Ph.D. Thesis

MOLECULAR LEVEL INVESTIGATIONS ON THE SARCOMER DYNAMICS (FRANK-STARLING RELATIONSHIP) IN DIFFERENT MAMMALS UNDER PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

István Ferenc Édes MD

Tutor: Zoltán Papp MD, PhD
Associate Professor, Head of Division

UNIVERSITY OF DEBRECEN
MEDICAL AND HEALTH SCIENCE CENTER
FACULTY OF MEDICINE
INSTITUTE OF CARDIOLOGY
DIVISION OF CLINICAL PHYSIOLOGY

DEBRECEN, 2008

1. Introduction

1.1. The mechanisms of cardiac contraction

Myofibrillar events during contraction may be understood as a dynamic and cyclic process between actin (thin filament) and myosin (thick filament) . One way of looking at this phenomenon is the so-called three-stage model of thin filament activation (Figure). 1) In a relaxed muscle (when Ca^{2+} concentration is low) tropomyosin blocks the “active” surface on actin that is required for the cross-bridge interaction (blocked state) 2) After depolarization, free cytoplasmatic Ca^{2+} levels increases, and Ca^{2+} binds to troponin C. 3) Binding of Ca^{2+} initiates a conformational shift in the tertiary structure of troponin C, due to which the inhibition of actin by troponin I decreases, furthermore the tropomyosin thread displaces above the actin groove. Thus it exposes the “active” surface actins, where myosin may bind to (closed state). 4) In the so called closed state, loose cross-bridges are formed between the myosin heads and the free active actin surfaces (weak force transmission - force generation). It is hypothesized that with increasing sarcomere length (SL), the thick and thin filaments move closer together, thus decreasing the cross-section (lattice spacing) of the myofilaments, which favors formation of weak cross-bridge formations and promotes the development of the closed state. 5) In the presence of Ca^{2+} the so called open state develops, where the tropomyosin thread further shifts in the direction of the actin groove, and the free surface of the actin becomes totally free. All of these events favor the development of strong cross-bridge formation and force generation (Figure). 6) During relaxation, opposite processes take place: Ca^{2+} dissociates from troponin C, (due to Ca^{2+} being pumped back into the sarcoplasmic reticulum at high speed), the original conformation of troponin C is restored, and the active surface of actin is again blocked (the tropomyosin thread shifts to its original relaxed state) (Figure 1).

Due to ß-adrenergic stimulation, a protein phosphorylation cascade occurs in the myocardium. Among the myofibrillar proteins the troponin I and the C-protein are phosphorylated following ß-adrenergic stimulation. The functional effect of troponin I phosphorilation has become more and more clear due to new experimental results. Current notion is, that phosphorylation reduces the Ca^{2+}-sensitivity of the myofibrills (the Ca^{2+} dependence of developed force and myosin ATPase activity shifts to the right). Troponin I phosphorylation increases the dissociation of Ca^{2+} from troponin C, and accounts for part of the observed positive lusitropic effects seen during ß-adrenerg stimulation. The opposite of this phenomenon works backwards as well. During pathological conditions (heart failure), due to the decrease in $ß_1$ receptor numbers and receptor uncoupling the basal phosphorylation level of troponin I is decreased, which may be regarded as a compensatory mechanism that increases the Ca^{2+}-sensitivity of the myofilaments. The effect of the phosphorylation of the C-protein on the contractility is the target of extensive research, but as of yet, only conflicting results are available.

1.2. Resting sarcomere length and contractile force

Since the discovery of the Frank-Starling law, the relationship between resting myofibrillar length (end-diastolic volume) and intraventricular pressure is well known. On the peak of the Frank-Starling (pressure-volume) relation, in both skeletal and cardiac muscle the resting sarcoemre length (SL) is about 2.3 μm. The ascending part of the relationship ranges up to SL 2.3 μm, and with higher SL values than 2.3 μm, a moderate decrease is already seen. At optimal SL (2.3 μm), the cross-bridge cycling between actin and myosin is maximized. Above this value (>2.3μm), the thin filaments start to slip out from the thick filaments (the number of cross-bridges decrease). Moreover, at shorter SL (<2.3μm) the thin filaments shift to the other side of the sarcomere, and partially overlap each other. This also decreases the formation of force generating cross-

bridges. In pathological conditions (severe dilatation) the SL rarely reaches values over 2.2-2.4 μm. Thus a failing heart is working at the peek of the Frank-Starling relationship. The massive connective tissue structure located within the heart (collagen and titin), inhibits the overstreching of the myocardium.

To explain the relationship between resting SL and contractile force, the development of the optimal actin-myosin configuration was proposed (optimal cross-bridge cycling). Newer evidence shows however, that apart from the optimal actin-myosin configuration, other mechanisms are also important for the determination of the contractile force: 1) Increasing resting SL decreases the cross sectional diameter of the myofibrils (in other words, elongated fibers are narrower) and the lattice spacing decreases. Thus, the distance between thin and thick filaments decrease and it favors the actin-myosin interaction and the development of weak cross-bridge-formations. 2) Elongation of SL increases the sensitivity of the Ca^{2+} receptor (troponin C) to Ca^{2+}, thus at the same Ca^{2+} transient levels increasing resting SL results in an augmentation in contractile force.

However, to understand the precise mechanism of action of the Frank-Starling relationship, further, detailed experiments are needed.

1.3. β-adrenergic receptor system, myofibrillar proteins and heart failure

With the progression of heart failure, activation of the sympathetic nervous system is evident, but neither the cause, nor the precise time of activation is known. In severe heart failure, there is a significant increase in plasma noradrenalin levels. The increased sympathetic activation in heart failure may be due to relative hypotension and consequential baroreceptor activation. Chronic increased sympathetic tone leads to deterioration of the myocardial function. The direct toxicity of catecholamines has long been suggested, but only little direct evidence to support this assumption. Furthermore, chronic elevation of catecholamines is linked to an increase in sarcolemmal

permeability, calcium overload and development of arrhythmias and an increased O_2 demand of the myocardium. There have been many reports on the downregulation of the ß-adrenergic system (ß-adrenergic receptor, G-proteins, adenyl-cyclase enzyme) in heart failure. Consequently, the ß-adrenerg activation is attenuated and in parallel the production of cyclic AMP (cAMP) is decreased. This pathological signal transduction is mainly due to a decline in $ß_1$-adrenerg receptor numbers (density), rather than desensitization caused by high serum cathecolamin levels. Due to the decrease in the $ß_1$ receptor system activation the cAMP-dependent phosphorylation processes attenuate. Because of all the above, the cAMP-dependent basal phosphorylation of troponin I decreases, that increases the Ca^{2+} sensitivity of the myocardium (shifting the force-pCa curve to the left).

Changes in myofibrillar proteins due to heart failure have long been the target of intensive research. It has been shown, that in advanced heart failure the myosin ATPase activity decreases. Moreover, in small mammals (mouse, rat) and in the human atrium the expression of the fast (α) myosin heavy chain (MHC) is substituted by the slow (β) MHC. As a result the contractile filaments work more efficiently, but the speed of the contraction is reduced (V_{max}). The myosin isoenzyme switch described in small mammals, only moderately affects the human myocardium, due to the abundance of ß MHC. The switch of the heart to a more economic mode can also be observed in humans. Fetal troponin T and atrial myosin light chain proteins are expressed in the failing human left ventricle that may also contribute to myofibrillar remodeling. However, the pathological changes in the myofibrillar protein composition/function in chronic heart failure are still not fully understood.

2. Research aims

1. The underlying mechanism of the Frank-Starling relationship has already been partially clarified (decreased lattice spacing and Ca^{2+} sensitization), but do not fully explain the well known relationship between volume and force. No or only few data are available on the regulation of the Frank-Starling relationship: 1) in various species with different MHC composition; 2) under various ischemic conditions (increased phosphate concentration and at low pH); 3) under different temperatures and 4) under pathological conditions (chronic heart failure). Moreover, only conflicting data are available on the relationship between SL and actin-myosin cross bridge cycling rate. Hence, we have decided to examine these questions in the present work.

2. The pathologic mechanical (contractile function) and biochemical (composition- and basal phosphorylaton of myofibrillar proteins and ß-adrenergic receptors) alterations in heart failure are not fully clarified and the available data are sometimes conflicting. Consequently, our aim was to examine the time course of pathological events in a well-defined transgenic heart failure animal model (Tgαq*44). We have carefully examined the alterations in: 1) the cardiomyocytes mechanics (isometric contraction, passive force, turnover rate of the actin-myosin crossbridge cycle etc.); 2) the composition of myofibrillar proteins (MHC expression and basal phosphorylation of myofibrillar proteins). Our aim was to obtain new data on the pathological changes that initiate/trigger the depression of the contractile function.

3. Materials and methods

3.1. Experimental tissue material

Adult human, murine, and porcine left ventricular cardiac tissues stored at -80°C were used in this study. Healthy human hearts, obtained from three general

organ donor patients (a 41-year-old man, a 46-year-old woman, and a 53-year-old woman) were explanted to obtain pulmonary and aortic valves as homografts for cardiac surgery. The donors did not show any sign of cardiac abnormalities and did not receive any medication except short-term dobutamine and furosemide. The experiments on human tissues complied with the Helsinki Declaration of the World Medical Association and were approved by the Hungarian Ministry of Health (no. 323-8/2005-1018EKU). Pigs received humane care in accordance with the Italian law (DL-116, Jan. 27, 1992), which is in compliance with the National Institutes of Health publication Guide for the Care and Use of Laboratory Animals, and the scientific project, including animal care, was supervised and approved by the Italian Ministry of Health.

Tgαq*44 mice were generated as described previously. Breeding pairs of homozygous Tgαq*44 mice and wild-type mice FVB were obtained from Prof Eva J. Neer (Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School) and bred in the Animal Laboratory of Polish Academy of Science Medical Research Centre in Warszawa. Homozygous Tgαq*44 mice and FVB mice, as wild type counterpart were used for experiments. All tissue samples were stored at -80 ^{o}C until assayed in permeabilized cardiomyocyte preparation or in the biochemical assays. These animal procedures conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Jagiellonian University Ethical Committee on Animal Experiments (No. 1/OP/2003).

3.2. Force measurements in permeabilized cardiomyocyte preparations.

The compositions of the relaxing and activating solutions used during the force measurements were calculated as described previously. The pCa, i.e., -log[Ca^{2+}], of the relaxing solution and the activating solution (pH 7.2) were 9 and 4.75, respectively. Force measurements were carried out by using two pairs of relaxing and activating solutions, one pair without added P_i and the other pair

with added P_i. The compositions of the solutions were as follows: the activating solution without P_i contained (mM) 1 free Mg^{2+}, 5 MgATP, 15 phosphocreatine, 7 calcium ethylene glycol-bis(2-aminoethyl ether)-N,N,N,N-tetraacetic acid (Ca-EGTA), and 100 N,Nbis[2-hydroxyethyl]-2-aminoethanesulfonic acid. The ionic equivalent was adjusted to 150 mM with KCl at an ionic strength of 186 mM. The composition of the P_i-free relaxing solution differed from that of the activating solution only in the use of 7 mM EGTA instead of Ca-EGTA.

Our experimental protocols also assessed the role of the use of the catalytic subunit of bovine heart PKA in our experimental system, with a relative phosphorylating effect of ~10 units/μg. To examine the role of PKA, isometric force measurements were conducted at a SL of 2.3 μm in cardiomyocytes of 10 and 14-month-old FVB and Tgαq*44 mice. After assessment of the initial Ca^{2+}-force relationships, the preparations were incubated in the PKA mixture for 40 minutes at 20 ^oC and the Ca^{2+}-force relationships were then determined again.

The technique employed for force measurements in a single myocyte-sized preparation has been detailed earlier. Briefly, a single myocyte was attached with silicone adhesive to two thin stainless steel needles while viewed by means of an inverted microscope. One needle was attached to a force transducer and the other to an electromagnetic motor, both connected to joystick-controlled micromanipulators. Sarcomere length adjustment was performed on a selected segment covering large part of the cardiomyocytes, where power spectra were obtained with the spatial Fourier transform of the cross striated pattern in relaxing solution. The diameters of the preparations were measured microscopically, in two perpendicular directions. The thickness of the preparation was determined by focusing on the lower and upper surfaces of the cardiomyocytes and measuring the displacement of the objective. The cross-sectional area was calculated by assuming an elliptical cross-section. Isometric force measurements were performed during repeated activation-

relaxation cycles at 15°C (and in some human cardiomyocytes also at 25°C), first at a SL of 1.9 μm and then at a SL of 2.3 μm, in every cardiomyocyte.

When the peak force was reached, the length of the cardiomyocyte was reduced by 20% within 1 ms, and 20 ms later the original length was restored. As a result of this intervention, the force dropped from the peak level to zero, thus allowing the determination of the total force level, and then started to redevelop. About 6 s after the onset of force redevelopment, the cardiomyocyte was returned to the relaxing solution, where a shortening to 80% of original preparation length with a long slack duration (10 s) was performed to assess the passive force level. The active isometric force was calculated by subtracting the passive force from the total isometric force. The force redevelopment after the restretch was fitted to a single exponential function in order to estimate the rate constant of force redevelopment (k_{tr}) at various [Ca^{2+}] levels:

$$[1] \quad F(t) = F_i + F_a(1-e^{-k}tr^t)$$

where F(t) is the force at any time t after the restretch at a given [Ca^{2+}], and F_i and F_a denote the initial force value after the restretch and the amplitude of Ca^{2+}-activated force redevelopment, respectively. Force records between F_i and complete force recoveries were included for k_{tr} determinations for all [Ca^{2+}] and species. The sampling rate was 20 Hz during the overall experiment, and during the k_{tr} determination it was 1 kHz.

Each experimental protocol began and ended with a control activation at saturating [Ca^{2+}] (pCa 4.75). This allowed force normalization and assessment of the rundown of the preparations through the comparison of changes in maximal Ca^{2+}-activated force (F_o). The F_o value used to normalize submaximal force levels was obtained by linear interpolation between successive maximal activations. If at the end of the experimental session the cardiomyocyte produced less than at least 80% of the maximum force of the first contraction, that preparation was excluded from the analysis.

3.3. Biochemical assays

Frozen left ventricular tissue samples of 43 mouse hearts were disrupted in a modified isolation solution (in mM; KCl 100, EGTA 2, MgCl2 1, ATP 0.1, imidazole 100, pH 7.0). The cytosol was separated by centrifugation (300 g, 1 min, supernatant) and 0.3 % Triton-X-100 in modified isolation solution (Sigma, St. Louis, MO, USA) was added to the pellet. Detergent soluble proteins were extracted for 5 min on ice with continuous agitation and separated by centrifugation from the contractile proteins (300 g, 1 min, supernatant: membrane proteins; pellet: permeabilized cardiomyocytes). Next, the protein concentration of the separated fractions were determined by the BCA method using BSA as standard. Endogenous PKA activity was then tested at room temperature in a reaction mixture containing 20 µl of the separated fractions, 4 µl modified isolation solution containing ^{32}P labelled ATP (final specific activity: 750 cpm/pmol) and 1 µl kemptide (1 mg/ml). Reactions were initiated by adding the ^{32}P-labelled ATP, then 10 µl of reaction mixtures were dropped onto phosphocellulose paper at 0 and 17 min. Papers were dried for 30 min and then washed three times with 0.5 % phosphoric acid and finally with acetone. Radiocativity on the papers was measured by scintillation counting.

To assess the phosphorylation level of troponin I (TnI) in the permeabilized cardiomyocytes a back phosphorylation assay was performed. Permeabilized cardiomyocytes were prepared as above. Reaction mixtures contained 40 µl of the isolated cardiomyocyte fraction (protein concentration 0.5 mg/ml in the modified isolation solution) 10 µl modified isolation solution containing ^{32}P labelled ATP (final specific activity: 1500 cpm/pmol) and 1 µl PKA (4000 U/ml). The reaction was performed at room temperature for 15 min. At the end, 50 µl of SDS sample buffer was added and the samples were boiled for 5 min. 30 µl of the boiled samples were loaded onto 10% polyacrilamide gels, separated and transferred onto nitrocellulose membranes. Phosphate incorporation into endogenous TnI was detected by autoradiography. Band

intensites (representing phosphate incorporation) were determined by densitometry using Image J software. Optical densities are shown on the figures in arbitrary units.

MHC isoforms ((α and β) were separated with a Bio-Rad apparatus using custom made gels (8 % acrilamide-bis acrylamide (27.5:1 ratio), 380 mM tris-HCl, 0.1% SDS, 5 mM TEMED, 2 mM AMPER). Gels were further evaluated by Image J software.

3.4. Statistics

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

$$[2] \qquad F = F_o\,[Ca^{2+}]^{nHill}/(Ca50^{nHill} + [Ca^{2+}]^{nHill}),$$

where F is the steady-state force at a given $[Ca^{2+}]$, while F_o, nHill and Ca50 (or pCa50) denote the maximal Ca^{2+}-activated force at saturating $[Ca^{2+}]$, and the slope and the midpoint of the sigmoidal relationship, respectively. Each experimental preparation was fitted individually, the fitted parameters were pooled, and the mean values are reported.

Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) and, where applicable, by Student's t-test. Values are given as means±SEM. Statistical significance was accepted at $P<0.05$.

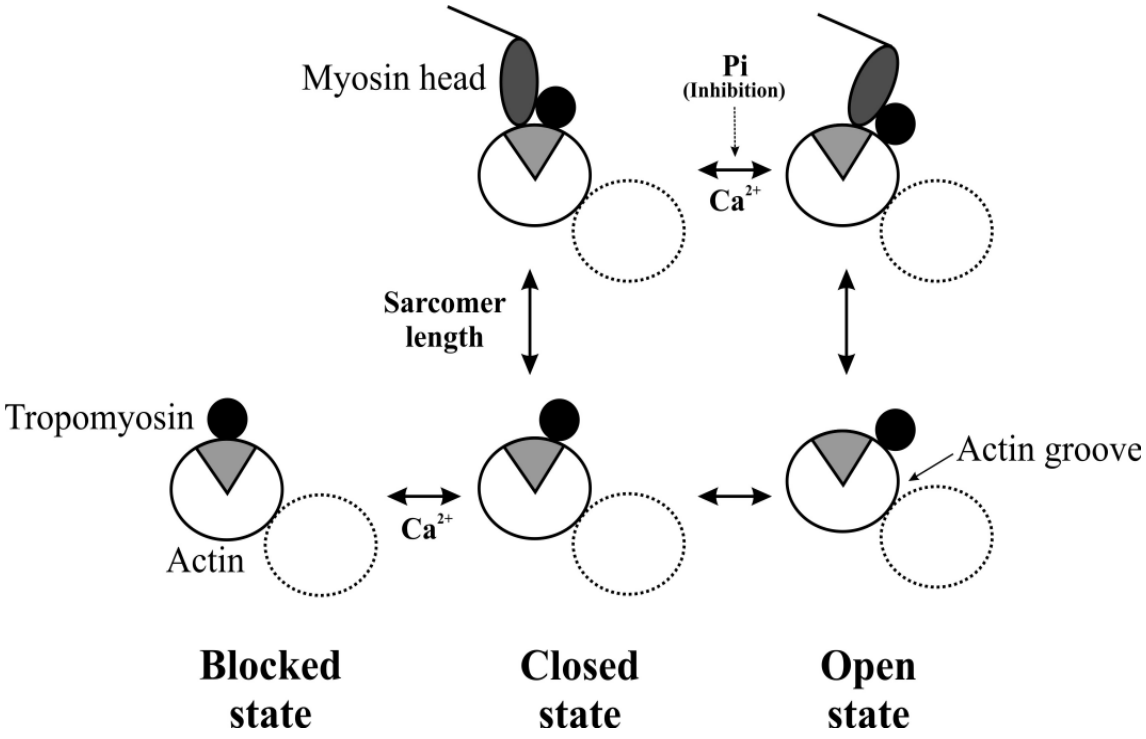

Figure. Three-stage model for thin filament activation. *In the blocked state tropomyosin inhibits (blocks) the active surface of actin (dark grey section of the circle) and hence prevents its binding to myosin. In the presence of Ca^{2+} troponin C is activated and this moves tropmyosin towards the groove of the actin double helix thereby allowing the weak interactions between actin and myosin (closed state). Ca^{2+} stimulates the transition towards the open state with high cooperativity and thus tropomyosin moves further in the direction of the actin grove and exposes totally the active surface of actin for myosin binding. This allows the development of strong cross-bridges. Increasing the resting length of the sarcomere decreases its cross sectional diameter and hence brings the thisn and thick filaments closer to each other actin and mysion filaments with a consequent increased probability for the transition between blocked and closed states. Inorganic phosphate (P_i) inhibits the transition towards the open sate. SL=sarcomer length; ATP=adenosin triphosphatet; ADP=adenosin diphosphate.*

4. Results

4.1. Effects of sarcomere length on contractile force and the rate of tension redevelopment in different species

In these experiments the mean values of the Ca^{2+}-activated force and Ca^{2+}-independent passive force have been measured in the cardiomyocytes of three different species (human, porcine and murine cardiomyocytes) at short and long SLs. Similar values were noted for the Ca^{2+}-activated force in all of them (SL 1.9 μm: 27.2±3.9 kN/m^2, 28.6±3.3 kN/m^2 and 30.3±3.0 kN/m^2; 2.3 μm: 56.6±3.9 kN/m^2 49.5±3.7 kN/m^2 and 51.8±6.9 kN/m^2 in the murine, human and porcine cardiomyocytes, respectively). At the 2.3-μm SL the passive force of murine myocytes was significantly higher than that in porcine or human cardiomyocytes (murine 10.5±0.6 kN/m^2, human 5.7±0.7 kN/m^2, and porcine 6.9±0.7 kN/m^2), in accordance with the previously suggested high resting stiffness in murine cardiomyocytes.

To compare the SL-dependent enhancement of isometric force production in the three investigated species, normalized force-pCa relations were determined under identical experimental conditions. The change in Ca^{2+} sensitivity of force production (i.e., the difference in pCa50 resulting in half the maximal force for an increase in SL from 1.9 μm to 2.3 μm) was very similar in human, murine, and porcine cardiomyocytes and corresponded to ~0.11 pCa unit. However, there were major differences when the k_{tr} values for the murine preparations were compared with those for the human or porcine cardiac preparations at all investigated [Ca^{2+}]. While the human and porcine cardiomyocytes exhibited comparable absolute $k_{tr,\ max}$ values (measured at pCa 4.75) at both SLs, the murine preparations displayed significantly higher k_{tr} values at both SLs (i.e., k_{tr} was 6–7 times higher in mice than in humans or pigs). k_{tr} decreased with [Ca^{2+}] at both SLs in all preparations. However, SL adjustment did not have a statistically significant effect on k_{tr} in any preparation

at any [Ca^{2+}]. At 1.9 µm of SL the $k_{tr,\ max}$ values were as follows: murine 7.44±0.15 1/s, human 1.33±0.11 1/s and porcine 1.02±0.05 1/s. Similar values at 2.3 µm SL were: 6.95±0.31 1/s, 1.19±0.07 1/s and 0.99±0.08 1/s. To compare the extent of the Ca^{2+}-dependent changes in k_{tr}, the k_{tr} values were normalized to their respective maxima during each experiment. This type of data representation suggests that the Ca^{2+} dependencies of k_{tr} are very similar in the three investigated species.

P_i is known to blunt the maximal Ca^{2+}-activated force production through direct interference with force-generating cross-bridge transitions. Accordingly, we studied the effects of 10 mM P_i on force production and k_{tr}. The maximal Ca^{2+}-activated force (F_o) was decreased by 10 mM P_i to ~60–65% of the P_i-free values in all three species (i.e., to 65±3%, 60±1%, and 61±1% in humans, mice, and pigs, respectively; P>0.05) at a SL of 1.9 µm. Moreover, the effect of 10 mM Pi on Fo did not depend on the SL.

To address the SL dependencies of Ca^{2+} sensitivity in the presence of P_i, the force values were also normalized to their respective maxima and the data were fitted to the Hill equation. This analysis revealed that, for all of the species, the change in pCa50 in the presence of P_i did not differ from that in the absence of P_i, i.e., it was ~0.1 pCa unit. However, 10 mM P_i induced a dramatic acceleration in the cross-bridge turnover kinetics in all preparations. The ratio of the $k_{tr,max}$ values in the presence and absence of P_i was ~1.6 in both humans and pigs, whereas in mice it was nearly 3 at both SLs. The SL had no significant effect on k_{tr} in the presence of P_i at any investigated [Ca^{2+}].

With increasing temperatures the P_i-induced acceleration in cross-bridge kinetics is augmented in skeletal myofibrils of the rabbit and cardiac myofibrils of the mouse. Hence, we set out to verify whether this effect is also present in human cardiomyocytes, using a more physiological experimental temperature. Moreover, we tested whether a change in temperature affected the P_i dependence of force production and the apparent independence of k_{tr} from SL. To these

ends, Ca^{2+} contractures were also evoked in the presence of P_i at 25°C. At a SL of 1.9 μm, maximal Ca^{2+}-activated force at 25°C in the absence of Pi was 35.2±4.2 kN/m^2 (n=10 human cardiomyocytes), while in the presence of 10 mM Pi it was 17.4±2.4 kN/m^2. Hence, these experiments showed that at a higher temperature the decrease in the maximal Ca^{2+}-activated force by P_i was somewhat larger in the human myocardium.

The amplitudes of the Ca^{2+} contractures at 25°C were also normalized, and the Ca^{2+} sensitivities of force production were determined at both SLs. Similar to the previous results, the shift in pCa_{50} did not change (pCa50 0.10±.02; $P<0.05$), despite the fact that k_{tr} was further increased with temperature (to ~5.2-fold when expressed as a function of k_{tr}, max in the absence of P_i at 15°C). k_{tr}, max at 25°C for human cardiomyocytes was 7.03±0.32 s^{-1}. However, no significant differences could be observed between the k_{tr} values of the two SLs at the same activating [Ca^{2+}] at 25°C in human cardiomyocytes.

To compare the activation dependencies of k_{tr} on species and P_i, k_{tr} was replotted against the force normalized to F_o under all experimental conditions at 15°C. k_{tr} increased nonlinearly with force at each SL and in all species to an apparent maximum at F_o. In general, at the shorter SL, k_{tr} tended to be slightly larger at equivalent submaximal force levels in all preparations and under all experimental conditions. The increase in k_{tr} at submaximal levels of force, when force was matched at both SLs, might be due to the higher [Ca^{2+}] needed to reach the same level of force at the shorter SL. In the absence of P_i and at a SL of 2.3 μm, the range of k_{tr} change for doubling of the force from half-maximal force to Fo was ~1.7-fold in humans, 2.2-fold in mice, and 2.4-fold in pigs. Ten millimolar P_i shifted the k_{tr} dependencies on force toward higher levels, but it did not change the characteristic slopes of these relationships at constant SL within the same species.

4.2. Late-stage alterations in myofibrillar contractile function in a transgenic mouse model of dilated cardiomyopathy (Tgαq*44)

A typical feature of the Tgαq*44 mice is the continuous overexpression of the $G_{\alpha q}$ protein. Consequently, a dilated cardiomyopathy (DCM) and congestive heart failure develops in these transgenic animals with age. The progression of the disease (i.e. DCM) is slow that enables the examination of the developed pathological changes both in the signal transduction pathways and the contractile structures. In our study we mainly focused on the mechanical function of the cardiomyocytes and the associated alterations in the myofibrillar proteins.

With ageing typical changes (suggestive for the development of DCM) were noted in the Tgαq*44 mice compared to the FVB animals. The heart/body ratio increased from the 14 months onward in the Tgaq*44 animals (14th month: 5.4±0.3; 18th month: 7.1±0.4 mg/g). Moreover, an increasing mortality was observed in the Tgαq*44 mice from the ages of 13-14 months onwards.

F_o and $F_{passive}$ were determined in the cardiomyocytes of the Tgαq*44 and FVB hearts at 4, 10, 14 and 18 months of age at both SLs of 1.9 and 2.3 μm. In association with the length-dependent regulation of myofibrillar force production (1) the means of F_o and $F_{passive}$ were both significantly higher at the SL of 2.3 μm than at 1.9 μm in the cardiomcytes of both Tgαq*44 and the FVB mice. Additionally, at a given SL, the cardiomyocytes of the FVB hearts did not exhibit statistical differences throughout the total time span in either F_o or $F_{passive}$ production. In contrast, at both SLs, the cardiomyocytes of the Tgαq*44 hearts produced similar forces only at 4, 10 and 14 months of age, whereas the mean value of F_o at 18 months of age was significantly lower (i.e. ~60% of that at 14 months of age) at both the short and the long SL than the different age groups of Tgαq*44 cardiomyocytes or the FVB animals at 18 months. Moreover, the

mean $F_{passive}$ in the cardiomyocytes of the 18-month-old Tgαq*44 mice was significantly higher by ~60% ($P<0.05$) than that in the FVB cardiomyocytes at the SL of 2.3 μm.

To investigate the Ca^{2+} sensitivity of isometric force production and to explore its dependence on SL, contractures were evoked by applying various submaximal [Ca^{2+}] in both the FVB and the Tgaq*44 cardiomyocytes at the SLs of 1.9 and 2.3 μm. Following force normalizations to their respective maxima, Ca^{2+}-force relationships were constructed from which the pCa_{50} values were determined. Our observations showed that the adjustment to a longer SL always led to an increase in pCa_{50} (and Ca^{2+} sensitivity) in both the FVB and the Tgaq*44 cardiomyocytes in every age group. Moreover, the magnitudes of the leftward shifts in the Ca^{2+}-force relationships were similar in each age group, with an average of ~0.11 pCa units. Furthermore, the pCa_{50} values of the Ca^{2+}-force relationships at identical SLs did not differ significantly in FVB and Tgaq*44 cardiomyocytes at 4 months and 10 months. In contrast, the Tgαq*44 cardiomyocytes at 14 and 18 months of age exhibited significantly higher Ca^{2+} sensitivities of force production at both SLs. In the 18-month-old mice, pCa_{50} was 6.25±0.01 and 6.13±0.02 for the long and the short SLs, respectively, in the FVB cardiomyocytes, and 6.38±0.01 and 6.24±0.01 for the long and the short SLs, respectively, in the Tgαq*44 cardiomyocytes.

Previous investigations had suggested that the apparent change in pCa_{50} upon the development of heart failure can be associated with a decrease in the level of contractile protein (troponin I) phosphorylation. In this context, the role of PKA is of particular interest as it is known to modulate pCa_{50} during sympathetic stimulation. Accordingly, we set out to study the effects of PKA-mediated phosphorylation in 10- and 14-month-old Tgaq*44 and FVB mice cardiomyocytes at the SL of 2.3 μm. Incubation in the presence of PKA did not have a significant effect on the 10-month-old specimens, where the initial Ca^{2+} sensitivity curves of the Tgaq*44 and the FVB cardiomycocytes were almost

superimposed. However, in the 14-month-old animals, where the initial Ca^{2+} sensitivity of the Tgaq*44 cardiomyocytes was significantly higher than that of the FVB cardiomyocytes, PKA diminished the apparent differences in Ca^{2+} sensitivity. PKA significantly reduced the pCa50 of the Tgαq*44 preparations (pCa50 was shifted to the right by ~0.15; $P<0.05$), whereas the PKA-induced rightward shift in the Ca^{2+}-force relationships of FVB mice was not significant.

The reversibility of the increase in Ca^{2+} sensitivity of force production in Tgαq*44 cardiomyocytes by PKA suggested the involvement of the ß-adrenergic system in the pathology. To investigate this possibility in detail, biochemical experiments were performed. Measurement of endogenous PKA activity revealed a decline after 10 months of age in the cytosol of the Tgαq*44 hearts. The decline in the endogenous PKA activity become significant in the 18-month-old mice, compared to the younger Tgαq*44 animals or to the FVB hearts. In accordance with the decline of endogenous PKA activity, the level of TnI back phosphorlyation was increasing in Tgαq*44 hearts (33±1 AU vs. 72±11 AU, in 4- and 18-month-old mice, respectively), suggesting lower initial phosphorylation levels of TnI. Moreover, these assays also indicated that TnI phosphorylation in Tgαq*44 hearts at 18 months of age was also significantly lower than that in FVB hearts between the 4 and 12 months of age.

Next, we tested whether the observed age-dependent alterations in force production and Ca^{2+} sensitivity of the Tgαq*44 cardiomyocytes are accompanied by changes in the turnover kinetics of the actin-myosin cycle. To this end, the rate constant of force redevelopment (k_{tr}) was determined in each FVB and Tgαq*44 cardiomyocyte within a wide range of $[Ca^{2+}]$ and at both short and long SLs. These investigations revealed that k_{tr} possessed a marked $[Ca^{2+}]$-dependence in both the FVB and the Tgaq*44 cardiomyocytes, i.e. k_{tr} clearly decreased with $[Ca^{2+}]$. However, these assays failed to reveal a

dependence of k_{tr} on SL, as the means of k_{tr} did not differ significantly from each other at the two SLs in any age group and at any [Ca^{2+}].

Moreover, our observations did not indicate significant differences in the cross-bridge cycling kinetics between the Tgαq*44 and the FVB cardiomyocytes until the age of 14 months, i.e. up to this time point the Tgαq*44 phenotype could be characterized by the same k_{tr} as in the FVB hearts. However, at 18 months the mean k_{tr} was significantly less in the Tgαq*44 than in the FVB hearts. The maximal value of k_{tr} ($k_{tr, max}$, measured at pCa 4.75) at the SL of 2.3 μm was 9.14±0.27 1/s in the FVB and 5.39±0.19 1/s in the Tgαq*44 cardiomyocytes. The changes in k_{tr} might reflected differential expression of contractile proteins in Tgαq*44 cardiomyocytes. To investigate this possibility, changes in MHC isoform expression were assessed in Tgαq*44 hearts along with the progression of DCM. These assays showed, that indeed, β- MHC expression increased from 4±4% to 51±3% in the Tgαq*44 hearts between the 4 and 18 months of age.

5. Discussion

5.1. Effect of sarcome length on the isometric force production and tension redevelopment

Our results indicate that k_{tr} varies with species, [Ca^{2+}], [P_i], and (in human cardiomyocytes) temperature, but not with SL in permeabilized cardiomyocytes. Furthermore, our results illustrate identical SL-dependent changes in the Ca^{2+}-activated force production for human, murine, and porcine cardiomyocytes, despite marked differences in the underlying crossbridge turnover rates. On the other hand, the Ca^{2+} dependencies of relative k_{tr} values appeared to be similar in these three species. Together, our data suggest similar characteristics for the

Ca^{2+} regulation of force and k_{tr}, whereas changes in SL are not accompanied by alterations in k_{tr} in the mammalian heart.

It is generally accepted that the species specificity of MHC composition and the high ratio of expressed fast α-MHC isoform versus slow ß-MHC isoform are responsible for the high crossbridge turnover rates of rodent hearts compared with large mammals. Our data suggest that the fast α-MHC does not characteristically alter the SL-dependent Ca^{2+} sensitization. Hence, the myosin isoenzyme switch during the development of chronic heart failure, which involves a relative increase in ß-MHC isoform expression at the expense of α-MHC expression, is expected not to interfere with this process. Moreover, our results suggest that the species differences in the expression of the contractile proteins do not give rise to changes in the extent of SL-dependent Ca^{2+} sensitization in the mammalian heart.

P_i reduces the overall free energy of MgATP hydrolysis; it reverses the Pi release step of the cross-bridge cycle through mass action and thereby decreases the proportion of cross bridges in the high-force conformation. Our data indicate that P_i reduced the force independently of the species and of the SL. Furthermore, P_i induced a small rightward shift in the Ca^{2+}-force relations under any of the investigated conditions. Overall, these data suggested that P_i release in the cross-bridge cycle for the different species studied is highly conserved. The P_i-induced accelerations in cross-bridge turnover are in accord with previous experimental observations. However, k_{tr} was increased to a greater extent by P_i in murine than in human or porcine cardiomyocytes. k_{tr} increased further when the temperature was raised to 25°C in the presence of P_i in human cardiomyocytes. Nevertheless, at a given $[Ca^{2+}]$ and P_i k_{tr} did not change with SL in any of the three species.

The molecular mechanism by which an increase in SL promotes Ca^{2+}-activated myofibrillar force production is not fully understood. It appears reasonable to suppose, however, that at a given submaximal free $[Ca^{2+}]$, an

increase in SL results in a cooperative amplification of the number of force generating cross bridges and increased binding of Ca^{2+} to the thin filament protein troponin C. The extent to which these two mechanisms are coordinated and how they affect the kinetics of the actin-myosin cycle is largely unknown. Theoretical considerations imply that the cooperative amplification of the number of force-generating cross bridges would decrease the turnover rate of the cross-bridge cycle, whereas an increased binding of Ca^{2+} to troponin C would increase this turnover rate. The present study revealed that at a given $[Ca^{2+}]$ k_{tr} was constant despite SLdependent alterations in force. In other words, in all three investigated species, variations in $[Ca^{2+}]$ and P_i (and in human hearts also in temperature) did not differentiate between these two scenarios. This might imply that the two processes are in exact opposition, but given the large variation in experimental conditions tested, this would seem highly fortuitous. Hence it appears that $[Ca^{2+}]$, and not SL, is the major determinant of k_{tr} in the mammalian heart. Moreover, our findings support the suggestion that the Ca^{2+} binding to cardiac troponin C does not directly modulate cross-bridge steps that are rate limiting to k_{tr}.

Our data are in support for the three-state model for the thin filament activation. This latter model postulates that two Ca^{2+}-controlled transitions of the thin filament would permit the separate regulation of a weak-binding cross-bridge attachment (a closed state) and force generation (an open state). To explain length-dependent activation, it was proposed that the size of the thin filament regulatory units in the closed state is length dependent (at higher SL the distance [lattice spacing] between the thin- and thick filaments is decreased). Our results lend support to the three-state model because they allow the development of a SL-dependent increase in force without apparent changes in cross-bridge kinetics.

Our observation that k_{tr} is unaffected by changes in SL is in accord with earlier observations in rat hearts and extends them to the human, murine, and

porcine myocardium. The findings that [Pi], [Ca^{2+}], temperature, and species differences were all involved in the modulation of k_{tr}, while SL was not, strongly suggest that kinetic alterations within the actin-myosin cycle are not prerequisites for the length-dependent Ca^{2+}-sensitization in the heart.

5.2. Late-stage alterations in myofibrillar contractile function in a transgenic mouse model of dilated cardiomyopathy (Tgαq*44)

The results of this study revealed that the myofibrillar remodeling of Tgαq*44 hearts resulting in PKC upregulation involved first an increased Ca^{2+} sensitivity of force production without any other mechanical alterations, which was subsequently complicated by significant reductions in F_o and k_{tr}. Surprisingly, the observed increased Ca^{2+} sensitivity of force production suggested a deficit of PKA-mediated intracellular phosphorylation rather than PKC activation during the development of DCM in this model. Differences in the expression and phosphorylation levels of contractile proteins and β-receptor coupling of rodent and human hearts limit direct extrapolation of our model experiments to humans. Nevertheless, the decreased PKA-mediated contractile protein phosphorylation is in accordance with a number of independent investigations on DCM in humans, and thereby points to a possible role of decreased PKA activation in the pathogenesis of DCM.

The advantage of the Tgαq*44 DCM model over most other existing ones relates to the way in which it reflects neurohumoral activation in the heart. Similarly to humans, the development of the resultant cardiac enlargement and failure demands a significant proportion of the total lifespan. It was earlier demonstrated that the phospholipase Cß activity begins to rise at around the age of 6 months in Tgαq*44 mice, i.e. much before the end-stage result of heart failure is seen, which is suggestive of an apparent temporal mismatch between Gαq protein activation and the development of DCM. Hence, besides the

activation of PKC enzymes, possible roles for additional pathways and/or mechanisms have also been implicated. Our observations illustrated that the mechanical parameters of myofibrillar force production were the same before and after the putative phospholipase Cß activation (i.e. at 4 months and 10 months). Alterations in the mechanical parameters and cardiac decompensation were both initiated around the age of 14 months, and progressed in a cumulative fashion thereafter. Hence, it is unlikely that protein changes in the cardiac myofilaments act as initiating factors of DCM development in Tgαq*44 mice.

Changes in contractile protein isoform expression and phosphorylation influence the cardiac contractile performance and, may thereby contribute to the decreased pump function during end-stage heart failure. While the impact of the frequently observed shift from α- to ß- MHC on the Ca^{2+} sensitivity of force production is debated, consensus has been reached in that an increased ß-MHC expression decreases the kinetics of the actin-myosin cross-bridge cycle. Results of previous investigations suggested that the increase in Ca^{2+} sensitivity during the development of heart failure is not due to the degradation or reexpression of contractile proteins in the failing ventricle, but pointed to the involvement of altered protein phosphorylation. Nevertheless, these results did not rule out the possibility that the expression of fetal troponin T isoforms and atrial myosin light chain-1 may also influence the Ca^{2+} sensitivity, but excluded the possibility that these are predominant changes involved in the alteration in Ca^{2+} responsiveness during the transition from compensatory hypertrophy to end-stage heart failure.

The phosphorylation status of contractile proteins is a function of several protein kinases and protein phosphatases, which may be altered under pathological conditions. Due to the large number of PKC isozymes and the propensity for target protein crossphosphorylation, it is rather difficult to define the relative contribution of each specific PKC isozyme to the control of myofilament force development and Ca^{2+} sensitivity. However, it was

previously recognized that only the expression levels of the PKCα and PKCδ isoforms increased, while that of PKCβ remained unchanged and that of PKCe decreased with age in Tgαq*44 hearts. It is noteworthy that myofilament phosphorylation by PKCε has little if any effect on the Ca^{2+} sensitivity of force production. Moreover, in contrast with our findings, increases in the activities of PKCα and PKCδ are expected to decrease rather than increase the Ca^{2+} sensitivity of force production. Although we cannot rule out a possible role for PKCα- and/or PKCδ-mediated myofilament phosphorylation in the ultimate reduction in F_o (in 18-month-old Tgαq*44 hearts), the increased Ca^{2+} sensitivity of force production at a stable F_o argues strongly against such involvements for PKCα and/or PKCδ at 14 months.

Previously, a significant decrease has been noted for the degree of TnI phosphorylation in failing as compared with non-failing human hearts. Accordingly, a shift from mono- and bisphosphorylated TnI to dephosphorylated TnI was found in failing human hearts, resulting in a positive correlation between the increased Ca^{2+} sensitivity and the percentage of dephosphorylated TnI. In agreement with previous observations, the incubation of myocytes with PKA decreased the Ca^{2+} sensitivity to a larger extent in failing Tgαq*44 hearts than in non-failing FVB cardiomyocytes, completely abolishing the difference in Ca^{2+} responsiveness between the non-failing and failing hearts. Moreover, PKA activity and TnI phopshorylation declined in Tgαq*44 hearts with age. Hence, our results suggest that the PKA-mediated phosphorylation of the contractile proteins is impaired during the development of DCM in Tgαq*44 hearts and this increases the Ca^{2+} responsiveness of the contractile apparatus from 14 months onward. Our data extend the findings of depressed ß-adrenergic response in human heart failure to the Tgαq*44 mouse heart, but are not consistent with increased PKC-mediated myofibrillar phosphorylation at the onset of DCM development. Collectively, mechanical data at 14 months of age and our

biochemical evidence support the formerly proposed antagonistic relationship between PKCα activation and ß-adrenergic responsiveness, and can be regarded as an additional argument against PKC-mediated myofilament phosphorylation as a key mechanism during DCM development in the Tgαq*44 model. Changes in PKA activity were observed in the cytosolic fraction of myocardial homogenates suggestive for alterations in the expression and/or regulation of PKA. However, the examination of the possible crosstalk between different signal pathways (PKA and PKC) in the Tgαq*44 mice was beyond the scope of this study.

While the molecular nature of declining F_o and increasing $F_{passive}$ at 18 months is unclear, the reduction in k_{tr} reflected a shift in the expression from the fast α-MHC isoform toward the slow ß-MHC isoform. Despite all these mechanical alterations, the length dependent increase in cardiomyocyte Ca^{2+} sensitivity was preserved throughout the entire lifespan of the animals. This suggests that the Frank-Starling mechanism is operational during the development of DCM in Tgαq*44 mice. Additionally, SL failed to modulate k_{tr} not only in the FVB, but also in the Tgαq*44 mice. Hence, these data confirm and extend our proposal from the healthy to the failing myocardium on the independence of the Frank-Starling mechanism from k_{tr}.

6. Summary

Our new important observation with the Frank-Starling regulation is the independence of the actin-myosin cross-bridge cycling rate (k_{tr}) from the SL. This observation has been found to be true in all examined species (human, porcine and murine myocardium). On one hand, the k_{tr} has been found to be dependent on the $[P_i]$, $[Ca^{2+}]$, temperature and the myofibrillar protein composition (MHC isoforms). On the other hand, k_{tr} has been found to be completely unaffected by SL under all experimental conditions. Based on these data, we propose that kinetic alterations of the actin-myosin cross-bridge cycling (k_{tr}) are not prerequisites for the Frank-Starling regulation (SL-dependent Ca^{2+} sensitization) under any examined experimental conditions.

We have shown that in the transgenic DCM mice (Tgαq*44) the decreased PKA activity is one of the most important underlying mechanisms responsible for the pathological alterations of the myofibrillar function (increased Ca^{2+} sensitization and decreased contractility). The Frank-Starling regulation (SL-dependent Ca^{2+} sensitization), was found to be fully operational during the development of DCM in Tgαq*44 mice. Additionally, SL failed to modulate k_{tr} not only in the FVB, but also in the Tgαq*44 mice. Hence, these data confirm and extend our proposal from the healthy to the failing myocardium (DCM) on the independence of the Frank-Starling mechanism from k_{tr}.

The observed biochemical alterations in the Tgαq*44 mice (reduced ß-adrenergic activation) correlate with the human findings on congestive heart failure, despite the species-dependent differences in the myofibrillar protein compositions.

7. Practical relevancies of the new scientific findings

1. The heart failure is a malignant disease with poor annual survival rate (the prevalence of the disease is about 1-2%). Consequently, the clarification of the underlying pathological events responsible for the depressed myocardial contractility is an important aim of research that enables the development of new approaches in drug therapy.

2. An important aspect of our research is the fact that human cardiomyocytes were used in the experiments. Consequently, our observations may be applied directly to the human myocardium.

3. The regulation of myocardial contractility is essential for the physiological heart function. The precise understanding of the exact mechanism of the contractile function is a prerequisite for the clarification of the pathological events in heart failure. Based on these assumptions, an open application (call for papers) has been advertised in the American Journal of Physiology. Our manuscript has been accepted in this journal under the key word of “regulation of myocardial contractility”.

8. List of publications

In extenso publications directly related to the Ph.D. thesis:

Edes IF, Czuriga D, Csanyi G, Chlopicki S, Recchia FA, Borbely A, Edes I, Velden JV, Stienen GJ, Papp Z. The rate of tension redevelopment is not modulated by sarcomere length in permeabilized human, murine and porcine cardiomyocytes. *Am J Physiol Regul Integr Comp Physiol. 2007;293:R20-9. (IF: 3,661)*

Edes IF, Toth A, Csanyi G, Lomnicka M, Chlopicki S, Edes I, Papp Z.: Late-stage alterations in myofibrillar contractile function in a transgenic mouse model of dilated cardiomyopathy. *J Mol Cell Cardiol. 2008 (in press) doi:10.1016/j.yjmcc.2008.07.001 (IF: 5,246)*

Additional *in extenso* publications and book chapter:

Szuk T, Gyongyosi M, Homorodi N, Kristof E, Kiraly C, **Edes IF**, Facsko A, Pavo N, Sodeck G, Strehblow C, Farhan S, Maurer G, Glogar D, Domanovits H, Huber K, Edes I. Effect of timing of clopidogrel administration on 30-day clinical outcomes: 300-mg loading dose immediately after coronary stenting versus pretreatment 6 to 24 hours before stenting in a large unselected patient cohort. *Am Heart J. 2007;153:289-95. (IF: 3,552)*

Édes I, **Édes IF**: A krónikus szívelégtelenség kialakulásának patomechanizmusa. *Orvosképzés 2008 in-press.*

Édes I. **Édes IF**, Nánási P: A szívműködés szabályozása. In: A belgyógyászat alapjai. *Medicina, Budapest, 2. kiadás, in press.*

Abstracts and proceedings in books:

Édes IF, Tapolyai M: Very Severe Office Hypertension Is Rarely Very Severe. *XVth Scientific Meeting of the Inter-American Society of Hypertension, Abstract Book, 2003;61:P3.*

Édes IF, Czuriga D, Csányi G, Chlopicki S, Recchia FA, Borbély A, Galajda Z, Édes I, van der Velden J, Stienen GJM, Papp Z. The rate of tension redevelopment is not modulated by sarcoemre length in permeabilized human, murine and porcine cardiomyocytes. *New Frontiers in Basic*

Cardiovascular Research, 7th Meeting, című rendezvény kongresszusi kiadványa, 2006;p45.

Édes IF, Csányi G, Chlopicki S, Édes I, Papp Z**.** Late stage alterations in myofibrillar contractile function in the TG(ALPHA)Q*44 model of heart failure. *Cardiol. Hung. 2006;36:A16.*

Édes IF, Czuriga D, Galajda Z, Borbély A, Édes I, Csányi G, Papp Z. The Frank-Starling law of the heart does not affect the actin-myosin turnover rate. *Cardiol. Hung. 2007;37:A14.*

Édes IF, Czuriga D, Papp Z, Borbely A, Csanyi , Chlopicki S, Edes I, Recchia FA, van der Velden J, Stienen GJM. Alterations in the turnover rate of the actin-myosin cycle are not responsible for the Frank-Starling law of the heart. *Eur Heart J 2007;28(S):872.*

Facskó A, Steiber Z, **Édes IF,** Tóth A, Édes I. Intramuscular accumulation of connective tissue substance in strabismus. *Szemészet 2007;144(S I.):37.*

Édes IF, Csányi G, Chlopicki S, Merkely B, Édes I, Papp Z. Progressively deteriorating myofibrillar contractile function in a transgenic mouse model of dilated cardiomyopathy (TGalphaQ*44). *Cardiol. Hung. 2008;38:B12.*

Czuriga D, Balogh Á, Pasztor ET, Recchia FA, Lionetti V, Czuriga I, **Edes IF**, Edes I, Chlopicki S, Papp Z. Myofibrillar alterations in animal models of heart failure. *New Frontiers in Basic Cardiovascular Research, 8th Meeting, című rendezvény kongresszusi kiadványa, 2008;p67-68.*