heart in post-infarction rat model**

We investigated the effect of SR33805 on cardiac contractile properties in post-MI rat model. Increased contractility by SR33805 was observed during *in vivo* echocardiographic and tissue Doppler measurements, which was in line with experiments on intact MI cardiomyocytes, where application of 10  $\mu\text{M}$  of SR33805 restored MI cell shortening without affecting the  $\text{Ca}^{2+}$ -transient amplitude. This positive inotropic effect of SR33805 can be explained by the observed increase in myofilament  $\text{Ca}^{2+}$ -sensitivity, which could be related to the concomitant slight increase in MLC2 phosphorylation and a more significant decrease in TnI phosphorylation after PKA inhibition induced by the drug.

It has been shown that increasing contractility after MI worsens cardiac injury and pump dysfunction. However, the increase in contractility was performed by over-expressing of  $\beta 2$ -subunit of the L-type  $\text{Ca}^{2+}$ -channel, which increased  $\text{Ca}^{2+}$  release. This therapeutic strategy is the opposite of that typically used clinically for treatment of HF. According to current guidelines, stimulation of the  $\beta$ -adrenergic system by catecholamines during MI is only acceptable in the case of acute HF, and only for a short period of time to support the circulation. In the long term, the use of  $\beta$ -blockers is recommended to preserve the reserve of pump function and reduce  $\text{Ca}^{2+}$ -induced arrhythmias. However,  $\text{Ca}^{2+}$ -sensitizing agents, with direct action on the myofilaments can theoretically be employed in chronic HF to improve contractile dysfunction. To our knowledge, this is the first investigation of SR33805's effects in pathological conditions.

Echocardiographic measurements confirmed, that an intraperitoneal bolus of SR33805 can improve LV contractility of rats *in vivo*. These data are in line with the results obtained in anaesthetized, open-chest dogs showing that intravenous administration of SR33805 resulted in a large, significant increase in segment shortening. This effect could not be explained by the vasorelaxant and  $\text{Ca}^{2+}$ -entry blocker properties of the SR33805, classically described with this drug, thus further investigations were required to clarify other potential mechanisms for

the beneficial effects of SR33805 on the myocardium. Interestingly, at the cellular level, SR33805 did not affect the  $\text{Ca}^{2+}$ -transient in failing cardiomyocytes, whereas it previously did in control cells. This effect could be due to a high dependency of SR33805-induced  $\text{Ca}^{2+}$ -blockade on membrane potential. Previously it was shown, that both in vascular and cardiac preparations, SR33805's  $\text{Ca}^{2+}$ -blockade was reduced in depolarized preparations. This could be the case in failing cardiomyocytes. Alternatively, HF-specific alterations in the intracellular metabolic environment can possibly also underlie the difference in the effects of SR33805 on the  $\text{Ca}^{2+}$ -transient between failing and control cardiomyocytes.

A potential risk of  $\text{Ca}^{2+}$ -sensitizers is the amplification of the diastolic dysfunction induced by an increase in  $\text{Ca}^{2+}$ -sensitivity to diastolic levels of  $[\text{Ca}^{2+}]_i$ . The only class of  $\text{Ca}^{2+}$ -sensitizer that reached clinical investigations is levosimendan, which did not alter the relaxation rate and even showed some lusitropic effects in both normal and failing dogs. During our investigations, there was no sign of relaxation prolongation after SR33805 treatment *in vivo*, as well as *in vitro*, at the cellular level, where no signs of increased passive tension in permeabilized cardiomyocytes or prolongation of  $\text{Ca}^{2+}$ -decline in intact myocytes were observed. Instead, a faster decline was obtained, suggesting a favoured  $\text{Ca}^{2+}$ -reuptake upon SR33805 treatment. Moreover, SR33805 induced an acceleration of contraction and relaxation velocities in MI cells and in control cells. Similar changes on contraction have been reported in myocytes overexpressing the specific cardiac MLC-kinase, in which MLC2 phosphorylation was increased. This suggests that changes of myofilament properties can affect the velocities of cell shortening/relaxation and that the increased phosphorylation level of MLC2 after SR33805 treatment, although modest, may participate in this effect. The mechanism of the accelerated  $\text{Ca}^{2+}$ -decay seen after SR33805 treatment is unclear. It is possible, that the drug can modulate intracellular  $\text{Ca}^{2+}$ -signaling by altering function of proteins regulating  $\text{Ca}^{2+}$ -homeostasis. The exact role of SR33805 in  $\text{Ca}^{2+}$ -homeostasis needs further investigations.

An interesting feature of SR33805 is the length dependency of its effects on myofilament  $\text{Ca}^{2+}$ -sensitivity. A modest, but significant effect on  $p\text{Ca}_{50}$  at short SL and a more pronounced effect at long SL was observed, resulting in a normalized length-dependent activation as indexed by  $\Delta p\text{Ca}_{50}$ . This result had previously been reported in control cells as well. Surprisingly, this effect is not shared with other  $\text{Ca}^{2+}$ -sensitizers (EMD 57033, CGP-48506 and calmidazolium), which blunt the length-dependent activation in permeabilized preparations. The length-dependent activation of the myofilaments form the cellular basis of the Frank–Starling law of the heart, and is a prerequisite of the normal heart function. The

molecular mechanism of this phenomenon, and the effect of SR33805 on length-dependent activation are unclear. SR33805 may restore myofilamentary stretch-dependent activation of MI cardiomyocytes through increasing MLC2 phosphorylation, however, based on its modest effect observed, we suspect that other mechanisms could also be involved in the large myofilament  $\text{Ca}^{2+}$ -sensitization.

$\text{Ca}^{2+}$ -sensitizers can improve contraction by affecting troponins and/or directly facilitating cross-bridge cycling. From our present investigations, a direct effect of SR33805 on troponins or cross-bridge kinetics cannot be excluded and has to be determined in future experiments. With biochemical measurements we confirmed, that TnI phosphorylation on PKA sites was increased in the MI myofilaments, consistent with a hyperadrenergic state and the decrease of myofilament  $\text{Ca}^{2+}$ -sensitivity observed in MI at this stage of HF. During HF, the sympathetic nervous system activity increases in order to maintain an adequate cardiac output. This process leads to increased  $\beta$ -adrenergic receptor stimulation and PKA-mediated phosphorylation of several excitation–contraction coupling proteins. In the advanced stage of HF, the increased sympathetic nervous system leads to a desensitization of  $\beta$ -adrenergic receptors that induces down-regulation of the PKA signaling pathway. Our investigations suggest, that SR33805 inhibited PKA activity in cardiomyocytes from MI rats, which - through decreased TnI phosphorylation - could explain  $\text{Ca}^{2+}$ -sensitization of their myofilaments. The effect of SR33805 on TnI is unique in the family of  $\text{Ca}^{2+}$ -sensitizers, since some of them are known to affect TnI phosphorylation on PKA sites by the inhibition of phosphodiesterase activity. In contrast, our data suggested a direct inhibition of PKA by SR33805, which resembles rather the mechanism of  $\beta$ -blockers.

In conclusion, SR33805 increased myofilament  $\text{Ca}^{2+}$ -sensitivity in cardiomyocytes from MI rats by altering phosphorylation status of regulatory proteins of contractility. This action was achieved by direct inhibition of PKA, without any deleterious effects on  $\text{Ca}^{2+}$ -homeostasis. Accordingly, *in vitro* beneficial effects of SR33805 contributes to the *in vivo* observed enhanced contractility of the animals, which suggests a future therapeutic role for the drug in human HF.

## 6. Summary

At present, the molecular background of chronic heart failure is not yet completely understood. Therefore, revealing the pathophysiology of the disease in detail can contribute to development of new therapeutic approaches of heart failure.

During our experiments we investigated, whether changes in myofilamentary protein phosphorylation can be associated with the *in vivo* found regional contractile dysfunction in pacing-induced heart failure. Furthermore, on a post-ischemic rat model we aimed to evaluate the possible  $\text{Ca}^{2+}$ -sensitizing effect of SR33805, a potent  $\text{Ca}^{2+}$  channel blocker that has previously never been tested in pathological conditions of the cardiovascular system.

During our investigations we made the following key observations: 1) In the porcine model of pacing-induced heart failure an overall decrease in protein kinase A-mediated troponin I phosphorylation can be characterized close to the site of electrical stimulation, which exhibits a large degree of cell-to-cell variability. 2) Heterogeneous remodeling of the failing myocardium which accompanies pacing-induced heart failure alters the  $\text{Ca}^{2+}$ -sensitivity of myofibrillar force production and may thereby contribute to regional contractile abnormalities observed in the animals *in vivo*. 3) SR33805 modulates the phosphorylation status of regulatory proteins of myofilamentary contractile function in post-ischemic rat myocardium. This result correlates in part with direct inhibition of protein kinase A activity. 4) SR33805 thus, increases  $\text{Ca}^{2+}$ -sensitivity in isolated rat cardiomyocytes, thereby contributing to improved contractility of ischemic failing hearts *in vivo*.

Major conclusions: outcomes of the present investigations improve insight into molecular aspects of heart failure-associated regional contractile dysfunction, and illustrate the possibility for a new pharmacological  $\text{Ca}^{2+}$ -sensitizing mechanism in future heart failure therapies.

## 7. In extenso publications of the author



DEBRECENI EGYETEM EGYETEMI ÉS NEMZETI KÖNYVTÁR  
KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA

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Candidate: Dániel Czuriga

Neptun ID: THKBBP

Doctoral School: Kálmán Laki Doctoral School

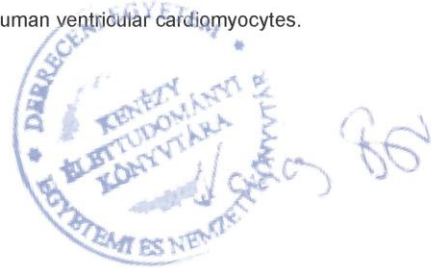
### List of publications related to the dissertation

1. **Czuriga, D.**, Tóth, A., Pásztor, E.T., Balogh, Á., Bodnár, A., Nizsalóczki, E., Lionetti, V., Recchia, F.A., Czuriga, I., Édes, I., Papp, Z.: Cell-to-cell variability in troponin I phosphorylation in a porcine model of pacing-induced heart failure.  
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List of other publications

3. **Czuriga, D.**, Paulus, W.J., Czuriga, I., Édes, I., Papp, Z., Borbély, A.: Cellular mechanisms for diastolic dysfunction in the human heart.  
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