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# MOLECULAR DETERMINANTS OF MYOFIBRILLAR CONTRACTILE FUNCTION IN THE HEART

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# ABBREVIATIONS

| | |
|---|---|
| **NYHA** | New York Heart Association |
| **SR** | sarcoplasmic reticulum |
| **MHC** | myosin heavy chain |
| **MLC** | myosin light chain |
| **RLC** | regulatory light chain |
| **HMM** | heavy meromyosin |
| **LMM** | light meromyosin |
| **ALC** | atrial light chain |
| **VLC** | ventricular light chain |
| **ATP** | adenosine 5'-triphosphate |
| **ADP** | adenosine 5'-diphosphate |
| **PKA** | protein kinase A |
| **PKC** | protein kinase C |
| **MyBP-C** | myosin binding protein C |
| **Tm** | tropomyosin |
| **TnI/TnT/TnC** | troponin I/troponin T/troponin C |
| **CHF** | chronic heart failure |
| **HCM** | hypertrophic cardiomyopathy |
| **DCM** | dilated cardiomyopathy |
| **CO** | cardiac output |
| **HR** | heart rate |
| **SV** | stroke volume |
| **$[Ca^{2+}]$** | calcium concentration |
| **pCa** | $- \lg([Ca^{2+}])$ |
| **$[cAMP]_i$** | intracellular cAMP concentration |
| **AP** | action potential |
| **$I_{Na}$** | fast sodium channel |
| **$I_{to}$** | transient outward current |
| **$I_{Ca\text{-}L}$** | L-type calcium channel |
| **$I_{K1}$** | inward rectifier potassium channel |
| **$I_K$** | delayed rectifier potassium channel |

| | |
|---|---|
| **RyR2** | ryanodine receptor |
| **SERCA2** | sarcoplasmic calcium ATPase |
| **PLB** | phospholamban |
| **CICR** | calcium-induced calcium release |
| **CaT** | calcium transient |
| **$P_i$** | inorganic phosphate |
| **SL** | sarcomere length |
| **PDE** | phosphodiesterase enzyme |
| **TAN** | total adenine nucleotide |
| **PCr** | phosphocreatine |
| **NCX** | sodium-potassium exchanger |
| **LV** | left ventricle |
| **PP** | protein phosphatase |
| **RAAS** | renin-angiotensin-aldosterone system |
| **ATII** | angiotensin II |
| **ET-**1 | endothelin-1 |
| **MMP** | matrix metalloproteinase |
| **ANP** | atrial natriuretic peptide |
| **BNP** | brain natriuretic peptide |
| **TNF-α** | tumor necrosis factor α |

# ABSTRACT IN ENGLISH

Chronic heart failure (CHF) is a prominent public health problem in the developed societies still accompanied by unacceptably high morbidity and mortality. Our knowledge on the pathophysiological alterations at the molecular level in CHF is, however, still incomplete. Increasing number of CHF patients also claims for novel therapeutic approaches. Thus, the studies summarized in this thesis examined these two distinct aspects of heart failure.

The central experimental method used throughout this work was the single cell force measurement in skinned cardiac myocytes. Following enzymatic or mechanical isolation of the myocytes their membranes were permeabilized with a detergent in order to allow precise control of both the intracellular medium composition and the sarcomere length. Skinned preparations were then attached to a highly sensitive force transducer and isometric contractures evoked by activating solutions with different calcium concentrations were recorded. A wide range of experimental methods from the molecular (e.g. SDS-PAGE, immunoblotting, PDE activity assays, real-time quantitative RT-PCR, etc.) to the whole organ (e.g. Langendorff-perfused heart, working-heart preparations) level completed the force measurements.

First, we focused on the behaviour of the Frank-Starling relationship in heart failure. The Frank-Starling law describes a most characteristic internal regulatory mechanism of the cardiac muscle that is increased blood volume in the cardiac cavities results in augmented contractile force. In rat left ventricle (LV) when stretching the sarcomeres from 1.9 μm to 2.3 μm we proved positive correlation between titin based passive tension, stretch-induced increase in $Ca^{2+}$ sensitivity and ventricular light chain 2 (VLC2) phosphorylation. The observed transmural gradient of both stretch sensitization and the accompanied increase in VLC2 phosphorylation was, however, blunted by the remodeling process in our rat model of CHF developed following myocardial infarction. Whether these alterations are compensatory changes or on the contrary, signs for decompensation of the myocardium remains to be elucidated.

The second study presented here analyzed the involvement of the cardiac myosin binding protein C (cMyBP-C) in protein kinase A (PKA)-phosphorylation and SL-mediated changes in the myofibrillar $Ca^{2+}$ sensitivity. Recent studies using a transgenic murine model that expressed the slow skeletal isoform of troponin I in the heart concluded that PKA induced reduction in $Ca^{2+}$ sensitivity was due, solely, to phosphorylation of cardiac troponin I (cTnI), not cMyBP-C. Specific role of cMyBP-C in the determination of contractile properties was therefore studied in our cMyBP-C knock out murine model. The absence of cMyBP-C reduced stretch sensitization and almost abolished the PKA-induced decrease in $Ca^{2+}$ sensitivity compared to wild type myofilaments. Thus, our results, in contrast with the aforementioned study, suggest that at least some of the PKA-phosphorylation mediated effects on myofibrillar $Ca^{2+}$-sensitivity require cMyBP-C.

Positive inotropic therapy represents an indispensable tool in the management of heart failure. Levosimendan, the first clinically approved $Ca^{2+}$-sensitizer was shown to have both $Ca^{2+}$ sensitising and phosphodiesterase (PDE) inhibitory potentials. In our further studies its mechanism of action in the heart was examined and compared with that of enoximone, a PDE III inhibitor. Up to 1 μM concentration levosimendan induced more potent positive inotropic response in isolated guinea pig hearts than enoximone. Izoenzyme selectivity (PDE III over PDE IV) of levosimendan proved to be higher than that of other PDE inhibitors, including enoximone. Furthermore, levosimendan inhibited only PDE III and not PDE IV at concentrations at which it increased myofibrillar $Ca^{2+}$ sensitivity. Provided that dual inhibition of PDE III and PDE IV is a prerequisite of the increase in the intracellular cAMP concentration $[cAMP]_i$ our results support the hypothesis that in the clinically relevant concentration range levosimendan exerts positive inotropy through a $Ca^{2+}$ sensitising mechanism. In contrast, up to the concentration of 10 μM enoximone failed to increase $Ca^{2+}$ sensitivity. Thus it acts most likely via PDE inhibition with limited PDE III versus PDE IV selectivity and concomitant elevation in $[cAMP]_i$. Interestingly, the clinically observed long-lasting beneficial hemodynamic effects of levosimendan are at least in part attributable to its biologically active metabolite OR-1896 that possesses a $Ca^{2+}$ sensitising potential similar to that of levosimendan.

In conclusion, our works furnished further evidences for the existence of transmural gradients of passive and active mechanical properties in the ventricular myocardium, revealed a new, exciting regulatory mechanism involved in the short-term regulation of stretch-sensitization and challenged the presumed exclusive role of cTnI in the $Ca^{2+}$ desensitization of myofilaments induced by β-adrenergic stimulation. Comparison of levosimendan and enoximone firmed previous hypothesis that levosimendan acts predominantly as a $Ca^{2+}$ sensitizer in the myocardium and characterization of OR-1896 suggested its significance as the mediator of long-term circulatory effects of levosimendan.

**KEYWORDS:** chronic heart failure, Frank-Starling law, myofilament contractility, stretch sensitization, intramural regionalisation, myosin binding protein C, troponin I, positive inotropy, $Ca^{2+}$-sensitization, levosimendan, OR-1896, enoximone, phosphodiesterase inhibition

# RESUME EN FRANÇAIS

L'insuffisance cardiaque chronique (ICC) qui cause, même de nos jours, une mortalité et morbidité inacceptable, est un problème sanitaire très important dans les pays du monde développé. Malgré tout, nos connaissances concernant les changements pathophysiologiques au niveau moléculaire au cours de l'ICC sont toujours incomplètes. En outre, l'augmentation du nombre des malades qui sont atteints d'ICC demande de trouver des traitements nouveaux. Les recherches présentées dans ce travail examinent ces deux différents aspects de l'insuffisance cardiaque.

Au cours de ce travail les expériences ont été réalisées essentiellement par la mesure de la force sur les cellules isolées du myocarde. Après isolation mécanique ou enzymatique, nous avons perméabilisé les cellules cardiaques par un traitement détergent afin de contrôler précisément la composition de l'espace intracellulaire et la longueur des sarcomères (LS). Après avoir fixé les préparations perméabilisées à une jauge de contrainte de haute sensibilité, nous avons enregistré les contractions isométriques provoquées par les solutions activantes contenant différentes concentrations en calcium. Outre la mesure de la force, nous avons utilisé aussi des techniques de biologie moléculaire (SDS-PAGE, immunoblot, activité PDE, PCR quantitative en temps réel, RT-PCR, etc. ) et physiologiques (cœur isolé perfusé en Langendorff, etc.).

D'abord nous avons examiné les propriétés de la loi Frank-Starling dans l'insuffisance cardiaque. La loi Frank-Starling décrit l'une des spécificités les plus typiques du myocarde telle qu'une élévation du volume sanguin dans les cavités cardiaques induit l'augmentation de la force de contraction développée par le myocarde. En étirant de 1,9 µm à 2,3 µm les sarcomères des myocytes isolés du ventricule gauche de rat on a constaté une corrélation positive entre la tension passive dépendante de la titine, la sensibilité au $Ca^{2+}$ des myofilaments et la phosphorylation de la chaine légère de la myosine ventriculaire (VLC2). Au cours du remodelage, une diminution des gradients transmuraux de tension passive et de phosphorylation de VLC2 a été observée accompagnant le développement de l'insuffisance cardiaque dans notre rat modèle après infarctus du myocarde.

Dans un second volet de ce travail nous avons analysé la fonction de la protéine C (cMyBP-C) dans les changements de la sensibilisation myofibrillaire au $Ca^{2+}$ provoqués par la proteine kinase A (PKA) et par l'augmentation de SL. Des travaux récents, utilisant un modèle transgénique de souris exprimant dans le myocarde l'isoforme de troponine I qui se trouve dans les muscles squelettiques lents, suggèrent que la phosphorylation de la troponine I cardiaque (cTnI) est à elle-seule suffisante pour diminuer la sensibilité au $Ca^{2+}$ provoquée par la PKA. Dans notre modèle de souris cMyBP-C knock-out, nous avons examiné la fonction de cMyBP-C dans la détermination des propriétés contractiles. L'absence de cMyBP-C d'une part diminue la sensibilisation à l'étirement par rapport au contrôle et d'autre part a presque supprimé la diminution de la sensibilité au $Ca^{2+}$ provoquée par PKA. Nos résultats suggèrent donc que les changements de la sensibilité au $Ca^{2+}$ lors de l'addition de PKA impliquent aussi cMyBP-C.

La thérapie inotropique positive est l'un des éléments les plus nécessaires dans le traitement de l'ICC. Le levosimendan, le premier « *$Ca^{2+}$-sensitizer* » cliniquement introduit, a à la fois des propriétés de sensibilisation au $Ca^{2+}$ et d'inhibiteur des phosphodiestérases (PDE). Dans ce travail, le mécanisme d'action du levosimendan dans le cœur a été comparé à celui de l'enoximone, un inhibiteur de la PDE III. Dans le cœur du cobaye isolé, le levosimendan jusqu'à une concentration de 1 µM a un effet inotrope positif plus marqué que l'enoximone. La sélectivité de l'isoenzyme PDE III comparé à PDE IV du levosimendan est plus forte par rapport aux autres inhibiteurs de PDE, y compris l'enoximone. De plus, dans le domaine de concentrations où le levosimendan avait déjà un effet sensibilisateur au $Ca^{2+}$, il avait un effet inhibiteur seulement sur PDE III, mais pas d'effet sur PDE IV. Puisque l'augmentation de $[cAMP]_i$ nécessite l'inhibition simultanée des PDE III et PDE IV, nos résultats suggèrent que dans le domaine de concentrations cliniquement utiles, le levosimendan provoque son effet inotrope positif en tant que « *$Ca^{2+}$-sensitizer* ». Par contre jusqu'à la concentration de 10 µM, l'enoximone n'a pas montré d'effet sensibilisateur au $Ca^{2+}$. Ainsi le mécanisme d'action le plus probable de l'enoximone est l'inhibition PDE de sélectivité modérée qui est accompagnée de l'augmentation de $[cAMP]_i$. Enfin, l'effet hémodynamique prolongé du levosimendan observé cliniquement pourrait être dû , pour le moins en partie, à son métabolite biologiquement actif le OR-1896, qui démontre un potentiel sensibilisateur au $Ca^{2+}$ similaire à celui du levosimendan.

Pour résumer, notre travail a renforcé avec de nouveaux arguments l'existence d'un gradient transmural des qualités mécaniques actives et passives caractéristiques de myocarde. Nous avons aussi montré un nouveau mécanisme dans la régulation à court terme de la sensibilisation par l'étirement et mis en question la fonction présumée exclusive de cTnI dans la dé-sensibilisation au $Ca^{2+}$ lors de la stimulation ß-adrénergique. La comparaison des effets du levosimendan et de l'enoximone a confirmé que le levosimendan exerce son effet en priorité comme « *$Ca^{2+}$-sensitizer* » du myocarde tandis que l'analyse d'OR-1896 a posé la question de sa fonction dans la formation des effets à long terme exercés sur la circulation par le levosimendan.

**MOT CLES :** Insuffisance cardiaque chronique, Loi de Frank-Starling, Contractilité cardiaque, Sensibilisation par l'étirement, Régionalisation transmurale, Protéine C, Troponine I, Inotropisme, Sensibilisation au $Ca^{2+}$, Levosimendan, OR-1896, Enoximone, Phosphodiestérase.

# ABSZTRAKT MAGYARUL

A krónikus szívelégtelenség (CHF), mely még napjainkban is elfogadhatatlanul magas morbiditással és mortalitással párosul, kiemelkedő jelentőségű közegészségügyi problémát jelent a fejlett országokban. Ennek ellenére, a CHF-ben molekuláris szinten bekövetkező patofiziológiai változásokra vonatkozó ismereteink mindmáig hiányosak. Továbbá, a CHF-ben szenvedő betegek növekvő száma új kezelési lehetőségek kidolgozását sürgeti. A bemutatásra kerülő tanulmányok a szívelégtelenség e két eltérő aspektusát vizsgálták.
Munkánk során központi kísérleti módszerként a permeabilizált szívizomsejteken végzett erőmérést alkalmaztuk. Enzimatikus, illetve mechanikai izolálást követően a szívizomsejteket detergens kezeléssel permeabilizáltuk. Ilymódon megvalósítható mind az intracelluláris tér összetételének, mind pedig a szarkomerhossznak a precíz kontrollja. A permeabilizált preparátumokat ezt követően nagyérzékenységű erőmérőhöz rögzítettük és a különböző kalcium tartalmú aktiváló oldatok által kiváltott izometrikus kontraktúrákat rögzítettük. Az erőmérés mellett számos molekuláris biológiai (SDS-PAGE, immunoblot, foszfodieszteráz-aktivitás mérés, real-time RT-PCR, stb.), valamint élettani (Langendorff-szív, working-heart preparátum) kísérletes módszert alkalmaztunk.
Elsőként a Frank-Starling törvény tulajdonságait vizsgáltuk szívelégtelenségben. A Frank-Starling törvény a szívizomnak azt az egyik legjellemzőbb sajátosságát írja le, miszerint a szívüregek fokozódó telődése a szívizomzat kontraktilis erejének növekedését idézi elő. Patkány bal kamrából (BK) származó myocyták szarkomerjeit 1,9 µm-ről 2,3 µm-re nyújtva pozitív korrelációt találtunk a titin-függő passzív erő, a stretch-szenzitizáció, valamint a kamrai könnyűlánc 2 (VLC2) foszforilációja között. A stretch-szenzitizáció és vele párhuzamosan a VLC2 foszforiláció kamrafalon belüli grádienseinek csökkenése volt azonban megfigyelhető a remodelláció során, mely szívinfarktust követően a szívelégtelenség kialakulását kísérte patkány modellünkben. Annak eldöntése, hogy ezek a változások kompenzatorikus természetűek-e, vagy ellenkezőleg, a szívizomzat dekompenzációjának jelei, további vizsgálatokat igényel. A következőkben a miozin-kötő C fehérje (MyBP-C) szerepét elemeztük a protein kináz A (PKA), illetve a szarkomerhossz (SL)-növekedés hatására bekövetkező myofibrilláris $Ca^{2+}$-érzékenység változások kialakulásában. A közelmúltban közölt, a szívizomban a troponin I lassú típusú vázizomban található izoformáját expresszáló transzgén egér modellben végzett vizsgálatok arra a következtetésre jutottak, hogy a PKA hatására bekövetkező $Ca^{2+}$-érzékenység csökkenésben kizárólag a kardiális troponin I (cTnI) foszforilációja játszik szerepet, a cMyBP-C-jé nem. Ezért cMyBP-C knock out egér modellünkben vizsgáltuk, milyen szerepet tölt be a cMyBP-C a kontraktilis tulajdonságok meghatározásában. A cMyBP-C hiánya a vad típushoz képest csökkentette a stretch-szenzitizáció mértékét és szinte megszüntette a PKA hatására bekövetkező $Ca^{2+}$-érzékenység csökkenést. Eredményeink tehát, szemben a fent említett vizsgálatokkal, arra utalnak, hogy a PKA hatására bekövetkező $Ca^{2+}$-érzékenység változások, legalábbis részben, a cMyBP-C-n keresztül valósulnak meg.
A pozitív inotróp terápia a szívelégtelenség kezelésének egyik nélkülözhetetlen eleme. A levosimendanról, mely az első klinikailag bevezetett $Ca^{2+}$-érzékenyítő, bebizonyosodott, hogy rendelkezik mind $Ca^{2+}$-érzékenyítő, mind foszfodieszteráz (PDE)-gátló hatással. A következőkben a levosimendan hatásmechanizmusát vizsgáltuk a szívben és összehasonlítottuk a PDE III-gátló enoximonéval. Izolált tengeri malac szívben 1 µM-os koncentrációig a levosimendan hatékonyabb pozitív inotróp szernek bizonyult az enoximone-nál. A levosimendan PDE IV-hez viszonyított PDE III izoenzim-szelektivitása is magasabb volt a többi PDE-gátlóénál, beleértve az enoximone-t is. Emellett abban a koncentráció-tartományban, ahol a levosimendan már $Ca^{2+}$-érzékenyítő hatással rendelkezett, csak a PDE III-ra volt gátló hatással, a PDE IV-re nem. Feltéve, hogy az intracelluláris cAMP koncentráció ($[cAMP]_i$) emelkedésének előfeltétele a PDE III és a PDE IV együttes gátlása, eredményeink arra utalnak, hogy a klinikailag releváns koncentráció-tartományban a levosimendan $Ca^{2+}$-érzékenyítőként fejti ki pozitív inotróp hatását. Ezzel szemben 10 µM koncentrációig az enoximone nem mutatott $Ca^{2+}$-érzékenyítő hatást. Így az enoximone legvalószínűbb hatásmechanizmusának a mérsékelt szelektivitású PDE gátlás és az ezzel együttjáró $[cAMP]_i$ emelkedés tekinthető. A levosimendan klinikailag megfigyelt hosszútávú hemodinamikai hatásainak hátterében, legalábbis részben, az OR-1896, a levosimendan biológiailag aktív metabolitja állhat, mely a levosimendanéhoz hasonló $Ca^{2+}$-érzékenyítő potenciállal rendelkezik.
Összefoglalásképpen elmondható, hogy munkánk további bizonyítékokkal támasztotta alá a szívizomzatot jellemző passzív és aktív mechanikai tulajdonságok kamrafalon belüli gradienseinek létezését, a stretch-szenzitizáció rövidtávú szabályozásában újszerű mechanizmust tárt fel és megkérdőjelezte a cTnI feltételezett kizárólagos szerepét a β-adrenerg stimuláció hatására bekövetkező $Ca^{2+}$ deszenzitizáció kialakulásában. A levosimendan és az enoximone összehasonlítása megerősítette azt a korábbi elképzelést, hogy a levosimendan a szívizomban elsősorban $Ca^{2+}$-érzékenyítőként fejti ki hatását, az OR-1896 vizsgálata pedig felvetette annak szerepét a levosimendan keringésre gyakorolt hosszútávú hatásainak kialakításában.

**KULCSSZAVAK:** krónikus szívelégtelenség, Frank-Starling törvény, myofilamentális kontraktilitás, stretch-szenzitizáció, kamrafalon belüli regionalizáció, miozin-kötő C fehérje, troponin I, pozitív inotrópia, $Ca^{2+}$-érzékenyítés, levosimendan, enoximone, foszfodieszteráz-gátlás

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# INTRODUCTION

**Heart failure**, accompanied by unacceptably high morbidity and mortality, is a prominent public health problem in the developed societies (Cowie *et al.*, 1997). In the United States the estimated number of patients with heart failure is 4.6 million and an equal number with asymptomatic left ventricular dysfunction can be surmised (Braunwald & Bristow, 2000) from whom more than 250.000 die annually. Despite the novel therapeutic tools and recently reassessed approaches in the management of patients with heart failure the outcome is still disappointing: the 4-year survival of patients after diagnosis in the NYHA (New York Heart Association) class I-II is 65 % decreasing to 50 % in the class III. Moreover, the surviving rate of patients in the NYHA class IV is only 50 % after 1 year (Alpert *et al.*, 2002). These rigorous facts unambiguously emphasise the need for investigations in order to get further insight into the pathomechanism of heart failure. New recognitions may enable us to improve present therapeutic strategies and, possibly, to develop novel, more effective ones attaining the supreme aim i.e. not just to treat but to cure heart failure.

During my PhD training I could participate in the research activities of two laboratories that have, however, diverse priorities in the field of heart failure research but work with this end in view. The works presented here provide further details concerning both the pathomechanism and the pharmacological treatment of heart failure. With the French group we focused on the examination of the **Frank-Starling mechanism** at the cellular level. **Passive and active contractile properties** changed gradually and in parallel through the healthy myocardial wall but not in heart failure. In the background, titin- and VLC-2 associated regulatory mechanisms could be identified. In another set of experiments, impact of **cMyBP-C** on the myofilament activation was studied in a knock out mouse model. Marked alterations in active contractile properties and in the response to β-adrenergic stimulation proved existent role and importance of cMyBP-C in the regulation of myofilament activation.

On the other hand, in Hungary we attempted to clarify the mechanism of action within the myocardium for **levosimendan**, the first clinically introduced representative of the $Ca^{2+}$-sensitizing agents. **Inotropic**, **$Ca^{2+}$-sensitizing** and **PDE-inhibitory potentials** were studied both at the organ level and in isolated model systems. Paralleling levosimendan with **enoximone**, a PDE-inhibitor, made their distinct inotropic mechanisms clear. Characterization of **OR-1896**, a metabolite of levosimendan, in these systems provided feasible explanation for the clinically observed long lasting inotropic potential of levosimendan.

Our observations are summarized and discussed in four sections within the ‘Results’ chapter of this thesis.

# THE HEART AND THE CARDIAC CONTRACTION

## The heart

Independently of nationality a vast number of traditional phrases as well as literary and artworks refer to the heart as a metaphor of life and soul. The central and most protected position of the heart inside the thoracic cavity symbolises perfectly its indispensable role in the maintenance of the circulation and life. The structure of this organ is specialised gorgeously in all details to adapt its pumping function to the all-time needs of the whole body. A brief review about the anatomy and function of the heart is presented below.

### Macroscopic anatomy of the heart

The heart can be considered as two pumps working parallel beside each other still separated, serving the systemic and the pulmonary circulation and maintaining a one-way flow of the blood (Fig. 1). Deoxygenated blood enters the right atrium via the inferior and the superior venae cavae that drain the lower and upper half of the body, respectively and also via the coronary sinus that drains the myocardium itself. The blood is then conveyed into the right ventricle through the tricuspid valve during the filling period (diastole). The contraction of the right ventricle ejects the blood forward into the pulmonary vascular tree. The highly specialized tissue structure of the lungs allows rapid gas exchange thus the blood that re-enters the heart through the left atrium is oxygenated. The atrium and the ventricle are separated by the bicuspid- or mitral valve. Its cusps as well as those of the tricuspid valve are linked to long muscular projections of the myocardium (papillary muscles) through thin, tendinous structures (chordae tendineae). These structures hinder the cusps from folding back into the atria during the ejection phase (systole) of the ventricles. Blood leaves the heart through the aortic valve entering the ascending aorta, the initial section of the systemic circulation. It is also this region, namely the aortic root where the left and right coronary arteries originate to supply with blood well-defined regions of the myocardium.

Besides basic structural similarities, according to the different characteristics of the two circulatory systems the right and left sides of the heart hold distinct features. For instance, the right atrium is capable to compensate for fast volume changes better than the left one. Additionally, because of the higher vascular resistance of the systemic circulation the thickness of the left ventricular wall exceeds three times that of the right one and has a concentric shape opposite with the semilunar form of the right ventricle assuring more powerful contraction. The wall of the heart includes a musculature of cardiac muscle for

contraction, a fibrous frame for attachment of the valves and an internal conduction system for synchronization of muscle contraction. In general, the myocardial wall is divided into three main layers; the endocardium, the myocardium and the epicardium.

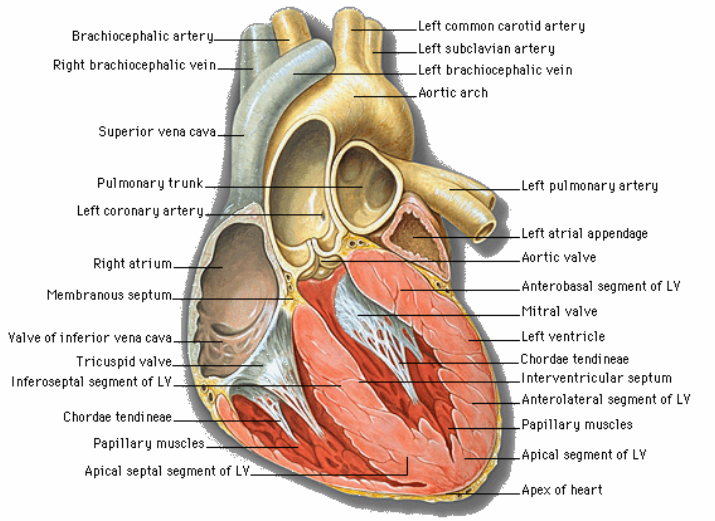



**Figure 1.** Gross anatomy of the heart (from http://www.info.med.yale.edu).

The **endocardium**, the innermost layer of the myocardial wall being very similar in structure to the endothelium that covers the inner surface of the vessels assures a continuous lining of the circulatory system. It consists of an inner layer of endothelium and subendothelial connective tissue, a middle layer of connective tissue and smooth muscle cells, and a deeper layer, also called subendocardial layer, of connective tissue. The impulse-conducting system of the heart is located in the subendocardial layer of the endocardium. Recent studies proved metabolic activity and suggested important regulatory functions of the endocardium on the left ventricular contraction. The **myocardium**, the cardiac muscle, is the principal component of the heart. Interestingly, direction of myocardial fibres is not homogenous in the ventricular wall. The inner and outer layers of the myocardium run approximately longitudinally along the long axis of the heart while in contrast central fibres run at right angle, circumferentially. The intervening layers exhibit intervening patterns (Fig. 2). Due to the complex pattern of the fibre run during systole both the length and the diameter of the ventricular cavity are reduced. The whole heart is almost entirely surrounded by a thin, fibrous, sac-like structure, the pericardium. The visceral layer of the pericardium covers the surface of the heart and together with the underlying connective tissue it is called **epicardium**. The blood vessels and nerves that supply the heart lie in the epicardium and are surrounded by adipose tissue that cushions the heart in the pericardial cavity. The parietal layer of the pericardium is in contact with the

surrounding tissues. A single layer of mesothelial cells lines the inner surface of the pericardium. A small amount of lubricating fluid fills the virtual cavity between the two layers allowing smooth movement of the heart among the neighbouring organs during contraction and relaxation.

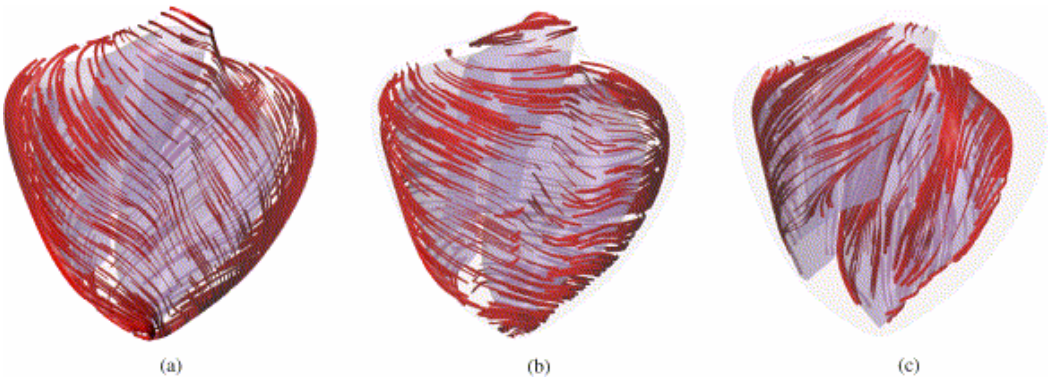



**Figure 2.** Fibre orientation in a model of pig ventricle. Fibre orientations are illustrated (a) on the epicardium, (b) at the mid-wall, and (c) on the endocardial surfaces (Stevens *et al.*, 2003).

The cardiac muscle is capable of contracting in a rhythmic manner without any direct stimulus from the nervous system. Specialized cardiac muscle fibres forming an impulse **conducting system** synchronize the contraction of the heart (Fig. 3). The pace of this beating action is initiated in the sinoatrial (SA) node, a group of specialized cardiac muscle cells located near the junction of the superior vena cava and the right atrium.

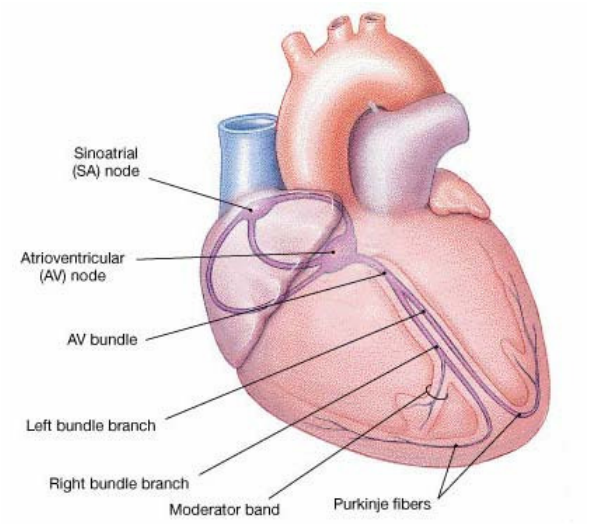



**Figure 3.** Schematic diagram of the cardiac impulse-conducting system (from http://www.biosbcc.net).

The impulse initiated in the SA node spreads then along the atrial cardiac muscle fibres and long internodal tracts composed of modified cardiac muscle fibres. It is then picked up at the atrioventricular (AV) node and conducted with some delay across the fibrous skeleton to the ventricles by the atrioventricular (AV) bundle (of His). The bundle divides into smaller right and left bundle branches and then into Purkinje fibres. The AV bundle, the bundle branches and the Purkinje fibres are modified cardiac muscle cells that are specialized to conduct impulses. During normal cardiac function the contraction is initiated in the atria forcing blood into the ventricles. Then, a wave of contraction in the ventricles begins at the apex of the heart forcing blood from the heart through the aorta and pulmonary trunk.

**Microscopic anatomy of the myocardium**

**The myocyte**

Shape and size of the individual **myocytes** vary according to their location within the heart. Ventricular myocytes are long, narrow cells and cylindrical in shape. Their size (50-100 µm in length and 10-25 µm in diameter) exceeds that of atrial myocytes, which are smaller (~20 µm in length and 5-6 µm in diameter) and exhibit elliptical shape. The myocyte is bounded by its external membrane often termed as **sarcolemma** that also serves as a diffusion barrier around the contents of the cell. This phospholipid bilayer is similar in composition to most external cell membranes. Integral molecules in the sarcolemma exhibit various functions and act as ion channels, -exchangers, -pumps or receptors. The outer surface of the sarcolemma is occupied by the outermost layer of the myocyte, the **glycocalyx**. It is composed chiefly of complex carbohydrates (polysaccharides), often associated with proteins (glycoproteins). Due to their negative electrical charges glycoproteins trap positively charged ions (e.g. $Ca^{2+}$) thus buffering the myocytes from the much higher external calcium concentration and thereby protecting them. The glycocalyx provides also the connection towards the components of the extracellular matrix (collagen, fibronectin, laminin, etc.).

The **intercalated discs** represent the major sites of attachment between cardiac muscle cells. Myocytes within a fibre may join with two or more cells through intercalated discs, thus creating a branched network of fibres (Fig. 4). The three components of these junctions are located in different parts of the intercalated discs and have different functions. The adherent plates (**fasciae adherentes**) serve to hold myocytes together at their ends to form the functional fibre. **Maculae adherentes** or **desmosomes** are points of connection with high solidity that provide a site at which the thin filaments of the terminal sarcomere may anchor

onto the plasma membrane as well as allow mechanical continuity and prevent the myocytes from splitting under the strain of regular repetitive contractions. The **gap junctions** are composed of microchannels (formed by the apposition of two connexons) passing through the intercalated discs and provide ionic community between myocytes allowing contractile signals to spread over the fibres. At these sites distance between the adjacent cell surfaces is reduced to 1 or 2 nm. The connexons are built up of six circularly arranged integral membrane proteins (connexins) and transport small molecules and ions between cells. They also act as low-resistance electrical pathways. Hence, gap junctions allow the myocardium to function as a syncytium while retaining cellular integrity and individuality. It is to note that connexons are very sensitive to abnormal increase in cytosolic calcium concentration, a condition that often occurs in ischemia. By closing the direct connection they are capable to close up to seal off the damaged cell hence precluding spread of cell injury.

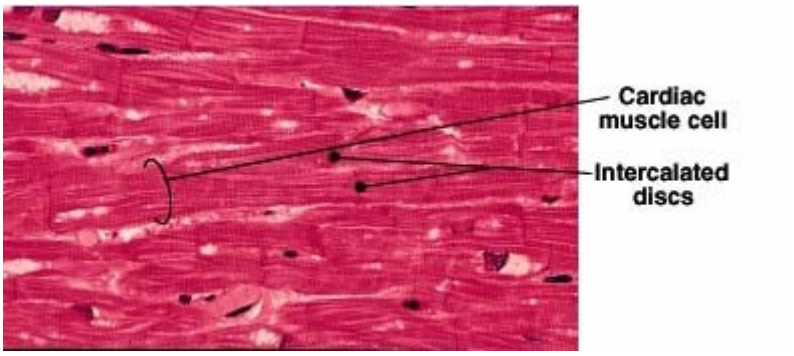



**Figure 4.** Anastomosing muscular fibers of the heart forming a syncytium shown in a longitudinal section (from http://cwx.prenhall.com).

The sarcolemma mostly at the Z lines penetrates into the intracellular space forming a series of tubelike invaginations called the **transverse tubular system** (Fig. 5). Being an extension of the surface area the T tubules increase the cell surface at least by 30 % thus facilitating the spread of the excitatory stimulus to within the cell and ensure adequate oxygen and nutrient supply of the myocyte. In addition, T tubules contain L type calcium channels that exhibit prominent role in the process of calcium-induced calcium release.

The **smoothed-surfaced endoplasmic reticulum (sER)** of the myocytes usually termed as **sarcoplasmic reticulum (SR)** segregates calcium thus playing a crucial role in the regulation of cardiac contraction and relaxation. Storage of $Ca^{2+}$ occurs at the highly charged 55-kDa storage protein **calsequestrin** and **calreticulin**, another protein similar in structure. The fine network of SR spreads throughout the myocytes and consist of ramifying tubules called **longitudinal** or **network SR**. This is the part of the SR that contains $Ca^{2+}$pumps in abundance

and concerns with the uptake of the $Ca^{2+}$ from the cytosol to initiate relaxation at the start of the diastole. The $Ca^{2+}$ then flows along the longitudinal SR towards its distal regions. The terminal bulbous swellings of the SR (**subsarcolemmal cisternae** or **junctional components**) at the level of Z discs interact with the T tubules forming structures called **triads** in skeletal or **diads** in cardiac muscle. The close physical contact between the cisternae and the sarcolemma is facilitated by the **electron-dense foot structures**, also called as **junctional channel complexes**. These connections allow short and fast journey for $Ca^{2+}$ between the extracellular space and the $Ca^{2+}$ release channel of the SR. At the beginning of the systole this relatively small amount of $Ca^{2+}$ entering through the voltage-gated L-type $Ca^{2+}$ channels triggers opening of the $Ca^{2+}$ release channels (ryanodine receptors) in the subsarcolemmal cisternae. The release of $Ca^{2+}$ ions from the SR into the cytosol then leads to the activation of myofilaments.

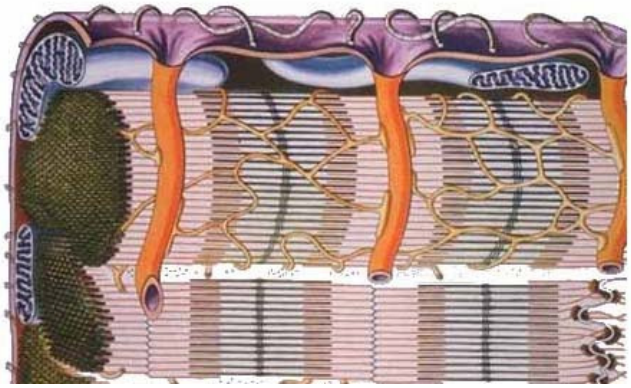


**Figure 5**. Schematic illustration showing location of T-tubules and sarcoplasmic reticulum in the cardiac muscle. The SR is more irregular in arrangement and usually contacts the T tubules in a 1:1 relation forming a diad. This junction of terminal cisternae with the T tubule is located at the Z line. (from http://www.mednote.co.kr).

The cytoplasm**,** in muscles also called the **sarcoplasm** contains the intracellular fluid (**cytosol**) together with all the proteins in it. The cytosol provides the cellular functions a fluid microenvironment of appropriate ion composition. Powerhouses of the myocytes, the **mitochondria** are wedged between the myofibrils so that the chief source of energy supply is close to the chief site of energy use. Besides generation of ATP with oxidative phosphorylation cardiac mitochondria are also able to accumulate $Ca^{2+}$. This potential becomes important when $Ca^{2+}$ overload occurs, in particular during severe lack of oxygen. Myocytes have usually one **nucleus** that is located near the centre of the cell, accounts for ~5 % of the cell volume and contains nearly all of the genetic information that is necessary to

maintain and repair its structure. The genetic information coded in the DNA is transferred to mRNA, which supplies the template for protein synthesis on the ribosomes either in the cytosol or on the surface of the **rough-surfaced endoplasmic reticulum (rER)**. The activated amino acids required are transported to the ribosomes by the tRNAs. The **Golgi apparatus** is functionally associated with the endoplasmic reticulum and functions in the posttranslational modification, packaging, and sorting of proteins utilized in the lysosomes or secreted by the cell as well as handles the distribution of newly synthesized membranes and membrane-associated proteins.

**The sarcomere and the contractile apparatus**

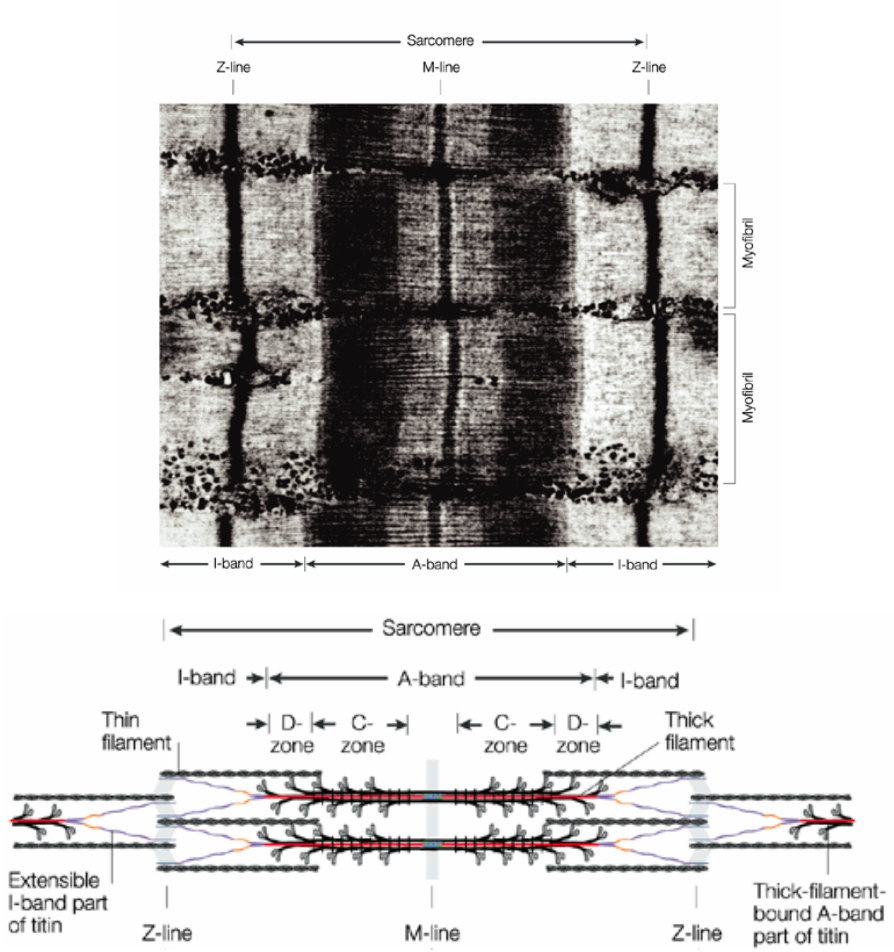



**Figure 6.** Micrograph (upper panel) and schematic structure (lower panel) of the sarcomere, basic unit of contractile apparatus in striated muscles (Tskhovrebova & Trinick, 2003).

Cross-striations of longitudinal sections of muscle fibers are evident when examining either hematoxylin and eosin-stained preparations with light microscopy or unstained ones with phase-contrast or polarization microscopy. In case of either preparation alternating light and dark bands can be observed; they are termed the I band and the A band (Fig. 6). In polarizing microscopy, dark bands are birefringent, therefore anisotropic and termed the **A band**. On the contrary, the light bands are monorefringent, therefore isotropic and termed as **I band**. A narrow zone of contrasting density bisects both of these major bands: the **Z line** ('Zwischenscheibe' – between disc) is a dense zone that bisects the I band and the **H zone** ('Helle' - light) is a light zone that bisects the A band, with the dark **M line** in its middle.

The **sarcomere**, bordered by two consecutive Z lines is the basic contractile unit of the striated muscle. In relaxed mammalian muscle it measures 2-3 μm but can also tolerate stretch to more than 4 μm. Two types of microfilaments **(myofilaments)** occupy the bulk of the muscle cell cytoplasm and are associated with cell contraction: *thick filaments* (~15 nm in diameter), composed of mainly myosin and *thin filaments* (6-8 nm in diameter), composed primarily of actin (Fig. 7). As sarcomeres in adjacent myofibrils and in adjacent myofibers are in register the entire muscle exhibits cross-striation pattern.

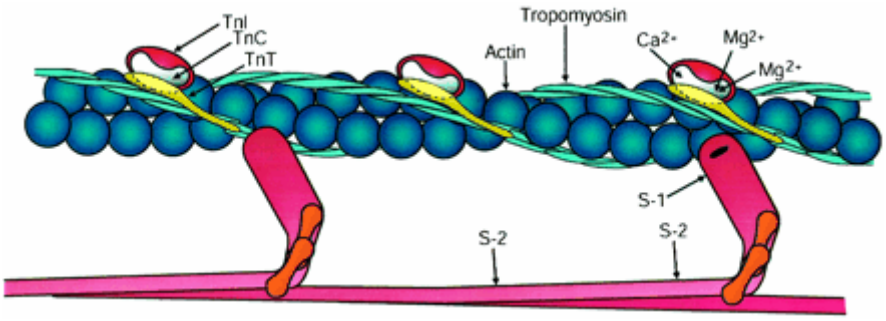



**Figure 7.** Schematic diagram of thick and thin filaments (TnI – troponin I, TnT – troponin T, TnC – troponin C, S-1 – myosin subfragment-1, S-2 - myosin subfragment-2; from (Moss *et al.*, 2004)).

**Thick filaments**

Thick filaments are about 1.5 μm long and are restricted to the A-band of the sarcomeres. They consist of predominantly myosin and myosin binding proteins. **Myosin** is an asymmetric hexamer, consisting of a pair of heavy chains (MHCs, MW=200 kDa) and two pairs of non-identical light chains (MLCs), the essential light chain (LC1, MW=21-25 kDa) and the regulatory light chain (LC2 or RLC, MW=25-37 kDa) (Fig. 8). The two *MHCs* fold to form a globular head or motor domain (S1 subfragment) and a rod-like coiled-coil domain

(the myosin rod). The rod is divided into the ~100 nm-long light meromyosin (LMM) and the ~50 nm-long S2 subfragment. The S1 subfragment is composed of the neck region that exhibits the binding sites for MLCs and the motor domain. The latter also holds the binding sites for actin as well as that for ATP and thus the enzymatic (ATPase) activity, while the rod retains the ability to self-assemble into filament-like structures. Myosin heads are arranged approximately helically on the filament with an axial repeat of 42.9 nm.

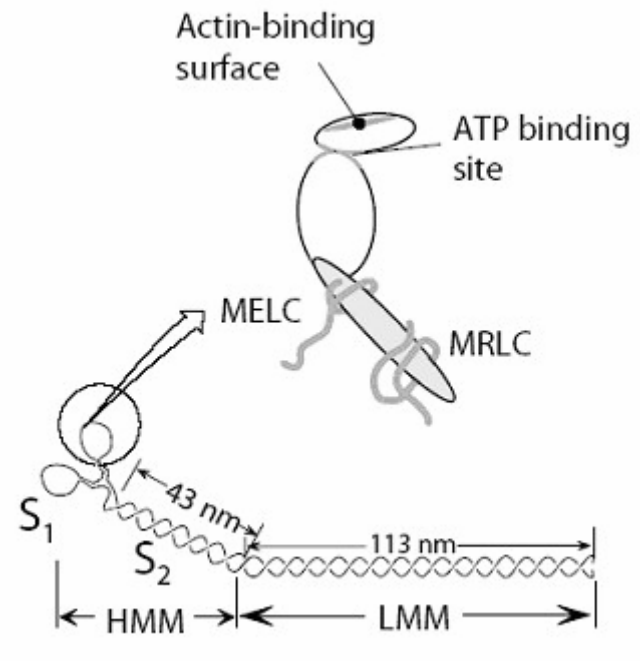



**Figure 8.** The myosin molecule and the details of the S1 head domain. (ATP-adenosine triphosphate, HMM-heavy meromyosin; LMM-light meromyosin; MELC-myosin essential light chain; MRLC-myosin regulatory light chain; S1, S2-subfragment 1 and 2 of HMM). (from http://www.proweb.org/myosin)

Two isoforms of MHC (α-and β-MHC) are expressed in the myocardium. Their expression rates show interspecies differences, vary with localisation within the heart and can also be influenced both by aging and by pathology. MHCs appear to be the major determinants of ATPase activity of the myocardium; hence there is a causal relationship between myosin isoform composition and contractility. It was shown that hearts expressing α-MHC have higher shortening velocity than those expressing β-MHC. Interestingly, although small rodent adult ventricles contain almost exclusively α-MHC, larger animals and primate ventricles consist largely of β-MHC. In all developing mammalian ventricles, β-MHC is predominant. In the human fetal and adult ventricles the ratio of β-MHC is 92 % and 96 %, respectively. *MLCs* bind to the neck region of the MHC and supposed to stabilize it during force

transmission. Phosphorylation of the RLC is thought to play a role in the regulation of contraction. In vertebrate striated muscle under normal physiological conditions myosin light chain kinase is activated during contractile activity, resulting in phosphorylation of the RLCs. Electron microscopic and optical diffraction studies showed that LC2 phosphorylation reversibly perturbs the relaxed arrangement of myosin heads on the surface of the native thick filaments (Levine *et al.*, 1995;Levine *et al.*, 1996), a sign of their partial release from the filaments' surfaces. Increased mobility of myosin heads may well explain the observed physiological effects on force generation (Metzger *et al.*, 1989;Sweeney *et al.*, 1993), since mobile heads are more likely to interact with their binding sites on the thin filaments than relaxed, unphosphorylated heads that remain close to the thick filament backbones. The **M-band** is the myosin head-free central bare zone region of the sarcomeric A-band and appears as a series of dark transversal lines. It is responsible for the regular packing, the perfect lateral alignment of the thick filaments and for the uniform distribution of the tension over the myofilament lattice in the activated sarcomere. Beside the C-terminal of the titin filaments and the myosin heavy chain tails that are anchored here, two closely related proteins, **myomesin** (~185 kDa) and **M-protein** (~165 kDa) are the most characteristic structural components of the M-band. Although the molecular organisation of the M-band is not yet clear; these members of the Ig-superfamily are the major candidates for the role of M-band bridges. (Agarkova *et al.*, 2003). The finding that both molecules bind to titin and myosin supports the integral structural role of these proteins. M-band is also a targeting site for numerous metabolic enzymes, e.g. the muscle isoform of creatine kinase (M-CK), enolase, adenylate kinase and phosphofructokinase (Keller *et al.*, 2000;Lange *et al.*, 2002). In addition, recently several other proteins (e.g. **DRAL/FHL-2**, **MURF-1**, **MURF-2**, **Smpx/Csl**, **obscurin**) have been identified at the M-band and supposed to be involved in various signalling processes, for instance, between the sarcomere and the nucleus. The M-band is thought to be an important element of the sarcomeric stabilization system characterized by a dynamic structure possibly exhibiting different composition in different regions of the heart (Agarkova *et al.*, 2003).

The **myosin binding protein-C** (MyBP-C, ~137 kDa) belongs to the immunglobulin (Ig) superfamily and is composed of repeated Ig and fibronectin domains (Fig. 9). It is localized in the inner two-thirds of the A-band, the so-called C zone and restricted to 7 to 9 axial bands that are approximately 43 nm apart in each half-sarcomere. Results from *in vitro* studies indicated importance of MyBP-C in the assembly and maintenance of the overall sarcomere structure. The relevance of MyBP-C function is further supported by the fact that various

gene mutations of MyBP-C result in familial hypertrophic cardiomyopathy (Carrier *et al.*, 1997;Watkins *et al.*, 1995). Given that almost a half of the crossbridges lie outside the C zone, the majority of crossbridges cannot be regulated by direct interaction with MyBP-C, suggesting mechanical coupling of crossbridges within the thick filament. Interestingly, both PKA and calmodulin kinase (CAMK) can phosphorylate the cardiac isoform of MyBP-C, certainly with different site specificity. Thus the involvement of MyBP-C in the fine regulation of myofibrillar contraction is highly feasible.

Biochemical studies showed that PKA-phosphorylation abolishes the MyBP-C-myosin S2 interaction, subsequently modulating contractility (Kunst *et al.*, 2000). PKA phosphorylation of the MyBP-C changes the extension of the myosin heads from the backbone and increases order (Kunst *et al.*, 2000;Weisberg & Winegrad, 1996;Weisberg & Winegrad, 1998).

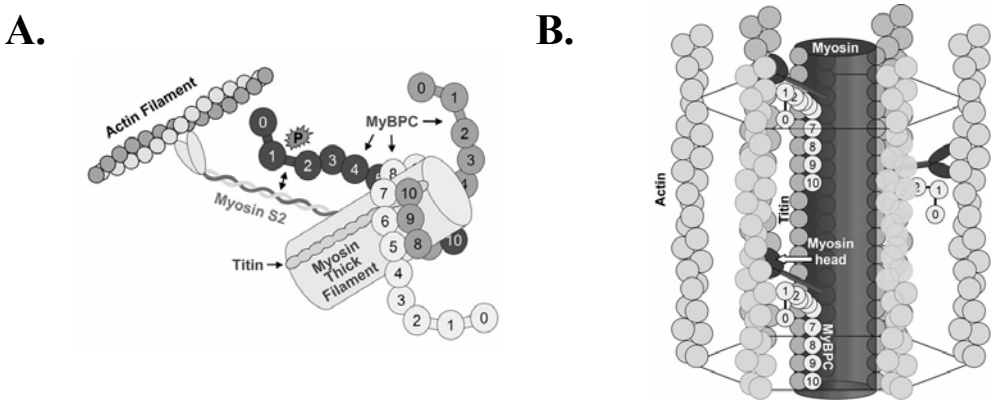



**Figure 9.** Schematic diagram of the trimeric collar (A) and the axial model (B) of cardiac MyBP-C. (Oakley *et al.*, 2004)

These changes suggest a critical role for MyBP-C in the setting of the probability of initial crossbridge attachment to thin filaments (weak attachment). Consequently, PKA-phosphorylation of MyBP-C may influence the number of force generating strongly bound crossbridges and thus the contractile force. This idea was, however, challenged by Konhilas *et al.* who demonstrated that MyBP-C phosphorylation by PKA does not alter calcium responsiveness of the murine myocardium (Konhilas *et al.*, 2003). The multi-modular **H-** (~86 kDa) and **X-proteins** described in vertebrate skeletal muscle are further minor components of the thick filaments. Being homologues of MyBP-C they are composed predominantly of Ig and fibronectin domains and localised also in the C-zone, however, with yet undefined functions.

**Thin filaments**

The ~1 μm long thin filaments attach to the Z line and extend into the A band to the edge of the H zone. **Actin** is the main constituent of thin filaments of muscle fibers. The actin filaments are composed of G-actin monomers (MW= 42 kDa), each possessing a binding-site for myosin. Under the conditions that exist in the cytoplasm the unpolymerized G-actin globular subunits can exchange bound ADP for ATP and they polymerise to form helical, two-stranded filaments (F-actin). The actin filaments have polarity, i.e. all actin monomers orient with their nucleotide binding cleft towards the same direction, designated the minus end. Growth of the actin filament occurs at the positive end while dissociation is dominant at the negative one. Capping proteins bind at the ends of the actin filaments. Depending on whether these proteins are associated to the positive or the negative end they may either stabilize the filaments or promote disassembly, respectively. Such a capping protein, the *tropomodulin* binds the negative end and thus stabilizes the filaments. Actin filaments may also associate into bundles via cross-linking proteins, like the dimeric *α-actinin*, a component of the Z-disc. The **Z discs** that border the sarcomeres cross-link and thus organize the overlapping thin myofilaments from opposing sarcomeres into a highly ordered, three-dimensional lattice (Fig. 10).

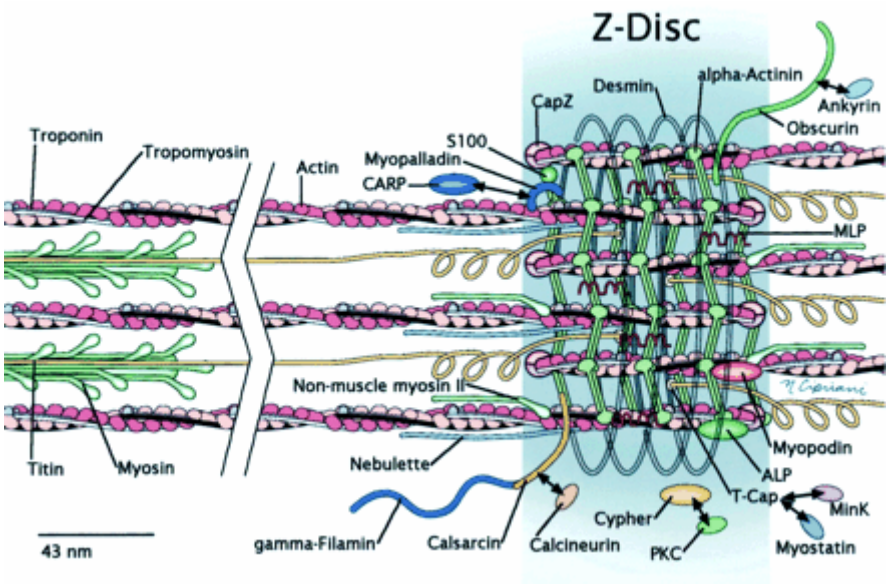



**Figure 10.** Cartoon of the molecular network that makes up the Z line (CARP – cardiac ankyrin repeat protein, T-Cap – telethonin, MLP – muscle specific LIM protein, PKC – protein kinase C, ALP – actinin-associated LIM-protein, MinK - the β-subunit of the slow activating component of the delayed rectifier potassium current ($I_{Ks}$) channel; from(Pyle & Solaro, 2004).

In addition, they occupy a unique position, both from structural and functional aspects, at the interface of the sarcomere, the cytoskeleton, the sarcoplasmic reticulum and the sarcolemma. These ~100-nm wide discs are situated in the centre of the I-band and serve as anchoring structures for numerous structural (**actin**, **CapZ**, **α-actinin**, **titin**, **T-cap** or **telethonin**, **actinin-associated LIM-protein** (**ALP)**, **cardiac ankyrin repeat protein** (**CARP)**, **nebulette, obscurin**, **desmin**, etc.) and functional (**PKC**, **PKA**, **calcineurin**, **calsarcin–1**, **cypher-1**, **S-100A1**, etc.) proteins. This diversity of proteins is indicative of a versatility of functions and a pivotal role in the cellular homeostasis and in the short- and long-term regulation of cardiac contractility. Indeed, beside serving as a way station for numerous signalling molecules (**NFATc**, **myopodin**, **calcineurin**, etc.) Z-disc is presumed to be involved, among others, in $Ca^{2+}$ signalling and ion channel regulation, in sensing mechanical strain in cardiomyocytes and in the development of myocardial hypertrophy, myopathies and heart failure (Pyle & Solaro, 2004).

Activation of the actin filaments is under the tight control of a regulatory protein complex. The thin filament regulatory strand in striated muscle is composed of tropomyosin and troponin, the $Ca^{2+}$ binding protein complex that occurs at regular intervals of 38 nm along the tropomyosin. Within the regulatory strand, **tropomyosin** (Tm) occurs as a linear array of coiled-coil dimers in which adjacent dimers overlap and interact in head-to-tail fashion winding around the actin filaments (Wolska & Wieczorek, 2003). According to the model by Smith & Geeves in the relaxed state cTnI binds to actin thus immobilizing Tm on the thin filament thus impeding strong actin-myosin interaction (Smith & Geeves, 2003). The release of cTnI from actin by the $Ca^{2+}$-TnC complex permits movement of Tm that results in crossbridge binding and induces a kink in the Tm-Tn chain. Recent studies in skinned fibres support the idea that the spread of activation along the thin filament involves head-to-tail interactions between Tm molecules. Indeed, C-terminal truncation of Tm has virtually abolished near-neighbor interactions and thus the cooperative manner of myosin S-1 binding (Pan *et al.*, 1989). Additionally, according to the findings of Regnier et al., it is presumable that much of the cooperativity in the activation of force is attributable to interactions between adjacent functional groups along the thin filament, most probably via Tm-Tm interactions (Regnier *et al.*, 2002). Interestingly, Tm isoform content exerts significant effects on the contractile properties ($Ca^{2+}$ sensitivity of force, twitch relaxation, etc.) as demonstrated in transgenic models expressing various amounts of β-Tm in the myocardium (Palmiter *et al.*, 1996;Wolska *et al.*, 1999).

Current model of thin filament activation distinguishes 3 different activation states of the thin filament each corresponding to a different position of Tm in relation to actin: a *blocked* state in the absence of $Ca^{2+}$, a *closed* state in the presence of $Ca^{2+}$ and an *open* state in the concurrent presence of $Ca^{2+}$ and strongly bound crossbridge (Fig. 11). Recent 3D reconstructions of thin filaments in different regulatory states have provided support for this idea (Craig & Lehman, 2001;Vibert *et al.*, 1997).

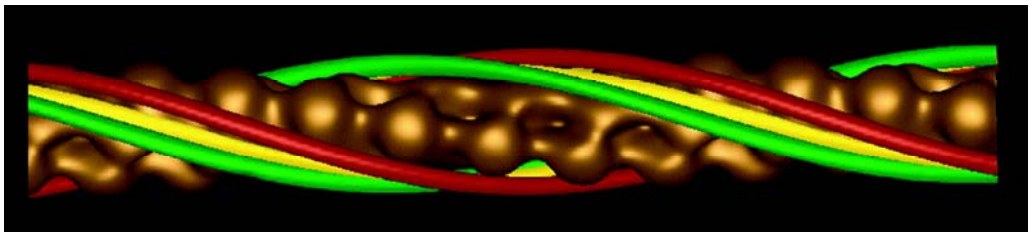


**Figure 11.** Three dimensional reconstruction of the surface view of a thin filament demonstrating Tm position changes under relaxed (red), in $Ca^{2+}$-activated (yellow) and in rigor (green) states (Craig & Lehman, 2001).

**Cardiac troponin C (cTnC)** (Fig. 12) consists of three $Ca^{2+}$ binding sites, two of them exchanging $Ca^{2+}$ much too slowly to occur within a heartbeat (Pan & Solaro, 1987). Kinetic analysis of the third binding site proved sufficiently rapid exchange of $Ca^{2+}$. Thus it is likely that only one binding site is involved in the regulation of myofilament contraction. Recent studies using the fluorescent probe 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS) observed two phases of the activation following $Ca^{2+}$ association: a $Ca^{2+}$ dependent fast phase ($Ca^{2+}$ binding event) and a slower, $Ca^{2+}$ independent phase (a conformational change triggered by the $Ca^{2+}$ binding) (Dong *et al.*, 1996;Dong *et al.*, 1997). On the basis of these results and the data on $Ca^{2+}$ binding kinetics of Hazard et al. (Hazard *et al.*, 1998) it is presumable that the regulatory site of cTnC rapidly binds and releases $Ca^{2+}$ and induces relatively slow conformational changes of cTnC.

The **cardiac troponin T (cTnT)** is the largest and longest component of the troponin complex having the most diverse and extensive interactions with its neighbours on the thin filament. The C-terminal region of cTnT forms the $Ca^{2+}$-modulated complex with cTnC, cTnI and Tm, whereas the N-terminal region anchors cTn complex to Tm through interactions with the C-terminus of the Tm and extends over the end-to-end overlap regions of adjacent Tm molecules (Solaro & Rarick, 1998). $Ca^{2+}$ binding to cTnC weakens the interaction between cTnT and Tm. The resulting movement of the regulatory strand drives the thin filament from the blocked to the open state thus allowing transition of weakly bound crossbridges to

strongly bound force-generating states (McKillop & Geeves, 1993). Interestingly, recent studies strongly suggested that troponin isoform composition might act as a molecular mechanism to tune myofilament function to meet the work demands on the ventricle (Solaro & Rarick, 1998).

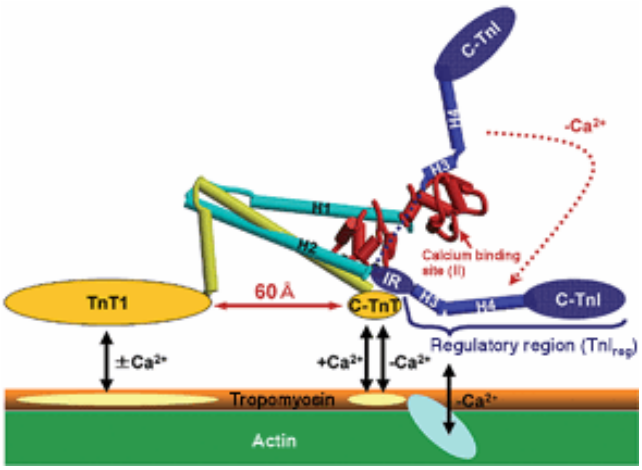



**Figure 12.** Changes in the structure of troponin and interaction with other proteins in response to $Ca^{2+}$ binding and release (predicted) TnT1, cTnI and cTnT serve as tabs for binding to tropomyosin and actin (Takeda *et al.*, 2003).

The third component of the troponin complex is the **cardiac troponin I (cTnI).** An essential function of the cTnI is to interact with actin thereby inhibiting actomyosin ATPase activity at low cytosolic [$Ca^{2+}$]. The importance of this function is supported by the highly conserved sequence of the inhibitory region among muscle types. Within the thin filament, in the presence of Tm maximum ATPase inhibition can be observed at a 7:1 molar ratio of actin : cTnI. The periodic distribution of cTnI along the Tm –actin filaments (Ohtsuki & Shiraishi, 2002) indicates that beside the amplification of cTnI inhibitory action on actomyosin ATPase Tm also serves to direct cTnI to its optimal location on thin filament during sarcomere assembly.

**The myocyte cytoskeleton**

The **microtrabecular lattice** or **cytoskeleton** is the term that summarizes all those proteins that connect sarcomeres to each other, contractile systems to the sarcolemma and cells to each other and the surrounding extracellular matrix. Additionally, it appears to anchor most of the cytoplasmic organelles and thus due to its three-dimensional architecture it provides a structural substratum along which directed cytoplasmic transport may occur and on which cytoplasmic reactions may eventuate. Despite its relatively small volume compared to that of

other cell organelles the cytoskeleton is essential in the maintenance of sarcomere stability during contraction and in the transmission of contractile force to the sarcolemma and to the extracellular matrix. The components of the cytoskeleton can be grouped as follows: endosarcomeric (inside the sarcomere) and exosarcomeric (outside the sarcomere) cytoskeletal proteins. Although numerous build up the cytoskeleton only the most important components are summerized below.

**The intrasarcomeric cytoskeleton**

The rod like **α-actinin** molecules cross-link actin filaments at the site of insertion into the Z discs. The α-actinin is an anti-parallel homodimer peptide that binds F-actin via the N terminus of each monomer. Its presumed role is to maintain 3D organization of the thin filaments, to transmit longitudinal force among the neighbouring sarcomeres and to form polar arrays of actin filaments where they are bound to the cell membrane.

In the skeletal muscle nebulin is likely to play a crucial for the assembly of the mature Z-disc. **Nebulette** is the functional homolog of up to 100 kDa of nebulin's C-terminal segment and it is predicted to substitute for the Z-disc integrative function of the skeletal muscle protein nebulin in the heart muscle. It is involved in the assembly of the sarcomere and also anchores microfilaments to the plasma membrane. Furthermore, similarly to nebulin, nebulette may play the role of Z-disc terminator and could determine the ending of the Z-disc structure and the transition to the I-band (Millevoi *et al.*, 1998). Nebulin is supposed to participate in thin filament length regulation. Although this regulation may exist also in cardiac muscle, nebulette is too short to span up to the pointed end of the thin filament, hence its impact on thin filaments is likely to be limited to a narrow region near the Z-disc.

The giant protein **titin** (also known as **connectin**) is the third most abundant protein in the striated muscle after actin and myosin. The molecule is formed by a flexible filament of a single polypeptide that is ~1 μm long and 3-4 nm wide with a molecular weight of up to ~4 MDa (Maruyama *et al.*, 1984;Trinick *et al.*, 1984;Wang *et al.*, 1984) that makes titin the largest polypeptide described so far in nature. Studies in the last decade proved diverse developmental, mechanical and catalytic functions that make titin a key component of the striated muscle. Differential expression of the human titin gene gives rise to a spectrum of isoform between the size of ~0.6 MDa and 3.7 MDa corresponding to molecular length of 0.2 μm-1.2 μm. Titin is largely composed of domains that are similar to immunoglobulin (Ig) and fibronectin type-III domains. In addition to the ~300 Ig and fibronectin domains titin also contains a kinase domain near the carboxyl terminus, potential phosphorylation sites at both

ends and several unique sequences. The molecules extend half of the sarcomere from the Z- to the M-line and are aligned parallel to the long axis of thick and thin filaments. Titin is attached to thick filaments that are arranged on a hexagonal lattice in the A-band while in the Z-disc it is attached to thin filaments arranged on a tetragonal lattice. The precise arrangement how six titin molecules per half sarcomere can satisfy these symmetries is still unclear (Cazorla *et al.*, 2000a;Liversage *et al.*, 2001).

Distinct regions of the peptide (Z-line, I-band, A-band, M-line) illustrate different structural properties that correspond with variable functions. According to its functional diversity titin interacts with numerous structural and regulatory proteins as indicated in Fig. 13.. Amino-terminal regions of titin molecules from adjacent sarcomeres overlap in the Z-line while carboxy-teminal regions join in the M-line. Ig domains interspaced with unique sequences constitute these overlapping regions. In the Z-line titin interacts with numerous proteins e.g. T-Cap/telethonin, α-actinin, obscurin, and near the edge of the Z-line, actin. On the other hand, myomesin and M-protein bind titin at its other extremity, in the M-line.

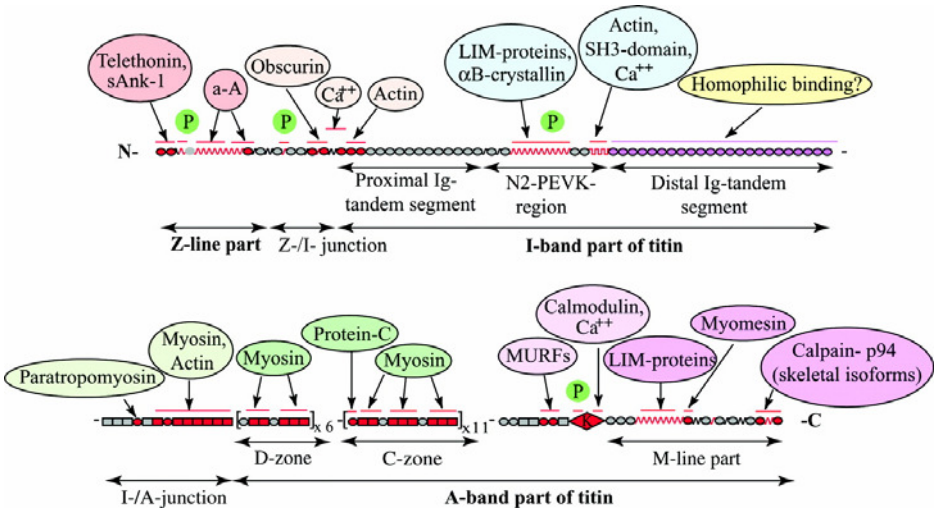



**Figure 13.** Schematic structure of the shortest cardiac titin isoform (see abbreviations in the text; from (Tskhovrebova & Trinick, 2004)).

A-band section (~2.1 MDa) that is highly conserved in sequence contains the largest part of the molecule and is an integral part of the myosin filament. Here, super-repeats (Labeit *et al.*, 1992;Labeit & Kolmerer, 1995) of Ig and fibronectin domains are 25-30 nm and 43 nm long, the latter correlating well with the repeat distance of the myosin heads on the thick filaments. These structural correlations predict intimate association between titin and thick filament molecules, namely myosin, MyBP-C and M-line proteins. The I-band region is composed of solely Ig domains and unique sequences. Between the conserved proximal (amino-terminal)

and distal I-band segments the joining part is isoform dependent and varies in size and structure. This segment has basically three main sub-regions: a sequence with variable number of Ig domains, the N2 region with alternating Ig domains and unique sequences and the PEVK region named after its preponderance of proline (P), glutamate (E), valin (V) and lysine (K) residues. The N2 region has two alternative forms: N2A that is characteristic in cardiac and skeletal muscle and N2B that can be found only in the cardiac muscle. The myocardium may also express the N2BA isoform that contains both N2A and N2B sequences. This isoform contains additional Ig domains and a longer PEVK segment. Alternative splicing results in variable PEVK segments and thus in a diversity of N2BA isoforms. Interestingly, recently a fetal cardiac titin isoform containing additional spring elements in the tandem Ig and PEVK region has been described. This isoform predominates the fetal and neonatal myocardium and gradually disappears during postnatal development (Lahmers *et al.*, 2004). Being longer and also more compliant than the adult isoforms fetal titin represents a possible way of adjustment of diastolic filling properties through the regulation of titin's spring composition.

Extensive research of the last decade revealed large diversity of titin's functional properties. It was presumed that titin acts as a molecular template (the *ruler hypothesis*) that determines the length of the thick filament (Whiting *et al.*, 1989). This hypothesis was supported by the findings that cells expressing defective titin molecules lack thick filaments, thus titin is vital for normal **sarcomere formation** (Peckham *et al.*, 1997b;Peckham *et al.*, 1997a;van der Ven *et al.*, 2000) and its expression precedes or occurs simultaneously with sarcomere assembly (Isaacs *et al.*, 1992;Komiyama *et al.*, 1993;Soeno *et al.*, 1999;van der Ven *et al.*, 1999). Additionally, data suggest that titin provides **scaffolding** not only for most structural proteins in the sarcomere, but also for numerous cytoplasmic proteins. Attributes of vertebrate titin and invertebrate titin-like molecules indicate that despite their physiological heterogeneity these molecules might be unified by the ability to connect thick filaments axially to Z-lines. Furthermore, they stabilize thick filaments' position relative to thin filaments centralizing them in the sarcomere during the cycles of contraction and relaxation. Within the normal operating range the I-band portion of titin performs this function. It is also the titin-generated passive tension that together with collagen determines the upper limit of the physiological sarcomere length range in the heart. Indeed, the main source of **passive tension** developed by muscle stretch originates also from titin and now it is widely accepted that titin plays a substantial role in determining passive myocyte mechanics (Granzier & Irving T.C., 1995). Exclusive structure of the I-band region enables titin to maintain sarcomere integrity and

operate in a wide sarcomere length range. The composition results in a highly flexible structure and in the absence of external force this I-band region is shortened to a near zero end-to-end length by thermally driven bending motions. Sarcomere extension beyond or its shortening to below the resting or slack sarcomere length results in straightening and extension of tandem Ig segments of the I-band titin from the initially coiled conformation (Fig. 14.). Further extension induces unfolding of the peptide in a definite order according to the mechanical stability of its different domains. Unfolding initiates in the unique PEVK sequence and is followed by the N2B regions thus preventing Ig-domains from unfolding (Linke *et al.*, 1998b;Linke *et al.*, 1998a;Minajeva *et al.*, 2001;Trombitas *et al.*, 2000). Ig and fibronectin domains unfold finally, in a hierarchical sequence. Although the likelihood of Ig and fibronectin unfolding *in vivo* is relatively low, it cannot be completely excluded (Goulding *et al.*, 1997). Myocytes exhibit viscoelasticity as revealed by passive force hysteresis in stretch-release cycles. Although the exact molecular basis of titin's viscoelasticity is still unclear titin-actin interactions, unfolding of Ig-domains as well as inter- or intramolecular cross links between PEVK and N2B unique regions may be involved (Kellermayer *et al.*, 2001;Kulke *et al.*, 2001;Minajeva *et al.*, 2001;Trombitas *et al.*, 2003;Yamasaki *et al.*, 2001;Yamasaki *et al.*, 2002). Given stretch of N2B isoform results in a higher fractional extension of the shorter extensible segment than that in the N2BA isoform. Accordingly, myocytes that express mostly N2B titin develops much higher passive stiffness than those expressing N2BA isoform. The dominant expression of N2B titin in animals with high heart rates is supposed to allow rapid diastolic filling due to the high restoring force (Cazorla *et al.*, 2000a). Varying the coexpression ratio of stiffer and looser isoforms allows development of graded force levels without affecting the critical functions performed by titin's inextensible regions (Trombitas *et al.*, 2001). This mechanism of passive stiffness modulation in the ventricular myocardium was proved by the differences in titin composition observed during heart development, among myocardial tissue of various species and also within the ventricular myocardial wall (Bell *et al.*, 2000;Cazorla *et al.*, 2000a;Lahmers *et al.*, 2004).

In contrast to isoform expression that represents a relatively long-term regulation further mechanisms allow rapid and fine ways for tuning titin based passive tension. The finding that PEVK region of N2B titin binds F-actin suggests that interaction between titin and thin filaments may retard filament sliding and increase passive stiffness (Yamasaki *et al.*, 2001). Furthermore, S100A1, a soluble calcium-binding protein found at high concentration in the myocardium inhibits actin-PEVK interaction in a calcium-dependent manner (Yamasaki *et*

*al.*, 2001). Increase of stiffness in rat trabeculae was associated to $Ca^{2+}$ decay in rat myocardium (Stuyvers *et al.*, 1997) suggesting that dynamic interaction of titin and actin indeed contributes to passive stiffness and that the interaction is modulated by the cyclic changes in the $Ca^{2+}$ level. Dynamic PEVK-actin interaction was however not confirmed in myocardium that expresses high level of N2BA titin (Campbell *et al.*, 2003), indicating isoform specific differences between actin binding properties. Phosphorylation of N2B unique sequence by protein kinase A (PKA) reduces passive tension (Yamasaki *et al.*, 2002) presumably for allowing more complete ventricular filling at higher heart rate induced by β-adrenergic stimulation.

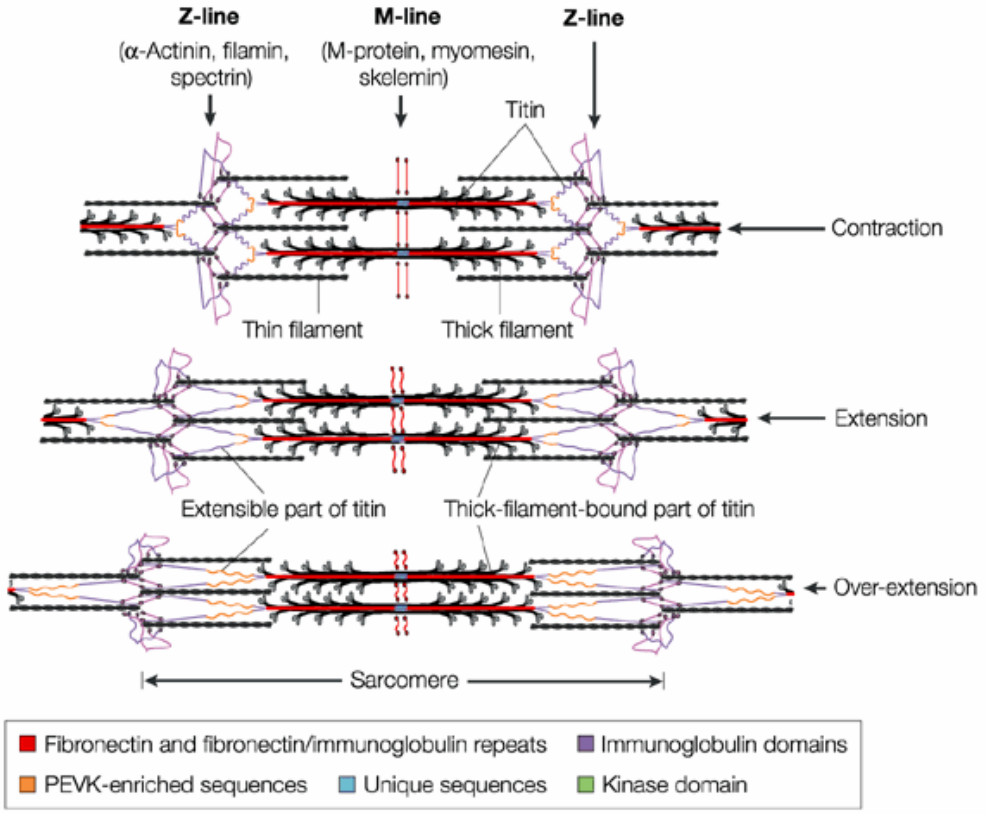



**Figure 14.** Sarcomere structure at different degrees of extension (Tskhovrebova & Trinick, 2004).

Furthermore, $Ca^{2+}$ increases titin-based passive tension through its direct effect on the E-rich motif (Labeit *et al.*, 2003). Considering that the E-rich motif is present in the N2BA titin, but not in the N2B isoform this difference may act as a further possible mechanism through which differential splicing events may influence passive tension (Bang *et al.*, 2001;Freiburg *et al.*, 2000;Greaser *et al.*, 2002;Labeit & Kolmerer, 1995). To sum up, high level of N2B results in elevated baseline passive stiffness that can be lowered by PKA phosphorylation and

Ca/S100. In contrast, calcium binding may elevate the relatively low passive stiffness at higher ratio of N2BA (Granzier & Labeit, 2004).

Beside its essential role in the determination of passive stiffness titin influences **active mechanical properties**, too. $Ca^{2+}$-sensitivity increased in proportion to titin-based passive force (Cazorla *et al.*, 2001). Moreover, similarly to $Ca^{2+}$-activated maximal force (Fukuda *et al.*, 2001), the SL-dependent increase in $Ca^{2+}$-sensitivity decreased in parallel with passive tension irrespectively of its root cause (e.g. mild trypsin degradation of titin or varying the SL history before activation using hysteresis property of titin) (Cazorla *et al.*, 1999;Cazorla *et al.*, 2001). The effect of titin on active tension was predicted to alter likelihood of actomyosin interaction either through the modulation of interfilament lattice spacing or through a direct strain on thick filaments affecting cross-bridge mobility (Cazorla *et al.*, 2001;Fukuda *et al.*, 2001). Moreover, below slack length, titin's restoring force further inhibits actomyosin interaction thus promoting rapid abolition of contraction in early diastole (Helmes *et al.*, 2003).

Titin was thought to act as a structural element of the sarcomere and a molecular spring is nowadays also considered as a **biomechanical sensor** and a signalling molecule. Early hypotheses were first confirmed by the discovery of a kinase domain at the carboxy-terminal, M-line end of the molecule. Since then evidences proved that both Z-line and M-line are involved in the control of tension- and protein-turnover related processes. Different connections of titin in the Z- and M-line regions not only contribute to structural integrity but also provide site-specific links with regulatory systems. The interaction between telethonin and potassium channel subunit minK/isk (Furukawa *et al.*, 2001), furthermore that between titin and the small ankyrin-1 (aANK1) (Kontrogianni-Konstantopoulos & Bloch, 2003) or obscurin (Bagnato *et al.*, 2003) connect **Z-line** part of titin to T-tubules and the SR, respectively. Links between membrane systems and titin point to a tension-related feedback mechanism between contractile and excitatory systems (Furukawa *et al.*, 2001) as well as to titin's role in the positioning of the SR and T-tubular membrane systems in close proximity of the I-band region of the sarcomere. Furthermore, telethonin interacts with calsarcin-3 (Frey & Olson, 2002), the muscle growth factor myostatin and muscle LIM protein (MLP) indicating titin's involvement in myogenic differentiation and stretch sensing (Arber *et al.*, 1994;Knoll *et al.*, 2002;Nicholas *et al.*, 2002). In the central **I-band region** N2B and N2A elements are hotspots for molecular interactions with various signalling systems. N2B binds DRAL/FHL-2 that anchors metabolic enzymes (creatine kinase, adenylate kinase, and phosphofructokinase) ensuring sufficient ATP for crossbridge cycling close to the overlap regions of the sarcomeres

(Lange *et al.*, 2002). Further interaction with αβ-crystallin indicates participation in differentiation-apoptosis and ischemic stress signalling response (Bullard *et al.*, 2004). By its interaction with the protease calpain-3 in the skeletal muscle N2A element regulates protein degradation (Ono *et al.*, 2004). Ability of N2A to bind cardiac ankyrin repeat protein (CARP) as well as ankrd2 and diabetes ankyrin repeat protein (DARP) suggests titin's connection with fast acting muscle stress response pathways (Miller *et al.*, 2003). Some signalling molecules mentioned previously (calpain-3, DRAL/FHL-2, telethonin, obscurin) have binding sites in the **M-line region**, too (Kinbara *et al.*, 1997;Lange *et al.*, 2002;Mayans *et al.*, 1998b;Young *et al.*, 2001). Near its kinase domain titin interacts with members of the muscle-specific RING-finger-family of signalling proteins (MURF1 and MURF2) indicating titin's role in the regulation of contractile protein turnover (Mayans *et al.*, 1998a) and that of gene expression (McElhinny *et al.*, 2002). Accordingly, the Z-disc, M-line and I-band signalling hotspots sense titin-based passive tension, the Z-disc and M-line sense also actomyosin-based tension, and Z-disc receives inputs from the tensions transmitted via the sarcolemma (Huxley *et al.*, 1994;Wakabayashi *et al.*, 1994). All these inputs support an important versatile integrative role for titin between muscle stretch and short- or long-term adaptive signalling mechanisms.

Its enormous size makes titin a prominent target for mutations. In vitro observations that different mutations of titin seriously impair sarcomere assembly and function (Gotthardt *et al.*, 2003;Gregorio *et al.*, 1998;McElhinny *et al.*, 2002;Peckham *et al.*, 1997a;van der Ven *et al.*, 2000) correlate well with recent in vivo studies, which described mutations associated to **muscle pathologies**. Alterations of the I-band region, e.g. expression of truncated titin molecules (Gerull *et al.*, 2002), as well as mutation of the N2B region (Xu *et al.*, 2002) impair elastic properties of the myocardium and were found in dilated cardiomyopathy (DCM). Several mutations in the Z-line segment of titin may also result in DCM, suggesting defective tension transmission (Gerull *et al.*, 2002;Itoh-Satoh *et al.*, 2002). In patients with hypertrophic cardiomyopathy (HCM) titin mutations in Z-disc and N2B elements were reported (Itoh-Satoh *et al.*, 2002). Studies on the effects of chronic mechanical challenge in animal models of both DCM and HCM, as well as in patients with coronary artery disease proved the role of isoform type composition in the fine adjustment of titin-based myocardial stiffness. (Bell *et al.*, 2000;Neagoe *et al.*, 2002;Wu *et al.*, 2002).

**The extrasarcomeric cytoskeleton**

The nonbranching, hollow cylinders of **microtubules** are ~25 nm in diameter and composed of a linear chain of dimeric tubulin molecules (Fig. 15.A). The tubulin dimer is formed from

an α- and a β-tubulin molecule. The dimers polymerise in an end-to-end fashion, head to tail, in repeating pattern to form protofilaments. The microtubules serve as guides for molecular motors and involved in intracellular transport of chromosomes and secretory granules, cell migration and maintenance of cell shape.

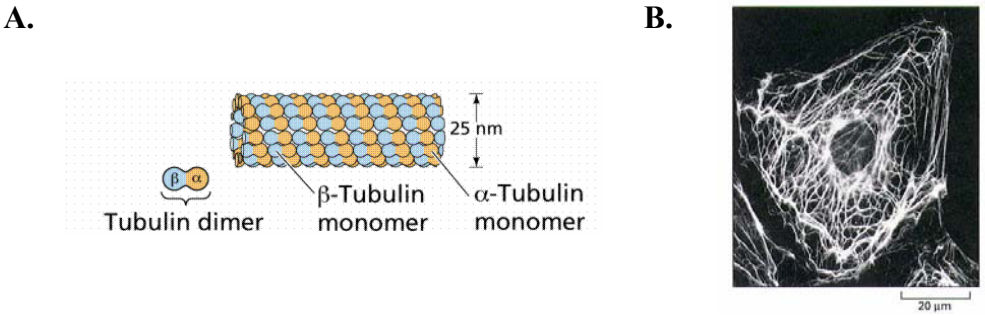



**Figure 15.** Tubulin components of the cytoskeleton (A) (www.emc.maricopa.edu) and the rope-like characteristics of intermediate filaments (B) (from http://www.cytochemistry.net).

**Intermediate filaments** (**IFs**) are less dynamic structures compared with myofilaments or microtubules hence they are believed to play primarily structural role within the cell and to comprise the cytoplasmic link of a tissuewide continuum of cytoplasmic, nuclear and extracellular filaments (Fig. 15.B). **Desmin**, the muscle specific IF protein is organized to form ringlike bands around the myofibrils at the Z discs, to provide lateral interconnections between the Z discs and to integrate the contractile apparatus with the sarcolemma and the nucleus. Its proposed function is to prevent sarcomeres from slipping and to maintain cross-striation during contraction. This idea is supportred by the observation that the lack of desmin from the sarcomere leads to severe disruption of muscle architecture (the loss of lateral alignment of myofibrils) and to degeneration (Milner *et al.*, 1996a). Beside its structural importance, functional role has also been attributed to desmin, since intermediate filaments were shown to contribute ~10 % to passive tension at physiological sarcomere length (Milner *et al.*, 1996b). In ventricular pressure overload hypertrophy and in decompensated congestive heart failure expression level of desmin is upregulated (Collins *et al.*, 1996) hence its increased importance in the determination of passive tension cannot be ruled out.

**AHNAK** is a 700 kDa ubiquitous protein expressed in a variety of cell types being a component of the subsarcolemmal cytoarchitecture. In cardiomyocytes it interacts through its carboxy-terminal domain with the regulatory $\beta_2$ subunit of the cardiac L-type $Ca^{+2}$ channel (Alvarez *et al.*, 2004) and can be phosphorylated by PKA suggesting its role in β-adrenergic signal transduction (Haase *et al.*, 1999). At the plasma membrane AHNAK is associated with

the annexin 2/S100A10 complex and regulates cortical actin cytoskeleton organization and cell membrane cytoarchitecture (Benaud *et al.*, 2004). In addition , it acts as a linker protein between cardiac L-type $Ca^{2+}$ channels and the actin-based cytoskeleton (Hohaus *et al.*, 2002). Recently, interaction of AHNAK carboxy-terminal domain with actin of the thin filaments suggested its stabilizing effect on muscle contractility (Haase *et al.*, 2004).

## Physiology of the heart

### Cardiac pump function

The myocardial contraction plays a crucial role in the maintenance and regulation of the circulation. It is to note at the same time that as the hemodynamic resistance against which the heart works is provided mainly by the tone of the peripheral arterioles contribution of the vasculature to the overall regulation of circulation is also remarkable. The cardiac pump function can be quantitatively characterized by the **cardiac output (CO)** that is defined as the amount of blood pumped out by the heart each minute. CO is the function of the **heart rate** (**HR**; the frequency of the heartbeat) and the **stroke volume** (**SV**; the volume of blood ejected from one ventricle with each heartbeat) and can be expressed as the product of these two amounts. Thus, cardiac activity strongly depends on the activity of the pacemaker and the myocardial performance, two determinants that almost invariably alter each other. Numerous intrinsic and extrinsic mechanisms allow the myocardium to adapt to the changes in hemodynamic conditions. The two principal **intrinsic mechanisms** exhibited by the completely isolated heart are the **Frank-Starling mechanism** and the **rate-induced regulation**. The former describes the observation that increased ventricular volume facilitates ventricular contraction and thus enables the ventricle to pump a greater stroke volume exactly matching cardiac output with venous return at equilibrium. The latter refers to augmented developed force in parallel with accelerating heart rate and is caused by a gradual increase in the intracellular [$Ca^{2+}$]. Furthermore, in the intact organism numerous **extrinsic factors** influence the myocardial activity. Beside the **nervous** (sympathetic and parasympathetic) and **hormonal** (adrenomedullary, adrenocortical and thyroid hormones, insulin, glucagone, etc.) control, changes in the concentration of **blood gases** (oxygen, carbon dioxide) as well as in the **pH** may alter the myocardial performance both in direct and more complex indirect ways. Some of these mechanisms are briefly summarized hereinafter.

### Excitation-Contraction Coupling

#### The cardiac action potential

Electrical behavior of the single myocytes could be revealed due to the development of microelectrode and patch-clamp techniques. It was shown that various phases of the cardiac **action potential (AP)** are associated with dynamic alterations in the permeability of the cell membrane, mainly to $Na^+$, $K^+$ and $Ca^{2+}$. Permeability to a given ion, in association with its transmembrane electrical and concentration differences determine the quantity of ion

diffusion and its direction across the membrane. The potential of the interior of the cell is about 90 mV lower than that of the surrounding medium. On the other hand, $[K^+]$ inside the cell far exceeds that outside the cell and the opposite is true for the sodium and calcium concentrations. As sodium and calcium conductances of the cell membrane are nearly negligible the **resting potential** is determined mostly by the $K^+$. According to its concentration gradient $K^+$ tends to diffuse from the inside to the outside of the cell against the electrostatic force. During the resting period $K^+$ diffusion takes place mainly through the inward rectifier $K^+$ channel ($I_{K1}$).

It is to note that two main types of action potentials exhibited by different cell types exist in the heart. The **fast response** occurs in the atrial and ventricular working myocytes and in the specialized conducting (Purkinje) fibers. In contrast, cells in the sinoatrial and atrioventricular nodes illustrate **slow response** that underlies natural pacemaker activity. Below events during the AP of the typical ventricular myocyte is summarized briefly (Fig. 16). In answer to any external stimulus that changes resting potential to the critical value (~ -65 mV) called the **threshold** the membrane depolarises, i.e. the potential difference rapidly reverses such that the potential of the interior of the cell exceeds that of the exterior by ~ 40 mV. The rapid **upstroke** of the action potential is designated as **phase 0** and characterised by a sudden and transient increase in the sodium conductance. Voltage-dependent opening of fast $Na^+$ channels ($I_{Na}$) allows $Na^+$ driven by both electrostatic and chemical forces to enter the cell. The reversal of the membrane polarity also called **overshoot** of the AP is the result of $Na^+$ influx driven by chemical forces that overwhelm the counteracting electrostatic forces.

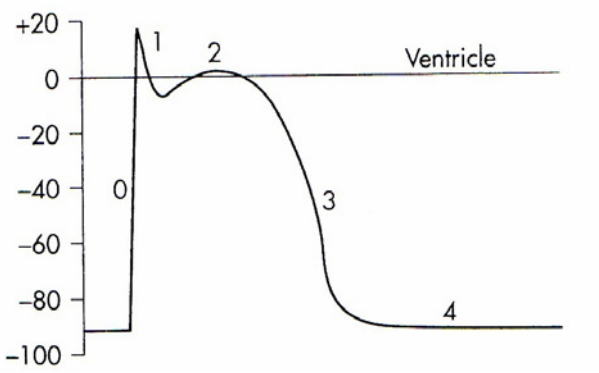



**Figure 16.** Typical action potential recorded from a ventricular myocyte (values are given in millivolts) (Berne & Lewy, 1998).

The upstroke is followed by a brief period of partial repolarization, the **early repolarization (phase 1)** ascribed to the sudden inactivation of $I_{Na}$ and activation of transient outward

currents ($I_{to1}$ and $I_{to2}$) carried by $K^+$ and $Cl^-$, respectively. The phase 1 notch is prominent in the Purkinje fibers as well as in the epicardial and midmyocardial ventricular cells and negligible in the endocardial region. Opening of predominantly the voltage-regulated L-type calcium channels ($I_{Ca\text{-}L}$) and the concomitant calcium influx result in a **plateau (phase 2)** that persists for ca. 0.1-0.2 sec. In this phase the influx of $Ca^{2+}$ is counterbalanced by the efflux of positive charge carried by $K^+$. $K^+$ exits through channels that conduct $I_{to}$, $I_{K1}$ and the delayed rectifier $K^+$ ($I_K$) currents. The $Ca^{2+}$ influx during the plateau phase is involved in the excitation-contraction coupling hence has a decisive impact on contractile force. During the plateau $Ca^{2+}$ influx gradually decreases in parallel with the increase of $K^+$ efflux. As soon as $K^+$ efflux exceeds $Ca^{2+}$ influx membrane potential starts to decrease, the **final repolarisation (phase 3)** begins. Two $K^+$ currents, $I_{to}$ and $I_K$ initiate the repolarisation. Interestingly, $I_{K1}$ is not involved in the initiation of phase 3 but once it has been started $I_{K1}$ does contribute to the repolarisation in a progressively predominating fashion. Following a brief period of hyperpolarization the cell membrane attains its original **resting state of polarization (phase 4)**. The excess $Na^+$ that entered the cell during phase 0 is eliminated by the electrogenic $Na^+/K^+$-ATPase compensating also for the $K^+$ loss during phase 2 and 3. Another electrogenic mechanism, the $Na^+/Ca^{2+}$ exchanger eliminates the excess $Ca^{2+}$. Furthermore, an ATP-driven $Ca^{2+}$-pump in the sarcolemma also plays a minor role. It is here to mention that the shape of the ventricular action potential also shows pronounced species-specific differences (e.g. rat vs. human).

**Excitation-contraction coupling**

The sarcolemma maintains a vast gradient of calcium ion concentration characterised by the extracellular value of $\sim 10^{-3}$ M and the intracellular values that range between the diastolic concentration of $\sim 10^{-7}$ M to the maximal systolic concentration of $\sim 10^{-5}$ M. Although the sequence of events that convert membrane potential changes into contractile force have already been described basically numerous details are yet to be elucidated. Once the electric impulse spread over the myocyte action potential is generated. Depolarisation of the T-tubule sarcolemma opens the L-type calcium channels ($I_{Ca\text{-}L}$) and induces influx of $Ca^{2+}$ ions into the intracellular space (Fig. 17). This relatively small amount of $Ca^{2+}$ acting on the foot region of the molecular complex causes configurational changes and activates the **ryanodine receptors (RyR2)** in the SR membrane. Opening of RyR2 $Ca^{2+}$ release channels liberates relatively large amounts of $Ca^{2+}$ from the SR. In this sense $I_{Ca\text{-}L}$ acts as a voltage sensor that promotes $Ca^{2+}$ release from the SR. It is to note that being the major $Ca^{2+}$ store within the myocyte SR

plays an indispensable role in the contraction regulation of all striated muscles. Four FK506 binding protein **FKBP12.6 (calstabin2)** bind each RyR2 complex at the outer side of the cytoplasmic domain. Beside decreasing open probability and stabilizing the closed state of the channel they are important for cooperative interactions among the subunits of the tetramer.

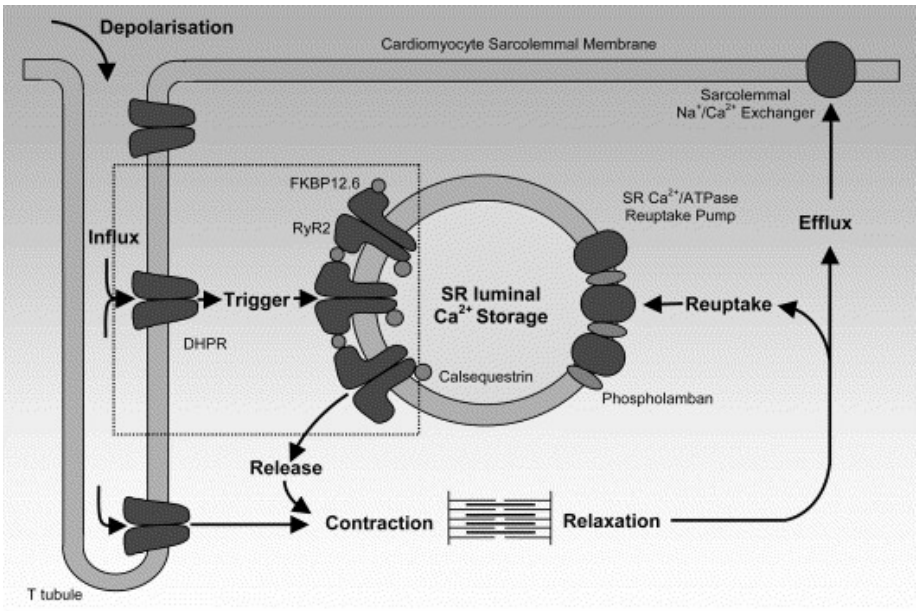



**Figure 17.** Key components of cardiac excitation-contraction coupling (DHPR-L-type voltage dependent (dihydropyridine sensitive) $Ca^{2+}$ channel, for further (Scoote & Williams, 2004).

The above model of **calcium induced calcium release (CICR)** in the myocardium in contrast to $Ca^{2+}$ release in the skeletal muscle does not require close proximity and mechanical association of the interacting molecules. The subsequent increase in the intracellular [$Ca^{2+}$] facilitates troponin-C-$Ca^{2+}$ interaction and activates the contractile filaments resulting in muscle contraction. Increase in intracellular [$Ca^{2+}$] at the same time activates also counteracting mechanisms. As soon as the removal of the $Ca^{2+}$ from the intracellular space exceeds its release from the SR, the increase in cytosolic $Ca^{2+}$ comes to an end and intracellular [$Ca^{2+}$] starts to decline. These cyclic variations in the concentration of cytosolic calcium are usually called **Ca-transient**. Decrease in the cytosolic $Ca^{2+}$ concentration allows dissociation of $Ca^{2+}$ from the troponin-C and the troponin complex starts to inhibit the actomyosin interaction. Relaxation results mainly from the uptake of $Ca^{2+}$ ions into the SR and only a minor quantity, to balance the $Ca^{2+}$ that enters the cell with each depolarisation, is expelled into the extracellular space. The **calcium-pumping ATPase (SERCA2)** located in the longitudinal SR membrane and stimulated by high cytosolic calcium concentration takes

up the majority of the cytosolic $Ca^{2+}$ via an energy-requiring process. SERCA2 is inhibited by its regulatory protein **phospholamban (PLB)**, a pentamer that colocalizes with the pump in a 1:1 molar ratio in the SR membrane. Two processes can remove $Ca^{2+}$ from the cell: the electrogenic **$Na^+/Ca^{2+}$ exchanger** that removes $Ca^{2+}$ ions in a 1:3 molar ratio using the transmembrane concentration gradient of $Na^+$ ions and of lesser importance the sarcolemmal **$Ca^{2+}$ pump** that is ATP consuming and can operate against a steep concentration gradient. Considering the relatively slow kinetics of exchange between the mitochondrial and the cytosolic $Ca^{2+}$, involvement of mitochondria in the beat-to-beat control of $Ca^{2+}$ movements is rather limited.

**Force generation – the cross-bridge cycle**

The term cross-bridge cycle refers to the repetitive attachment and detachment of myosin heads to and from the actin filaments. Each cross-bridge cycle produces a power stroke that drives the actin filament along the thick filament. The swinging-cross-bridge hypothesis of muscle contraction (featured in most text books) has recently been modified into the swinging-lever arm hypothesis. According to the new idea the myosin head is thought to bind to actin without rolling on the surface during the power stroke (as had been initially suggested). Rather, large movements are envisaged to occur at the distal (C-terminal) part of the myosin cross-bridge moving as a lever arm (Geeves & Holmes, 1999). Structural changes within the cross-bridges are associated with the strain of an elastic component present in the cross-bridges, which operates over a range of 8–10 nm (Huxley & Simmons, 1971). The α-helical neck domain is believed to function as a lever arm that swings relative to the catalytic domain. Thus, picometer changes in the active site of myosin S1 are magnified into nanometers of motion by rotation of the converter domain (Eisenberg & Hill, 1985;Holmes, 1997). The light chain binding domain, rather than the catalytic domain revealed tilting motions during length perturbations in the isometrically contracting muscle (Hopkins *et al.*, 1998;Irving *et al.*, 1995;Lombardi *et al.*, 1995). The elastic element of the cross-bridge may therefore reside in the lever arm (Howard & Spudich, 1996). Both the ATP- and ADP-$P_i$-loaded MHC bind (as the cross-bridge) to actin. Myosin undergoes changes in actin affinity and structure, being strongly attached to actin (having high affinity) or weakly attached (having low affinity) (Brenner, 1988;Eisenberg & Hill, 1985). Force is generated upon the transition from the weakly to the strongly attached state that is coupled to the $P_i$ release step (Dantzig *et al.*, 1992). Main structural and biochemical events in a cross bridge cycle are summarised in (Fig. 18).

At physiological ATP concentrations (3–5 mM), ATP binding to myosin is rapid and irreversible *(step 1)*. The subsequent detachment of actin from the actin-myosin·ATP (A~M·ATP) complex is similarly rapid and is caused by the opening between myosin's upper and lower 50-kDa regions *(step 2)*. A bending of the myosin neck region accompanies the hydrolytic cleavage of ATP *(step 3)*. Following ATP cleavage, myosin again binds weakly to actin at a high rate, but in the absence of $Ca^{2+}$ Tm sterically blocks access of the myosin head to strong binding sites on actin *(step 4)*. However, when $Ca^{2+}$ is bound to TnC, TnI detaches from actin, allowing the Tm/Tn complex to roll or slide over the thin filament surface. This way actin exposes additional weak binding sites and transiently exposes strong binding sites for binding to the complementary regions in myosin's 50-kDa domain *(step 5)*. The greater the $[Ca^{2+}]$, the greater the fraction of time the Tm/Tn complex allows myosin access to strong binding sites on actin. Consequently, the rate of strong cross-bridge attachment is dependent on $[Ca^{2+}]$ and Tm position.

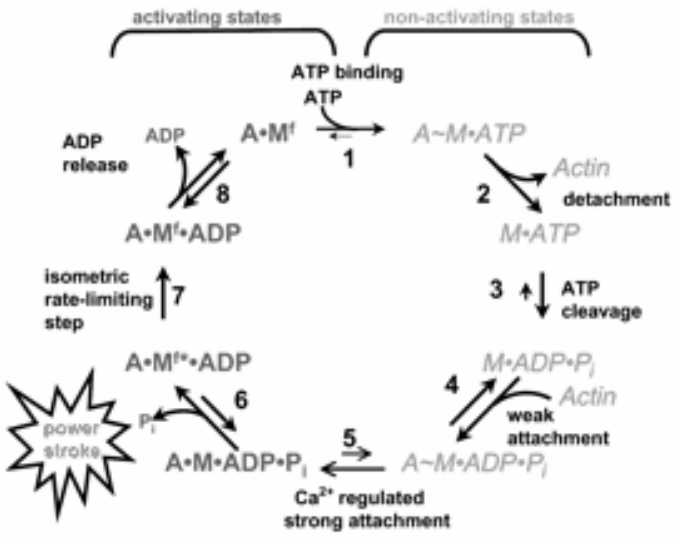



**Figure 18.** Eight main events of the cross-bridge cycle. "•" and "~" denote strong and weak binding between two species, respectively. "f" indicates cross-bridge exerting force; A, actin; M, myosin; $P_i$, inorganic phosphate (Gordon *et al.*, 2001).

Strong binding of myosin to actin is associated with movement of the upper and lower 50-kDa subdomains toward each other. This movement may allow the neck region of myosin to extend, allowing of $P_i$ release from the ATP binding pocket in myosin. Alternatively, the closure might promote $P_i$ release from the binding pocket, which then allows the extension of myosin's neck region. In any case, myosin neck extension is the power stroke that, in isometric muscle, stretches an elastic element by some 10 nm and produces force *(step 6)*. In

nonisometric conditions, shortening of the neck extension causes the thick and thin filaments to slide past each other. The subsequent irreversible isomerization *(step 7)* is strain sensitive; i.e., when the force on the cross-bridge is large, as in isometric contractions, the transition is slow being the rate-limiting step for the cross-bridge cycle. Finally *(step 8)*, ADP is released from $A{\cdot}M^{f}{\cdot}ADP$ (where f is a cross-bridge exerting force). This step is irreversible to form the rigor state, $A{\cdot}M^{f}$. Cross-bridges attach and exert force constantly during steps 7, 8, and 1 during isometric contraction, and force drops to zero when the cross-bridges detach in step 2. During shortening contractions the filaments slide past each other, the strain on the cross-bridge is reduced, and step 7 occurs more rapidly. The chemomechanical mechanism above implies that during isometric contraction, cross-bridges remain strongly attached to actin for a relatively long time (>100 ms/cycle). Strongly bound cross-bridges prevent Tm/Tn from returning to its blocked or closed position, maintaining the thin filament in a "switched on" position. In the absence of $Ca^{2+}$, cross-bridge detachment at the end of the cycle allows Tm/Tn to cover the strong myosin binding sites on actin and deactivate the thin filament.

**Cooperativity within the myofilament and calcium-dependence of force**

At resting SL the amount of $Ca^{2+}$ released from intracellular stores during a myocardial twitch is insufficient to saturate thin filament sites, so force and power are submaximal (Fig. 19.). During twitch the estimated ratio of activated crossbridges is only 25 %. Protein function within the thin filament is modulated by several factors, including the binding of myosin to actin, phosphorylation of myofibrillar proteins, and near-neighbor **cooperative interactions** extending from active to inactive regions of the thin filaments. None of these mechanisms is obviously required for myocardial contraction but all of them contribute to the force and speed of contraction.

The isometric force generated by the myocardium is a function of myoplasmic $[Ca^{2+}]$ and their relationship is typically described by the Hill equation: $F\,(Ca^{2+}) = F_0{*}([Ca^{2+}]^{nH} / (Ca_{50}{}^{nH} + [Ca^{2+}]^{nH})$ where F is the steady-state active force, $F_0$ denotes the steady isometric force at saturating $Ca^{2+}$ concentration, nH is the Hill coefficient and $Ca_{50}$ (or $pCa_{50}$) represents the $[Ca^{2+}]$ at which force is half-maximal, i.e. the midpoint of the relation. The Hill coefficient provides an estimate of the minimum number of binding sites involved in the regulation of force and is typically found more than 3 in the cardiac muscle. The discrepancy between the Hill coefficient and the presence of only a single regulatory $Ca^{2+}$-binding site on TnC suggests involvement of positive cooperative mechanism in the activation process.

Increased affinity of TnC binding sites for $Ca^{2+}$ may arise from an increase in either the number of crossbridges bound to actin or the amount of $Ca^{2+}$ bound to regulatory sites along the thin filament. Bremel and Weber were the first to propose that crossbridge binding increases $Ca^{2+}$ binding affinity of thin filaments (Bremel & Weber, 1972). Moreover, recent studies have reported conformational changes in TnC in response to strong crossbridge binding to the cardiac thin filaments (Hannon *et al.*, 1992). Although it is evident from studies (Gordon *et al.*, 2000) that crossbridge binding to thin filaments can increase the apparent $Ca^{2+}$ binding affinity of TnC in the cardiac muscle, it remains to be determined whether this increase in affinity significantly increases the force at any given $[Ca^{2+}]$ or is a consequence of increased numbers of crossbridges bound to the thin filament.

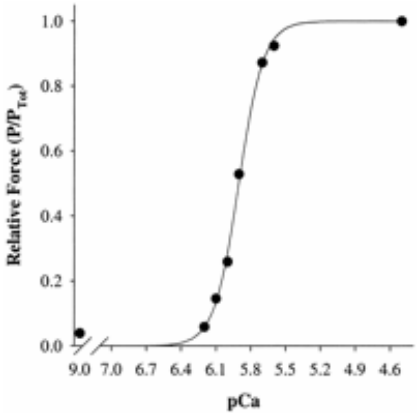



**Figure 19.** Tension-pCa relationship from rat skinned myocardium at 15°C. Force at each submaximal pCa-s was normalized to the maximal force obtained at pCa 4.75 (Moss *et al.*, 2004).

Crossbridge binding to actin also exhibits positive cooperativity as supported by numerous observations. Increased [MgADP] increases the $Ca^{2+}$ sensitivity of force (Lu *et al.*, 1993;Thirlwell *et al.*, 1994) presumably by increasing the number of strongly bound crossbridges at each $[Ca^{2+}]$ and thereby facilitating cooperative recruitment of additional crossbridges to force-generating states (Dantzig *et al.*, 1991). Conversely, an increase in $[P_i]$ reduces the $Ca^{2+}$ sensitivity of force by reversal of the powerstroke and the resultant decrease in the number of strongly bound crossbridges (Dantzig *et al.*, 1992). Unfortunately, the latter chemical interventions have direct effects on the developed force, too. The N-ethylmaleimide derivate of myosin S-1, NEM-S1, is devoid of this problem binding strongly to the thin filament without developing force. In biochemical experiments, NEM-S1 facilitates crossbridge binding to regulated thin filaments (Nagashima & Asakura, 1982) and potentiates

ATPase activity in the presence of $Ca^{2+}$ (Williams, Jr. *et al.*, 1988), supporting the idea that crossbridge binding exhibits positive cooperativity.

A further mechanism to consider is the cooperative signaling within the thin filament. Three-dimensional reconstructions of regulated thin filaments have shown that there are multiple positions of tropomyosin on the thin filament corresponding to the absence of $Ca^{2+}$, $Ca^{2+}$ bound to TnC, and both $Ca^{2+}$ and crossbridges bound to the thin filaments (Craig & Lehman, 2001;Vibert *et al.*, 1997). Such reconstructions provide a compelling context for regulation in which $Ca^{2+}$ and crossbridge-binding act synergistically to effect full activation of the thin filaments (Gordon *et al.*, 2000).

**Frank-Starling Relationship**

Due to the isolated heart experiments of Otto Frank and Ernest Starling it has been known for about a century that increased ventricular volume enhances the systolic performance, thus matching cardiac contractility to hemodynamic load (Fig. 20).

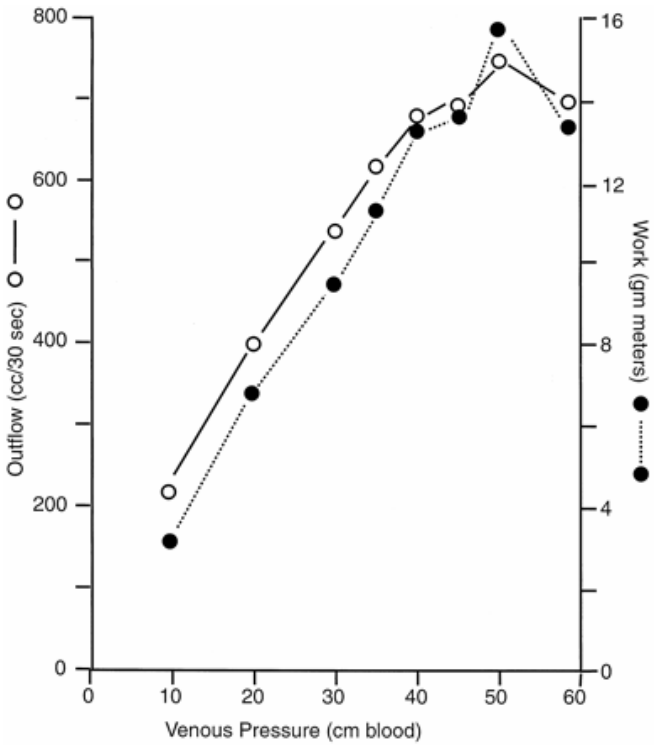



**Figure 20.** Effect of increasing superior vena cava pressure (venous pressure) on cardiac output and left ventricular work, data from (Howell & Donaldson, 1884).

At the level of the myofilaments, increases in sarcomere length within the physiological range are associated with increase in the $Ca^{2+}$ sensitivity of force (Kentish & Wrzosek, 1998), such

that at each submaximal [$Ca^{2+}$], force increases steeply as length is increased. This relationship often cited as Frank-Starling law is widely recognized as a physiologically important characteristics of the cardiovascular system.

At least two different mechanisms may result in augmented active force with increased fibre length. First, enhanced CICR from the SR leads to progressively increasing cytosolic $Ca^{2+}$ concentration $[Ca^{2+}]_i$. Although evidences suggest that CICR is enhanced with muscle length (Fabiato, 1980;Fabiato & Fabiato, 1975) its contribution to the length-dependent activation may be minor at best. This is because immediately after stretch active tension increases, whereas $[Ca^{2+}]_i$ barely changes (Allen & Kurihara, 1982). On the other hand, in experiments on chemically skinned cardiac muscle preparations where the contribution of altered cytosolic calcium-handling to length-activation is negligible the Ca-sensitivity of myofilaments is increased with length (Hibberd & Jewell, 1982). This is demonstrated both by a leftward shift of the force-pCa curve and by the observation that the maximal $Ca^{2+}$-acivated force obtained at saturating concentrations of $Ca^{2+}$ also exhibits length-dependence. In spite of the numerous studies conducted the precise molecular mechanism of length-dependent activation, still to be solved, is likely to be multifactorial.

A possible interpretation for the increase in $Ca^{2+}$-sensitivity may be the stretch-induced increase of $Ca^{2+}$-binding affinity of cardiac troponin C. This proposal is based on experiments with skinned cardiac muscle that showed diminished length-dependent leftward shift of the force-pCa curve following reconstitution with skeletal troponin C (Babu *et al.*, 1988;Gulati *et al.*, 1991). This idea was however challenged by others on the basis of studies using both reconstitution (Moss *et al.*, 1991) and overexpression models (McDonald *et al.*, 1995). Another interesting posing is that the length-dependent activation results from an increase in the fraction of force generating cross-bridges, which apparently increases myofibrillar $Ca^{2+}$-sensitivity. This view is supported by the finding that in the presence of MgADP length-dependent activation is attenuated (Fukuda *et al.*, 2000) most probably due to the diminished number of cross-bridges in the weakly binding state (Fukuda *et al.*, 1998;Shimizu *et al.*, 1992).

The lateral separation of thick and thin filaments (often referred as interfilament lattice spacing) is also considered as an important factor that influences the fraction of force-generating cross-bridges by the modulation of myosin attachment to actin (Fuchs & Wang, 1996;Fukuda *et al.*, 2000;McDonald & Moss, 1995). Increased sarcomere length is associated with reduced lattice spacing as revealed by recent structural analysis on skinned and intact cardiac muscle preparations using synchrotron X-ray (Irving *et al.*, 2000). Thus, changes of

lattice spacing may indeed be an important determinant of the length-dependent activation as supported by an earlier mathematical model analysis, too (Ishiwata & Oosawa, 1974). The mechanism, however, by which the reduction in lattice spacing results in increased $Ca^{2+}$-sensitivity is still ambiguous as pointed out by recent works (Konhilas *et al.*, 2002a;Konhilas *et al.*, 2002b;Konhilas *et al.*, 2003). These studies provided evidences in various experimental models that the lattice spacing and $Ca^{2+}$-sensitivity are not well-correlated suggesting additional mechanism(s) involved in length-dependent activation compared to activation induced by osmotic compression. For instance, changes in thick and thin filament geometry as well as contractile protein modulation by phosphorylation may act as important factors in the determination of $Ca^{2+}$-sensitivity. Phosphorylation of MLC2 increases myofibrillar $Ca^{2+}$-sensitivity (Clement *et al.*, 1992;Craig *et al.*, 1987), while dephosphorylation of TnI with stretch increases length dependent activation (Konhilas *et al.*, 2003). Recent results have led to a new hypothesis in which length dependence is a consequence of subtle changes in crossbridge orientation at long lengths, which increases the probability of crossbridge binding (Moss *et al.*, 2004). Additionally, several findings have suggested a special role of the giant sarcomere protein titin by sensing stretch and promoting actomyosin interaction (Cazorla *et al.*, 1999;Cazorla *et al.*, 2001;Fukuda *et al.*, 2001). The underlying mechanisms may involve the titin-based modulation of the lattice spacing and/or the strain-dependent changes in the thick filament structure as reviewed by Fukuda and Granzier (Fukuda & Granzier, 2004).

**Regional differences of physiological properties in the heart**

Numerous studies on different species and human showed that ventricular epicardium and endocardium differ with respect of **electrophysiological characteristics**. The epicardial cells exhibit shorter and spike-and-dome shaped action potential (AP) while the endocardial myocytes have longer AP with a less prominent repolarisation in phase 1 (Fig. 21).

The substantial differences in the duration and shape of the myocardial action potential is mostly attributed to the higher density of the transient outward current ($I_{to}$) in the epicardial layer compared to that in the endocardial one (Fedida & Giles, 1991;Litovsky & Antzelevitch, 1988;Wettwer *et al.*, 1994). Similarly, magnitude of $I_{to}$ was found to be responsible for the regional differences demonstrated in rat ventricular cells where the current density of $I_{to}$ was larger in the left ventricular free wall cells than in cells from the apex or the septum (Benitah *et al.*, 1993). In contrast, no such regional differences in the characteristics of the L-type calcium current ($I_{Ca\text{-}L}$), a main determinant of the cardiac excitation-contraction coupling could be demonstrated (Bryant *et al.*, 1997).

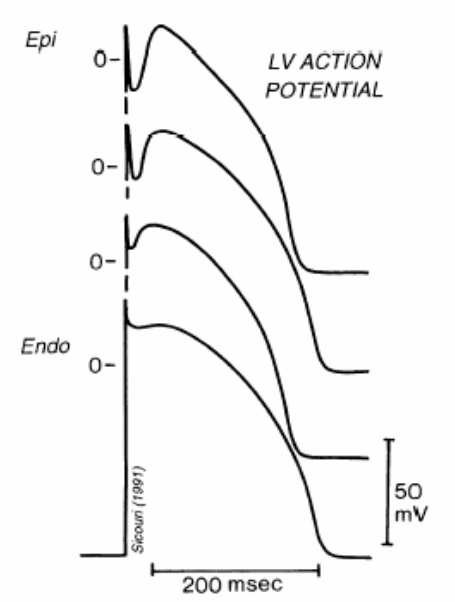



**Figure 21.** Epicardial versus endocardial action potential patterns. The prominent spike-and-dome pattern in the epicardial cells is the result of the activity of the early repolarizing potassium current ($I_{to}$) (Opie, 1998).

Studies on the **passive mechanical properties** of the myocardium found also regional differences. Following moderate stretch intact preparations from the endocardial myocardium were stiffer, i.e. developed higher passive tension than those from the epicardial layer. This feature was observed both in strips from bovine ventricle (Kang *et al.*, 1996) and in intact myocytes from rat, ferret and also guinea pig ventricle (Cazorla *et al.*, 1997;Cazorla *et al.*, 2000b). In addition, previous works of our group on skinned guinea pig myocytes observed regional differences of the **active mechanical properties**. Stretching myofilaments from 1.9 μm to 2.3 μm SL resulted in a more pronounced calcium-sensitization in the sub-endocardial than in the sub-epicardial layer of the myocardium as indicated by the difference in $\Delta pCa_{50}$ (0.19±0.01 and 0.09±0.01, respectively, unpublished data). According to earlier studies the **mechanical load** on myocytes, i.e. the resultant of forces that determine pre-strain of the myofilaments, is not homogenous through the ventricular wall. Streeter et al. suggested that at the end of the diastole it is the myocytes in the sub-endocardial layer that are subjected to the highest longitudinal and circumferential strains (Streeter, Jr. *et al.*, 1970). Transmural differences in the **end-diastolic sarcomere length** (1.91 μm in the sub-epicardium and 1.78 μm in the sub-endocardium) (MacKenna *et al.*, 1994;MacKenna *et al.*, 1996;Rodriguez *et al.*, 1993) as well as in the percentage of length shortening during systole (10-20 % in the sub-epicardium and 20-40 % in the sub-endocardium) (Naito *et al.*, 1996;Streeter, Jr. *et al.*, 1969)

were also reported. According to an interesting proposal the epicardium develops greater specific force than endocardium during early stages of systole, and as a result the endocardium is stretched (Davis *et al.*, 2001). The resulting stretch activation of endocardium would then produce a delayed force that contributes to the generation of myocardial power during ejection. This hypothesis was based on the observation that epicardium exhibits greater phosphorylation of the myosin regulatory light chain (RLC), which the authors associate with greater force at submaximal [$Ca^{2+}$]. On the other hand, lower level of RLC phosphorylation in endocardium was associated with a greater stretch activation.

## Neurohormonal regulation of myocardial performance

### Sympathetic influences

In brief, sympathetic stimulation of the myocardium, either humoral or neural, results in positive inotropic, lusitropic, chronotropic and dromotropic effects. $\beta_1$-receptor activation, mainly induced by epinephrine, causes G-protein mediated increase in cAMP concentration. Then, activation of the protein kinase A (PKA) leads to subsequent phosphorylation of both $Ca^{2+}$-handling (L-type Ca-channel, phospholamban, ryanodine receptor, etc.) and myofibrillar regulatory proteins (troponin-I, myosin binding protein-C, etc). Norepinephrine liberated from nerve terminals acts on $\alpha_1$-adrenergic receptors and has a modest inotropic effect by increasing cytosolic calcium concentration.

Several additional aspects of intracellular β-adrenergic signalling are to be mentioned in detail here. Enhanced PKA function requires elevated intracellular cAMP concentration $[cAMP]_i$. In theory, both increased adenylate cyclase activity and phosphodiesterase inhibition may result in elevated $[cAMP]_i$. Although PDE III is often cited as the mediator of the positive inotropic effect in the heart, other PDEI isoformes (PDE I, II and IV) are also present and therefore may contribute to $[cAMP]_i$ regulation in the human and guinea pig myocardium (Reeves *et al.*, 1987). For instance, a prominent role of the PDE IV on the regulation of $[cAMP]_i$ was also proved in rat isolated ventricular myocytes. In rat and rabbit heart marked increase in $[cAMP]_i$ could be achieved only if at least two PDE isoforms were inhibited simultaneously (Kelso *et al.*, 1995;Mongillo *et al.*, 2004;Shahid & Nicholson, 1990;Verde *et al.*, 1999). Accordingly, PDE inhibitors with poor selectivity are more potent elevators of $[cAMP]_i$ (Cone *et al.*, 1999;Shakur *et al.*, 2002). Increasing body of evidence supports the importance of functional compartmentation of cAMP-mediated signalling pathways (Mongillo *et al.*, 2004;Steinberg & Brunton L.L., 2001). Since PDEs play a critical role limiting the diffusion of cAMP within the cell, inhibition of their activity results in reduced cAMP

compartmentation (Jurevicius & Fischmeister, 1996) and allows effective interaction between different PDE isoforms.

**Parasympathetic influences**

Effects of the parasympathetic system reach the heart through vagal stimulation and are transmitted by acetylcholine that binds muscarinic receptors ($M_2$) in the myocardium. Major effects involve bradycardia and a modest negative inotropy. Activation of $M_2$ receptors indirectly inhibits the effects of sympathetic stimulation by hampering cAMP formation. Moreover, direct effects of cGMP also play a role in parasympathetic modulation.

**Other hormonal influences**

The proposed mechanism of **endothelin**-mediated increase in contractile force in myocytes involves a G-protein – phospholipase C (PLC) – protein kinase C (PKC) pathway. PKC stimulation increases $H^+$-efflux via the $Na^+/H^+$-exchanger. The intracellular alkalosis causes sensitization of cardiac myofilaments to $Ca^{2+}$. Influx of $Ca^{2+}$ via the $Na^+/Ca^{2+}$-exchanger may also contribute to endothelin-induced positive inotropy. Thus, in the left ventricular myocardium endothelin causes an increase in contractile force. At the same time it increases the time to reach 50% relaxation (Goldberg *et al.*, 2000;Pieske *et al.*, 1999). Effects of endothelin on human heart were shown to be small in comparison with those of adrenergic stimulation (Kaumann *et al.*, 1999). Initial observations emphasized negative inotropic effect of **nitrogen oxide (NO)**. This idea was supported by the finding that phosphorylation of troponin I by the NO-induced cGMP-dependent protein kinase results in a reduction in myofilamentary calcium sensitivity. Recently, favourable effects of NO on cardiac energetics left ventricular diastolic distensibility and endomyocardial fibrosis that clearly outweigh the negative inotropy led to the revaluation of NO's role in the failing myocardium (Paulus & Bronzwaer, 2004). Studies in intact animals and humans have demonstrated that **thyroid hormones** enhance myocardial contractility. The rates of ATP hydrolysis and of $Ca^{2+}$ uptake by the SR are increased in hyperthyreoidism. Thyroid hormones increase protein synthesis in the heart resulting in hypertrophy as well as affect the composition of myosin isoenzymes by increasing the ratio of those with the greatest ATPase activity. Vasodilation and decreased total peripheral resistance in hyperthyroid subjects result in increased cardiac output. Characteristically, these patients exhibit tachycardia, palpitations and arrhythmias, such as atrial fibrillation. Interestingly, thyroid hormones were shown to increase the density of β-adrenergic receptors in the cardiac tissue thus increasing the sensitivity of the myocardium to

the already increased sympathetic neural activity. **Insulin** has a prominent, direct positive inotropic effect on the heart. This effect can be observed even under normoglycaemic conditions and in the presence of β-adrenergic receptor antagonists. Moreover, β-adrenergic blockade potentiates the positive inotropic effect of insulin. The enhancement of contractility cannot be explained satisfactorily by the concomitant augmentation of glucose transport into the myocytes. **Glucagon** has potent positive inotropic and chronotropic effects on the heart that closely resemble those of the catecholamines. Although glucagon does not bind to β-adrenergic receptors eventually it activates the adenylate-cyclase. The subsequent rise in cAMP then increases $Ca^{2+}$ influx into the myocyte, facilitates $Ca^{2+}$ release and reuptake by the SR and decreases myofilament $Ca^{2+}$ sensitivity to facilitate relaxation.

The extracellular **adenosine 5'-triphosphate** acting on P2-purinergic receptors also influences myocyte function. ATP is degraded to adenine nucleotides and nucleosides so that the response of cardiomyocytes should be the integral of these multiple compounds. So far ionotropic $P2X_{1\text{-}7}$ and metabotropic $P2Y_{1,2,4,6,11}$ receptors have been cloned and their mRNA found in cardiomyocytes (reviewed in (Vassort, 2001)). However, precise functions of these proteins are not clear yet. ATP and its derivatives exert positive inotropy in rat papillary muscle (Legssyer *et al.*, 1988) and enhance cell shortening in various mammalian ventricular myocytes (Danziger *et al.*, 1988;Podrasky *et al.*, 1997;Scamps *et al.*, 1990). Beneficial effect of adenine nucleotides on contractility was also observed in postischemic papillary muscle. Beside the obvious role of ATP in the replenishment of intracellular high-energy phosphate stores (Shah *et al.*, 1974) positive inotropy in diseased cells is also attributable to an increase in $I_{Ca\text{-}L}$ (Hirano *et al.*, 1991;Scamps *et al.*, 1992;Scamps *et al.*, 1993). The spread of electrical activation should be reduced in the presence of ATP both by $I_{Na}$ depression and cell uncoupling after intracellular $Ca^{2+}$ release and acidosis. ATP was also shown to produce transient tachycardia at low doses or to slow heart at higher doses. Moreover, at higher doses it also induces atrioventricular and His bundle blocks with transient ectopic beats of AV junctional origin at the onset of the AV blocks (Fischer & Frohlicher, 1948). Interaction between the effects of catecholamines and ATP on $I_{Ca\text{-}L}$ (De Young & Scarpa, 1987;Zheng *et al.*, 1992) supported the hypothesis that the release of ATP under pathophysiological conditions could be arrhythmogenic (Kuzmin *et al.*, 1998;Scamps & Vassort, 1990). Interestingly, extracellular ATP markedly inhibits glucose transport (Fischer *et al.*, 1999). In addition, autocrine release of ATP mediated by stress was shown to induce hypertrophy of cultured rat neonatal myocytes, an effect that involved P2Y receptors and the activation of stretch-induced ERK (Uozumi *et al.*, 1998).

## Heart failure

During the last decades numerous attempts of different approaches tried to define heart failure. In the clinical practise heart failure is suitably defined as a clinical syndrome characterized by exertional symptoms and caused by heart disease (Opie, 1998). However, because of the inadequacy of this definition for experimentalists and the crucial role of depressed contraction-relaxation cycle as a result of diseased state, Opie suggests the following: "LV failure exists when the venous filling pressure is increased because of relaxation abnormalities or when myocardial ejection is impaired because the inotropic state is depressed".

Clinical picture of the fully developed heart failure is an admixture of three separate components. First, inadequate emptying of the atria results in **increased venous pressure** and pulmonary congestion. Second, **systolic failure** is characterized by decreased force development and poor inotropic reaction in response to exercise that results in impaired peripheral perfusion and muscular fatigue. Third, important **neurohumoral changes** occur in order to maintain the normal blood pressure. Sympathetic tone is increased that leads to increased vascular resistance and afterload. Activation of the renin-angiotensin-aldosterone system results in fluid retention with peripheral congestion and oedema so that preload is further increased. Indeed, excessive activation of the initially compensatory mechanisms further burdens the myocardium and rushes the heart into a vicious circle with poor prognosis.

Heart failure may develop either as a slow, chronic but yet progressive process or as a sudden, acute decline of the heart pump function being a consequence of chronic heart failure or accidental myocardial or hemodynamic alterations. Regarding the differences in the underlying pathophysiological mechanisms and in the therapeutic consequences the two entities are discussed separately below.

### Chronic heart failure

Development of chronic heart failure is a successive, long-term process. Primary alterations evoke complex compensatory response that is capable to maintain satisfactory cardiac function (Opie, 1998). These changes are transitionally useful and beneficial, however, in long-term they *per se* are able to set up a self-supporting process that leads to progressive decline of the myocardial function. The developed vicious circle finally ends up in inadequate cardiac performance manifesting in the typical clinical syndrome of heart failure. In the

followings we summarize the most important compensatory mechanisms of the myocardium in order to overcome the pathological demands.

**Initiating defects and myocardial reactions**

Increased **pressure load** on the left ventricle is most often caused of sustained severe hypertension or stenosis of the aortic valve. In parallel with the higher left ventricular pressure (LVP) – according to Laplace's law – left ventricular wall stress increases, as well. To decrease the wall stress and the consecutively increased oxygen demand, myocardial tissue develops concentric hypertrophy. With wall thickening, despite the increased intraventricular pressure, wall stress decreases and may even get normalized. It is to note that this delicate balance is rather susceptible to ischemia and exercise, moreover, characterised by an increased possibility of abnormal diastolic function. A characteristic triad, namely loss of distensibility, impaired relaxation and decreased early diastolic filling describes the diastolic dysfunction (Opie, 1998). The systolic function is, however, maintained in this phase.

Insufficiency of aortic or mitral valves leads to regurgitation and consequent **volume overload**. As dealing with a continuously increasing stroke volume the heart uses the upper limit of the Frank-Starling mechanism (discussed in details below). For the mechanism of ventricular dilatation "longitudinal hypertrophy" (Grossman *et al.*, 1975) and "slippage" of myocytes were proposed. As a result, enhanced early diastolic feeling accompanied by decreased LV stiffness (Zile *et al.*, 1993) results in improved diastolic function. In response to the increased chamber size a moderate and proportional hypertrophy retains the normal wall stress.

In the third group of defects not hemodynamic or valvular changes, but instead, damage of the myocardium initiates functional alterations. If the cause of the myocardial degeneration can be established (toxin, drug, hypoxia, etc.) the term secondary **cardiomyopathy (CM)** is used while in case no underlying pathology can be clearly identified, primary cardiomyopathy is the appropriate name. *Hypertrophic CM* is characterized by small cavity size and abnormally thick ventricular wall. Although the ejection fraction is high, because of a markedly decreased distensibility of the LV diastolic dysfunction predominates. Studies assume genetic defects (Takeda, 2003) in the background of myocardial hypertrophy. In *dilated cardiomyopathy* pressure generation fails as a consequence of myocardial degeneration caused by known (e.g. alcohol, myocardial infarction) or unknown (most probably viral infection) factors. Decreased ejection fraction results in volume overload and increased wall stress leads to consecutive compensatory hypertrophy, however, inadequate to normalize wall stress. In a rare form a

CM, LV relaxation is mechanically hampered because of e.g. an infiltrative process. In *restrictive cardiomyopathy* the infiltrate replaces the functional myocardium increasing the stiffness of the ventricular wall. Mechanical alterations, for example pericardial constriction, have the same consequences.

**Development of heart failure**

Once the compensating mechanisms reach their limits but provoking pressure or volume overload is still present the myocardium passes from the compensated hypertrophic state to decompensated dilated failure. As soon as hypertrophy is unable to compensate for the pressure or volume overload, the ventricle dilates and wall stress increases. When the reduction in myocardial power output fails to maintain sufficient cardiac output to meet the metabolic demands of the body, heart failure develops (Alpert *et al.*, 2002).

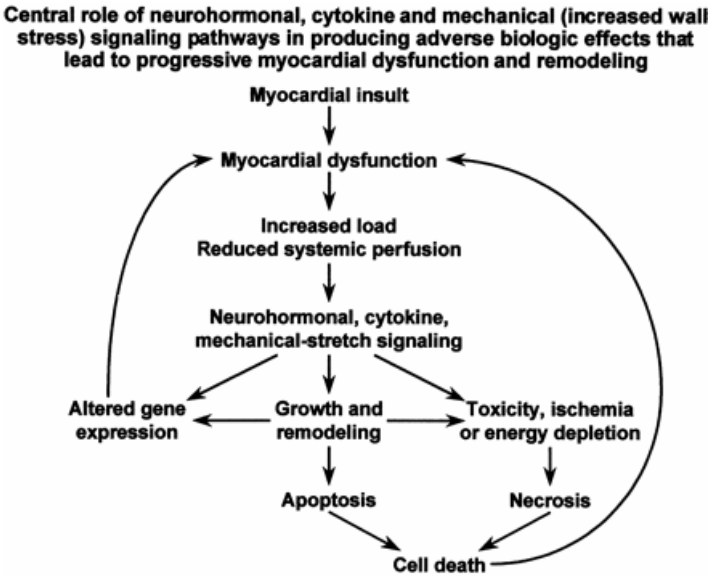



**Figure 22.** Vicious circle that develops in response to myocardial damage. Altered gene expression as well as cell death, either in a regulated apoptotic or an in unregulated necrotic way results in permanently declining myocardial function (Braunwald & Bristow, 2000).

In spite of the enormous number of studies addressing the process of the transition from compensated hypertrophy to decompensated failure this question is still far from being answered. It is now apparent that there is neither a unifying hypothesis to explain divergent findings nor a unitary common molecular alteration to account for the various types of failure (Opie, 1998). Specific and non-specific changes in different models act together to produce

experimental heart failure (Fig. 22). Below we attempt to delineate the most established mechanisms currently believed to be involved in the transition towards heart failure.

**Inadequate blood supply**

One of the first and most attractive hypotheses trying to explain the reasons of decompensation is that the vascular system is unable to keep up with the growing mass of hypertrophied myocardium causing a relative diminution in blood supply (Linzbach, 1960). The critical limit of heart weight seems to be approximately 500 g. In addition to the increased oxygen demand of the individual hypertrophied myocytes, a relative decrease of capillary surface area and increased distance between the capillaries contributes to the inappropriate blood supply of the myocardial tissue (Tomanek *et al.*, 1982).

**Impaired vascular reserve**

An important observation is that in LVH even in the absence of overt coronary artery disease coronary vascular reserve – the ratio of coronary resistance at rest and following maximal dilation – is diminished. This leads to an inadequate circulation during exercise especially in the endocardial layer that is subject to a higher wall stress (Hittinger *et al.*, 1990). Repetitive episodes of ischemia result in focal necrosis and fibrosis. Even if hypertrophy of myocytes regresses due to therapeutic interventions the depositions of collagen cannot be removed maintaining the altered tissue structure and impaired diastolic properties of the myocardium (Krayenbuehl *et al.*, 1983).

**Abnormalities in energy metabolism**

In patients with left ventricular hypertrophy increased oxygen consumption per unit mass of heart was accompanied by high systolic wall stress and impaired LV function (Strauer, 1983). In low-output, systolic heart failure such as dilated CM and ischemic heart disease, the diminished “external work” performed by the left ventricle is at the cost of virtually normal energy consumption. Thus, the dilated failing heart is energy-inefficient (Braunwald & Bristow, 2000).

Due to recently introduced methods such as biophysical tools (NMR, PET) and transgenesis there is a renewed interest in the old hypothesis suggesting energy-starvation of the failing myocardium. (reviewed in (Ingwall & Weiss, 2004). It was shown that [ATP] is ≈25 to 30 % lower in failing human myocardium compared to non-failing (Beer *et al.*, 2002;Starling *et al.*, 1998). Animal studies showed that the decrease in the level of ATP is caused by loss in the

total adenine nucleotide (TAN) pool (Liao *et al.*, 1996;Shen *et al.*, 1999). Interestingly, in the failing heart a mismatch between ATP synthesis and degradation results in a loss of ATP and TAN in spite of normoxic conditions (Bache *et al.*, 1999;Traverse *et al.*, 1999). Why [ATP] decreases by only ca. 25 %, what is the exact mechanism for TAN loss and how long the heart can tolerate the new steady-state remain questions to be answered.

The creatine pool decreases by 60 % in LVH and in the failing heart. The observation originally made in animal models (Zhang & Bache, 1999) was later confirmed also in humans (Nakae *et al.*, 2003). Decreased number of creatine transporters (Neubauer *et al.*, 1999) as well as changes in CK isozyme expression leading to lower [PCr]-to-[total-creatine] ratio (Saupe *et al.*, 2000) may account for a decreased [PCr]. In addition, reduced availability of high-energy phosphate stores may also be a result both of the decreased activity of creatine kinase (Nascimben *et al.*, 1996) and of mitochondrial damage in failing heart (Schultheiss *et al.*, 1995). Consequently, the [PCr]-to-[ATP] ratio is decreased in DCM and heart failure (Conway *et al.*, 1991;de Roos *et al.*, 1992) accompanied by a fairly normal value of the [ATP]-to-[ADP] ratio, however, at lower absolute concentrations. In patients with DCM the [PCr]-to-[ATP] ratio proved to be a more powerful predictor of overall and CV mortality than either the NYHA classification or the LV ejection fraction (Neubauer *et al.*, 1997). Lower [ATP] evokes massive changes in metabolic regulation that involve switch in the isozyme distribution of lactate dehydrogenase (Bishop & Altschuld, 1970), downregulation of enzymes controlling fatty acid oxidation (Garnier *et al.*, 2003;Osorio *et al.*, 2002) and increased glucose uptake and utilization observed in LVH (Allard *et al.*, 1994;Liao *et al.*, 2002).

Decrease in CK reaction velocity (Dzeja *et al.*, 1999) is only partially compensated for by acceleration in adenylate kinase reaction and glycolytic rate in the failing heart. As a consequence, decreased energy reserve contributes to the impaired contractile function. Reduction in the free energy transmitted by ATP slows $Ca^{2+}$ reuptake by the SR and impairs myofilament cross-bridge cycling thereby altering both ventricular relaxation and contraction (Braunwald & Bristow, 2000). Furthermore, dramatically reduced contractile reserve during exercise might lead to acute failure (Ingwall & Weiss, 2004). Additionally, increase in the cytosolic [ADP] by itself is sufficient to slow cross-bridge cycling thereby contributing to diastolic dysfunction (Tian *et al.*, 1997). Through the adenylate kinase reaction elevated [ADP] level induces increase in [AMP]. Elevated [AMP] leads to the activation of AMP dependent protein kinase (AMPK) that remodels metabolic pathways toward greater

metabolic efficiency (Hardie & Carling, 1997) but in contrast provokes also purine and hence TAN loss.

**Ventricular hypertrophy and extracellular remodeling**

Myocardial failure is mostly preceded by the hypertrophy of the myocytes and consequent increase in the muscle mass. This initially adaptive mechanism involves increase in the number of conractile elements, diminution of wall stress and increase in the stroke volume (Grossman *et al.*, 1975;Spann, Jr. *et al.*, 1967). Mediators of the hypertrophic process imply norepinephrine, angiotensin II, aldosterone, endothelin 1, FGF, TGF-β1, TNF-α, IL-1β and G protein 130-signalling pathways. These signals activate various effector molecules, e.g. PKC, MAPKs, Raf-1 kinases that induce fetal gene program and cardiac hypertrophy (Braunwald & Bristow, 2000;Mann, 1999). Despite initially compensatory effects, altered contractile properties caused by the qualitative changes in gene expression in time contribute to the decompensation of cardiac pump function. Complex structural changes within the myocytes (Schaper *et al.*, 1991), in the extracellular matrix, the connective tissue and the vascular system take place simultaneously and are collectively called remodeling.

A main aspect of ventricular remodeling is the marked structural change in the extracellular matrix composition characterised by excessive deposition of collagen. Although the presence of collagen, especially type I collagen in physiological amount helps to prevent myocardium from overstretching its accumulation in forms of perivascular and replacement fibrosis due to either ischemia or metabolic signals (e.g. angiotensin II and TGF-β1) is accompanied by adverse effects. Decreased distensibility and compliance with increased stiffness of the ventricular wall result in elevated oxygen demand at the same end diastolic volume further increasing the relative ischemia and promoting interstitial fibrosis (Mann, 1999;Opie, 1998).

During remodeling, inverse changes in the expression of matrix metalloproteinases (MMPs) and their inhibitors, TIMPs has been reported in failing human heart (Li *et al.*, 1998). The initial enhanced activation of MMPs cause collagen degradation that may allow of myocyte-realignment, LV wall thinning and dilation (Mann, 1999). Later, despite pronounced MMP activation (Janicki *et al.*, 2004), collagen deposition provoked by permanently increased neurohormonal activity dominates in which fibrogenic effects of angiotensin II, endothelin-1, aldosterone and catecholamines are well established (Brilla *et al.*, 1990;Brilla *et al.*, 1994;Guarda *et al.*, 1993;Silver *et al.*, 1990). Increased fibronectin deposition precedes collagen accumulation (Farhadian *et al.*, 1996) and seems to be component of a biological

cascade that includes sequential expression of TGF-β1, fibronectin and finally collagen (Swynghedauw, 1999). In human DCM the extracellular space proved to be enlarged containing increased amounts of laminin, fibronectin, collagen I, III and VI and chondroitin sulfate (Schaper *et al.*, 1995).

**Contractile proteins: expressional and functional alterations**

Clear evidence exists for cardiac **myosin** isoform switch in experimental heart failure (Miyata *et al.*, 2000;Nakao *et al.*, 1997;Schwartz & Mercadier, 1996). In human heart failure a diminution in the amount of α-MHC isoform is accompanied with an increase in the amount of β-MHC isoform (Lowes *et al.*, 1997). In fact, the failing adult heart reverts to a fetal gene profile by downregulating adult gene transcripts rather than by upregulating fetal genes (Razeghi *et al.*, 2001). If translated into protein expression the net effect of this alteration in gene expression appears to be a reduction in myofibrillar ATPase activity and contraction velocity (Pagani *et al.*, 1988). This change theoretically allows the heart to produce force at a lower cost albeit reaching the required tension more slowly (Narolska *et al.*, 2005;van der Velden *et al.*, 1998). The cost of this energetic benefit is decreased contractility and impaired maximal power production (Herron *et al.*, 2001). Ectopic expression of **atrial myosin light chain 1 (ALC1)** isoform in the ventricle partially replacing the endogenous **ventricular myosin light chain 1 (VLC1)** isoform, with subsequent increase in myocardial contractility, has been observed in human heart failure (Hirzel *et al.*, 1985;Morano *et al.*, 1997;Morano, 1999;Schaub *et al.*, 1998). Alterations in other contractile protein isoforms have uncertain functional consequences (Kitsis & Scheuer, 1996;Solaro & Rarick, 1998). Decrease in cardiac **α-actin** is accompanied by an increase in skeletal α-actin in heart failure (Shimoyama *et al.*, 1999). Thin filament proteins, including **troponin I**, are not thought to be modified in chronic overload. Yet, this question remains open due to the repeated finding of decreased ATPase activity without isomyosin shift in the crude myofibrils isolated from failing human myocardium (Leclercq *et al.*, 1976;Pagani *et al.*, 1988). As a possible mechanism, isoform shift in **troponin T**, namely the reexpression of the fetal TnT4c isoform has been observed in end-stage heart failure (Anderson *et al.*, 1991;Anderson *et al.*, 1995). The amount of **myosin binding protein C** remains unchanged in failing human hearts (Morano *et al.*, 1994).

Beside the long-term modulatory mechanisms like gene-expression, alterations of short-term regulatory processes involving phosphorylation of contractile proteins have also been examined in the failing heart. Calcium sensitivity of end-stage failing human myocardium (NYHA class IV) was significantly increased in comparison to donor myocardium (van der

Velden *et al.*, 2000;van der Velden *et al.*, 2003a), an observation that is well correlated with the increased percentage of unphosphorylated TnI in failing hearts (van der Velden *et al.*, 2003b). Additionally, endogenous degree of **ventricular myosin light chain 2 (VLC2)** phosphorylation was found lower in failing than in donor tissue (van der Velden *et al.*, 2003b) yet, failing myocardium exhibited enhanced contractile response to MLC-2 dephosphorylation (van der Velden *et al.*, 2003a).

The existence of Frank-Starling mechanism in heart failure is contradictory. Stretch-induced increase in $Ca^{2+}$-sensitivity was found either preserved (Holubarsch *et al.*, 1996;van der Velden *et al.*, 2000) or blunted (Schwinger *et al.*, 1994) in dilated cardiomyopathy

**Cytoskeletal changes**

Alterations in the concentration of a number of cytoskeletal components were found to be associated to heart failure. The excessive **microtubular polymerisation** in pressure overload has been shown to detrimentally affect systolic function (Tsutsui *et al.*, 1994). It has been proposed that microtubular network imposes a resisitive intracellular load on sarcomere shortening and, when in excess, impedes sarcomere shortening (Tsutsui *et al.*, 1993). It is to note, however, that microtubular modifications are load, chamber and species specific (Walsh, 1997). Concentration of certain endosarcomeric skeletal proteins like **α-actinin** and **myomesin** were found either decreased (Hein *et al.*, 2000) or unchanged (Wang *et al.*, 1999) in heart failure. Data on **titin** expression are conflicting showing either a decrease (Hein *et al.*, 2000) or an increase in titin content in the failing myocardium (Collins *et al.*, 1996;Morano *et al.*, 1994). It is to note, however, that in the latter studies no protease inhibitors were applied. The amounts of other cytoskeletal proteins such as **desmin**, **dystrophin**, **spectrin**, **talin**, **tubulin** and **vinculin** increase in end-stage failing human hearts (Hein *et al.*, 2000;Schaper *et al.*, 1991). In numerous cases in the background of human dilated cardiomyopathy phenotype gene mutation or deletion of different cytoskeletal components were revealed. Such examples are **metavinculin** (Maeda *et al.*, 1997), **dystrophin** (Towbin, 1998), **desmin** (Li *et al.*, 1999), **sarcoglycans** (Barresi *et al.*, 2000) and nuclear-envelope proteins **lamin A and C** (Brodsky *et al.*, 2000;Fatkin *et al.*, 1999). The observation that mutations of cytoskeletal proteins result in IDCM phenotype implies that qualitative or quantitative changes of extrasarcomeric proteins may also contribute to contractile alteration in secondary DCM (Braunwald & Bristow, 2000).

**Electrophysiological alterations**

Frequency of sudden cardiac death (SCD) in patients with heart failure is 6 to 9 fold higher than in the general population and responsible for approximately 50 % of the deaths in this group. Because of the electrical instability of the failing heart for background of this phenomenon lethal cardiac arrhythmias (such as ventricular tachycardia and fibrillation) are most commonly suggested. Numerous evidences have been raised to support this idea (reviewed in (Tomaselli & Marban, 1999;Tomaselli & Zipes, 2004).

Alterations in $I_{Na}$ are undoubtedly present and may play an important role in the development of arrhythmias, however, the detailed changes vary with the cause of HF (Tomaselli & Zipes, 2004). Downregulation of $I_{to}$ is the most consistent finding both in animal models (Kaab *et al.*, 1996;Rozanski *et al.*, 1997) and in failing human heart (Beuckelmann *et al.*, 1993;Wettwer *et al.*, 1994). As $I_{to}$ profoundly influences phase 1 repolarisation and thus the level of the AP plateau, consequently, it crucially affects also the subsequently activated currents.

Action potential (AP) prolongation is a characteristic feature of failing myocytes (Akar & Rosenbaum, 2003;Tomaselli & Marban, 1999). Thus, potassium currents, as major determinants of cardiac repolarisation are reasonably subjects for intensive studies. The delayed rectifier potassium current ($I_K$) plays a decisive role in the phase 3 repolarisation. Reduced $I_K$ density, altered kinetics and downregulation of both $I_K$ components ($I_{Ks}$ and $I_{Kf}$) have been reported in different animal models (Furukawa *et al.*, 1993;Tsuji *et al.*, 2000). The inward rectifier potassium current ($I_{K1}$), setting the resting membrane potential, is significantly reduced at negative voltages (Beuckelmann *et al.*, 1993). Nevertheless, changes in both $I_K$ and $I_{K1}$ are far not as unambiguous as those in $I_{to}$ and call for further studies. Expression and function of $Na^+$-$K^+$ ATPase is decreased in failing heart as suggested by the majority of observations (Dhalla *et al.*, 1991;Houser *et al.*, 1981). The consecutive reduction in the outward repolarizing current and increase in the intracellular [$Na^+$] contribute to prolonged AP and enhanced reverse mode of NCX, respectively. Additionally, albeit prolongation of the AP is likely to be necessary to produce ventricular arrhythmias it is the accompanying temporal and spatial dispersion of the repolarisation that determines whether arrhythmia eventuates or not (Tomaselli & Zipes, 2004).

**Defective calcium handling**

For several features of HF, including decreased developed force, prolongation of relaxation and blunted force-frequency relationship, among others, altered $Ca^{2+}$ handling in the

myocytes can also be accounted. Apparently, defective $Ca^{2+}$ homeostasis within the myocardium is the primary reason for SCD in heart failure (Tomaselli & Zipes, 2004). Due to its central role in the cardiac contraction $Ca^{2+}$ is able to influence both the mechanical and the electrophysiological properties of the myocardium. Density of the L-type $Ca^{2+}$ current is most often found unchanged (Beuckelmann & Erdmann, 1992;Mewes & Ravens, 1994), but its augmentation by β-adrenergic stimulation is attenuated (Ouadid *et al.*, 1995). As basic electrophysiological feature, whole cell current decay of $I_{Ca\text{-}L}$ is slower (Ryder *et al.*, 1993) most probably due to an elevation of availability and open probability of L-type calcium channels within active sweeps (Schroder *et al.*, 1998).

Decreased amplitude as well as reduced rate of decay of the Ca transient was observed in intact preparations and isolated cells (Gwathmey *et al.*, 1987;McIntosh *et al.*, 2000;O'Rourke *et al.*, 1999). However, because of marked regional differences within the myocardium, comparisons of Ca transient characteristics demand prudence (Laurita *et al.*, 2003;McIntosh *et al.*, 2000). In several animal models upregulation or re-expression of T-type Ca current ($I_{CaT}$) was shown (Laurita *et al.*, 2003;Nuss & Houser, 1993). In contrast $I_{CaT}$ could not be detected neither in normal nor in failing human ventricles therefore its contribution to the development and progression of heart failure in humans is rather unlikely (Beuckelmann *et al.*, 1991;Beuckelmann & Erdmann, 1992).

Numerous studies confirmed increased NCX functional capacity, mRNA and protein content as well as lower activity of SERCA-2a (de Tombe, 1998). Although SERCA-2a and PLB mRNA levels are consistently found decreased, several reports showed no change in the SERCA-2a and PLB protein quantity in failing human heart (Flesch *et al.*, 1996;Linck *et al.*, 1996;Meyer *et al.*, 1995;Schwinger *et al.*, 1995). Decreased SERCA-2a function (Beuckelmann *et al.*, 1995;Flesch *et al.*, 1996;Hasenfuss *et al.*, 1992;Linck *et al.*, 1996) has major consequences in the failing myocardium. First, increase in diastolic $Ca^{2+}$ concentration (Beuckelmann *et al.*, 1992) leads to improper relaxation. Second, despite no significant changes in calsequestrin protein levels (Arai *et al.*, 1993;Meyer *et al.*, 1995), $Ca^{2+}$ sequestration in the SR is defective. Indeed, a central factor limiting $Ca^{2+}$ transient amplitude in HF is a decrease of SR $Ca^{2+}$ content (Lindner *et al.*, 1998;Piacentino, III *et al.*, 2003). These alterations are partially compensated for by enhanced NCX expression and function (Tomaselli & Marban, 1999). Increased extrusion of $Ca^{2+}$ from the cytosol towards the extracellular space may also be responsible for decreased $Ca^{2+}$ pool in the SR leading to reduced $Ca^{2+}$ release during systole (de Tombe, 1998). In addition, a prominent increase in the function of the electrogenic NCX contributes significantly more depolarizing current thus

prolonging the late repolarisation phase of the AP and increasing the propensity for arrhythmias triggered by DAD and EAD (Lehnart *et al.*, 2004). In HF $Ca^{2+}$ release from the SR is also defective. The RyR macromolecular complex includes PKA, phosphatases (PP1 and PP2a), and FKBP12.6 (also called calstabin). In HF the RyR is hyperphosphorylated by the PKA attributed to hyperadrenergic state and loss of RyR-associated phosphatases (despite increased global myocyte phosphatases). Phosphorylation of RyR results in FKBP12.6 dissociation from the channel and altered RyR gating (Marx *et al.*, 2000;Reiken *et al.*, 2003). The altered RyR phosphorylation hypothesis is supported also by the results of single-channel recordings that showed increased overall RyR open probability in HF. At the cellular level this alteration of the RyR function contributes to both increased diastolic SR $Ca^{2+}$ leak and reduced SR $Ca^{2+}$ content (Bers *et al.*, 2003).

**Neurohormonal changes**

Recently, several investigations as well as the success of newly introduced (ACEIs, ARBs) or reassessed therapeutic approaches (β-blockers) revealed the flavour and emphasized the importance of functional alterations of neurohormonal regulatory systems in heart failure (Fig. 23). As a general view, short-term activation of these systems is clearly adaptive, however, persistent activation leads to maladaptive and detrimental effects on cardiac function (Braunwald & Bristow, 2000). Despite the complexity of changes and the partially controversial feature of findings several alterations are well established and considered to be of crucial importance.

The "adrenergic hypothesis", formulated in the early 1980's, was based on the observation that a chronically increased adrenergic drive contributes to the loss of myocardial reserve and progression of LV dysfunction through desensitization of signal transduction and adverse biologic affects on cardiac myocytes (Bristow, 1997). Indeed, since then numerous studies have confirmed the pivotal role of increased *β-adrenergic system* activity in the development of failing phenotype. Peripheral hypotension promotes enhanced release and maintains persistently high circulatory levels of catecholamines. As a consequence, by the selective downregulation of $\beta_1$-receptor subtype, β-receptor signalling pathways remove approximately 50-60% of their functional capacity (Bristow, 1997). Interestingly, $\beta_2$-receptor levels remained unchanged in most of the studies. In addition, functional receptors are desensitised through phosphorylation by G protein-coupled receptor kinases and by PKA or by an β-arrestin mediated mechanism. Moreover, increase in $G\alpha_i$ level and diminished expression of

$G\alpha_s$ may also contribute to decreased catecholamine responsiveness (Lohse *et al.*, 2003). Because of enhanced adrenergic drive intracellular [cAMP] remains increased (Bristow, 1997) but myocardial reserve and exercise capacity are attenuated (Fowler *et al.*, 1986). Detrimental effects of excessive β-adrenergic activation are mostly attributed to $\beta_1$-receptor stimulation and consequent PKA phosphorylation of target molecules (Lohse *et al.*, 2003). A most prominent cardiac target of PKA is phospholamban. Studies with PLB knock-out mice, however, showed that inhibition of PLB activity (similarly to the effect of PKA phosphorylation) has rather beneficial than unfavourable consequences (del Monte *et al.*, 2002;Hoshijima *et al.*, 2002). Recently, this conclusion has been questioned by the findings that inactivating mutations of PLB resulted in heart failure (Haghighi *et al.*, 2003;Schmitt *et al.*, 2003). These later findings call for caution when extrapolating results obtained in mouse models to the human.

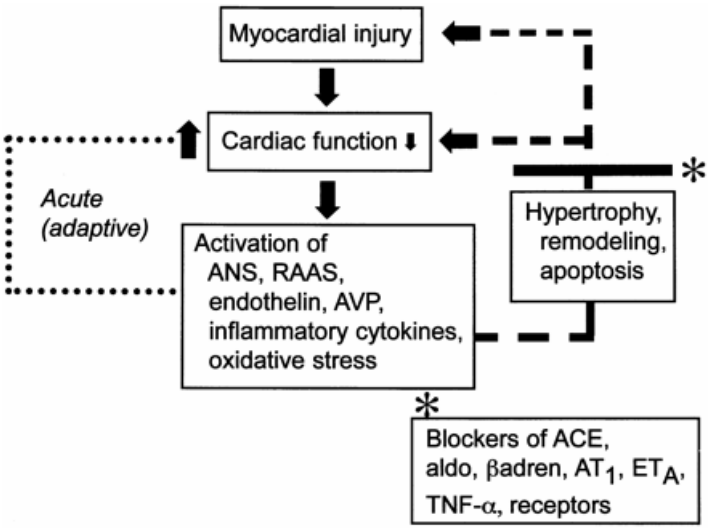



**Figure 23.** Interplay between cardiac function and neurohumoral and cytokine systems in chronic heart failure (ANS-sympathoadrenal system; RAAS-renin-angiotensin-aldosterone system; βadren-β-adrenergic receptor; AT1-angiotensin type 1 receptor; $ET_A$-endothelin type A receptor (Braunwald & Bristow, 2000).

In HF density of L-type $Ca^{2+}$ channel is reduced but due to the increased open probability basal L-type calcium currents are maintained (Chen *et al.*, 2002). The role of PKA-hyperphosphorylation induced FKBP12.6 dissociation from RyR in the generation of diastolic $Ca^{2+}$ leak and DADs is well established. A further support for this hypothesis was provided by the recent finding that the experimental drug JTV519 prevents arrhythmias and sudden cardiac death in FKBP12.6$^{+/-}$ haploinsufficient mice but not in FKBP12.6$^{-/-}$ knock-out mice via rebinding of FKBP12.6 to RyR (Wehrens *et al.*, 2004). Nevertheless, it is presumable that

elevated diastolic calcium concentration due to altered channel function may act as a final common pathway in the failing cells. In addition, functional defects in the intracellular $Ca^{2+}$ cycling become exaggerated during β-adrenergic stimulation, activating inward depolarizing currents and DADs as a likely trigger mechanism for cardiac arrhythmias (Lehnart *et al.*, 2004). $Ca^{2+}$ sensitivity of myofilaments is decreased by PKA through the phosphorylation of cTnI (Wattanapermpool *et al.*, 1995) and – presumably - MyBP-C (Winegrad, 1999). Recently, increased global protein phosphatase (PP) activity in heart failure was suggested (Neumann, 2002). Reduced expression of protein phosphatase inhibitor-1 observed in human heart failure (El-Armouche *et al.*, 2004) may explain enhanced PP effects. In addition, expression of constitutively active PPI-1 was able to rescue the function and β-adrenergic responsiveness of failing cardiac myocytes (Carr *et al.*, 2002).

A critically important aspect of enhanced β-adrenergic stimulation is its effect on myocyte loss. $Ca^{2+}$ overload causes damage in functional and structural elements by the activation of proteases (e.g. calpains) (Barta *et al.*, 2003;Papp *et al.*, 2000). Norepinephrine has been shown to be cytotoxic to cultured myocardial cells in concentrations that may be relevant in the failing heart (10-100nM)(Mann *et al.*, 1992). Of note is the observation that β-adrenergic stimulation may induce apoptosis. Proapoptotic effect was attributed to $\beta_1$-receptor mediated signalling and was dependent on reactive oxygen species. A role for CaMK activation was also raised. In contrast, $\beta_2$-receptors proved to possess antiapoptotic properties (Communal *et al.*, 1999;Remondino *et al.*, 2003;Zhu *et al.*, 2003).

Increased functional activity of the *renin-angiotensin-aldosterone system (RAAS)* is also characteristic in heart failure. Harmful effects of elevated circulating and tissue angiotensin II concentrations are numerous: myocyte hypertrophy and apoptosis, interstitial fibrosis, vascular and cardiac remodeling. Increased secretion of aldosterone stimulated by ATII facilitates cardiac remodeling and collagen deposition (Weber, 1997) leading to increased stiffness and thus impaired filling of the ventricles. Aldosterone was shown to decrease $I_{to1}$ density and increase $I_{Ca\text{-}L}$ density in the myocardium (Benitah *et al.*, 2001). Angiotensin II has also been implicated in the modulation of ionic currents, e.g. it decreases the amplitude of $I_{to}$ (Yu *et al.*, 2000), increases peak $I_{Ca\text{-}L}$ density (De Mello & Monterrubio, 2004;Kass & Blair, 1981) and increases both the activity and the mRNA exprression of the NCX (Krizanova *et al.*, 1997). In clinical studies inhibitors of the RAAS (ACEIs, ARBs, aldosterone antagonists) have all been found to be valuable in the management of HF (Pfeffer *et al.*, 2003;Pitt *et al.*, 1999;The CONSENSUS Trial Study Group, 1987). Because of the changes in adrenergic

activation observed after the administration of RAAS inhibitors an apparent interaction between the β-adrenergic system and the RAAS was assumed (Francis *et al.*, 1993;Gilbert *et al.*, 1993).

High level of circulating *arginine vasopressin* in heart failure contributes to increased preload (by water retention) and afterload (by vasoconstriction). Numerous signaling molecules, e.g. ATII, AVP, norepinephrine and IL-1 facilitate the secretion of *endothelin-1*. ET-1 is the most potent endogenous vasoconstrictor known exerting its effect through ET-A receptors. In addition, the contribution of ET-1 to pathological remodeling is also presumable (Kirchengast & Munter, 1999). *Nitric oxid (NO)*, a powerful vasodilator, is produced in the vascular endothelium. Unfortunately, vascular response to NO is attenuated in HF further enhancing predominance of vasoconstrictive effect.

**Role of cytokines**

All cell types residing in the normal heart are able to express inflammatory mediators, although not in a constitutive manner (Kapadia *et al.*, 1997). The possible role of the inflammatory mediators in the development of heart failure was raised by a number of clinical and experimental observations. First, many known aspects of the syndrome of HF, such as LV dysfunction and CM in humans, activation of the fetal gene program and myocytes apoptosis experimentally can be explained by the biological effects of cytokines (Mann, 2002). Second, expression pattern of cytokines in HF is similar to that of the classic neurohormones (e.g. ATII or NE) (Mann, 1999). TNF-α, IL-1β and IL-6 are persistently overexpressed in the failing heart (Torre-Amione *et al.*, 1996b) and their expression levels are in direct relation to the NYHA functional classification. Moreover, cytokines are activated already in NYHA class II heart failure, thus earlier than the classical neurohormones (Torre-Amione *et al.*, 1996a). In HF, circulating levels of cytokines (e.g. TNF, IL-6, sTNFR1 and sTNFR2) also have prognostic importance and are highly predictive of adverse outcomes (Rauchhaus *et al.*, 2000). Third, evidences support interactions between classic neurohormonal and cytokine systems. Pathophysiologically relevant concentrations of ATII are able to evoke cytokine synthesis through an NK-κB-dependent pathway (Mann, 2002) in accordance with the finding that ATII receptor type 1 antagonists consistently decreased circulating levels of TNF and cell adhesion molecules ICAM-1 and VCAM-1 in HF patients (Gurlek *et al.*, 2001). Interestingly, $\beta_1$-selective blockade of the adrenergic system prevented the proinflammatory mediator expression in a postinfarct model of LV remodeling, too (Prabhu *et al.*, 2000).

Recent hypotheses presume that acute and chronic elevation in cytokine concentration levels within the heart has distinct and partially adverse consequences. Acutely, cytokines modulate cardiac contractility, affect myocytes survival and apoptosis regulation initiating the restoration of function and wound healing while chronically, basically unfavourable features come to the front that contribute to myocyte phenotype transition, cardiac and vascular regeneration and ECM alteration causing eventually excessive chamber dilation and depressed cardiac pump function (Nian *et al.*, 2004).

Cellular remodeling is determined, at least in part, by the net balance between paradoxical effects of cytokines on myocyte survival and apoptosis. Cytoprotective effects of TNF-α may converge partially through NK-κB and activate cytoprotective gene expression (Malinin *et al.*, 1997;Pimentel-Muinos & Seed, 1999). Contrarily, TNF ligand binding to TNF-R1 and 2, members of the so-called "death receptors" family activates caspases thus promoting apoptotic events in the cell.

Long-term elevation in cytokine levels results in collagen deposition and interstitial fibrosis. Initially, TNF-α and IL-1β upregulate matrix metalloproteinases (MMPs) leading to increased collagen degradation. In addition, after myocardial injury short-term maximal concentration values of TNF-α, IL-1β, IL-6, TGF-β1 and are closely correlated to the subsequent deposition of type I and III collagens, especially TGF-β3 appearing to play an important regulatory role in late collagen accumulation (Deten *et al.*, 2001;Yue *et al.*, 1998). ATII-mediated collagen deposition may also be facilitated by AT1 receptor upregulation evoked by TNF-α (Peng *et al.*, 2002).

Vasodilator peptides, such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are overexpressed in chronic HF (Braunwald & Bristow, 2000). ANP and BNP are produced by overloaded ventricles (Drexler *et al.*, 1989;Tsuchimochi *et al.*, 1988;Vanderheyden *et al.*, 2004) and actually considered to be the best biological marker for ventricular overload (Takahashi *et al.*, 1992;Vanderheyden *et al.*, 2004). In contrast to cytokines discussed in the previous paragraph, ANP and BNP mediate a mostly beneficial, counterregulatory effect through vasodilation and reduced sodium reuptake, as well as renin and aldosterone secretion.

**Apoptosis**

Apoptosis is an energy requiring active form of cell death orchestrated by a complex and precisely regulated genetic program (reviewed in (Gustafsson & Gottlieb, 2003;Sabbah,

2000). Previous observations suggested its role and possible importance in a variety of cardiovascular diseases, including also human end-stage heart failure (Narula *et al.*, 1996;Olivetti *et al.*, 1997). A number of factors that either evoke cardiac dysfunction or are activated as compensatory mechanism in HF may initiate apoptosis (Fig. 24).

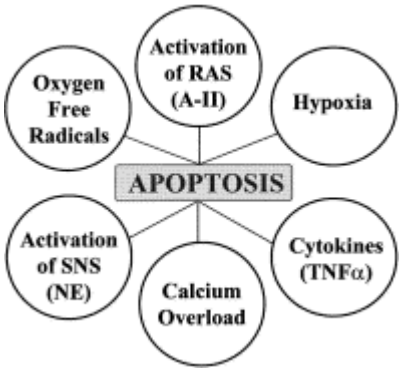



**Figure 24.** Pathophysiologic factors implicated in cardiomyocyte apoptosis of the failing heart. RAS, rennin-angiotensin system ; A-II, angiotensin-II; NE, norepinephrine; TNF-α, tissue necrosis factor α. (Sabbah, 2000)

Hypoxia (Long *et al.*, 1997;Tanaka *et al.*, 1994), chamber dilation and myocardial stretch (Cheng *et al.*, 1995), sustained pressure overload and ventricular hypertrophy (Capasso *et al.*, 1990), mitochondrial injury or cytochrome c release (Golstein, 1997;Sabbah *et al.*, 1992), increased $O_2$-derived free radical species (Gottlieb *et al.*, 1994), enhanced activity of RAAS (Kajstura *et al.*, 1997) and β-adrenergic system (Communal *et al.*, 1998), intracellular $Ca^{2+}$ overload (Orrenius *et al.*, 1989), cell necrosis and consecutive fibrosis (Liu *et al.*, 1995b;Sharov *et al.*, 1996), TNF-α (Krown *et al.*, 1996), the cell surface antigen Fas and its ligand, FasL (Yamaguchi *et al.*, 1999) and finally cysteine proteases, like caspase-2 and 3 (Heinke *et al.*, 2001;Laugwitz *et al.*, 2001) were all shown to initiate or contribute to the apoptotic process in the heart. Particularly interesting and promising are the findings that under experimental conditions both the blockade of angiotensin II type I receptor (losartan) and that of β-adrenergic receptor signalling (propranolol) were able to prevent completely apoptosis induced by angiotensin II (Kajstura *et al.*, 1997) or norepinephrine (Communal *et al.*, 1998), respectively. Despite the numerous evidences that support function of apoptotic events during cardiac remodeling the precise contribution of programmed cell death to the progression of heart failure is still to be established.

**Acute heart failure**

Heart failure may also develop suddenly, particularly as a complication of acute myocardial infarction or as an acute exacerbation in patients with transitionally compensated chronic heart failure. In acute heart failure, the finite adaptive mechanisms that may be adequate to maintain the overall contractile performance of the heart at relatively normal levels become maladaptive when trying to sustain adequate cardiac performance. The acute insult to the heart results in failure of the pumping action, low cardiac output and a low blood pressure, inadequate for the body's requirements. Acute heart failure may be due to left sided heart failure (left ventricular failure), right-sided heart failure or a combination of left and right known as congestive cardiac failure. There are numerous potential causes of acute heart failure e.g. acute myocardial infarction (sudden loss of functional myocardium), heart valve dysfunction (acute regurgitation due to the rupture of valve leaflets or supporting structures, etc.), high blood pressure, cardiomyopathy, infections (infective endocarditis with acute valve incompetence), severe arrhythmias, etc. In new onset heart failure usually no preceding myocardial remodeling occurs. In addition, structural and functional alterations of the myocardium in decompensated chronic heart failure are concordant with those reviewed above. Therefore, possible myocardial alterations in acute heart failure are not detailed here.

## Therapeutic approaches in congestive heart failure

### Conventional treatment of acute heart failure

The past 40 years brought remarkable evolution and at times radical changes in the management of heart failure patients. First approaches applying diuretics and digitalis focused on the amelioration of the symptoms of volume overload. Inotropic agents and vasodilators provided new therapeutic facilities from the 1970s. More recently, experimental and clinical results led to the recognition that impairment of the neurohormonal systems, in particular the renin-angiotensin-aldosterone system and the sympathetic nervous system, plays crucial role in the development and progression of myocardial dysfunction (Endoh, 2001;Miller, 2003). This process is continuously maintained and intensified by a vicious circle as a consequence of the damaged neurohormonal regulation. Interruption of this course results in cardiac unloading and suppression of cardiac hypertrophy. Indeed, number of clinical trials demonstrate beneficial impact of angiotensin-converting enzyme inhibitors (ACEIs) and β-blocking agents (BBs) on cardiac remodeling ensuring long-term cardiac protection in this wise.

Unfortunately, however, acute decompensation that is a most life-threatening situation necessitates immediate and direct support of cardiac contractility. Despite the increasingly extensive use of mechanical assist devices positive inotropic drugs remain the most commonly accessible therapeutic tools. Traditionally used cardiotonic agents (digitalis, catecholamines) and even the more recently developed PDE inhibitors (milrinone and enoximone) support cardiac contractility through intracellular mechanisms that induce positive inotropy in effect by increasing diastolic and systolic intracellular $Ca^{2+}$-concentration. Accordingly, all these approaches possess serious therapeutic drawbacks of similar fashion. Intracellular $Ca^{2+}$ overload impairs diastolic relaxation, causes arrhythmias and cell injury. Large increase in intracellular [$Ca^{2+}$] may even diminish contractile force (Allen *et al.*, 1985;Capogrossi *et al.*, 1988), a phenomenon termed as '$Ca^{2+}$ overload'. In addition, enhanced $Ca^{2+}$-handling increases energy consumption and thus oxygen demand exacerbating ischemia in the myocardium. Furthermore, narrow therapeutic window, significant side-effects, long plasma half-life and differences in individual elimination rates (digitalis), moreover development of drug tolerance (tachyphylaxis; catecholamines) further complicate application of these drugs. All these concerns necessitated the invention of alternative possible therapeutic strategies and the development of novel inotropic agents lacking detrimental consequences of intracellular calcium-overload.

**Mechanisms of inotropy**

To achieve this end, attention has been turned towards myofibrillar proteins. Mechanisms that may enhance force - even if in theory - are briefly reviewed below. From a physiological point of view the main possible functional states, i.e. force-generating and non force-generating states, of the cross-bridges can be described by a most simplified two-state model of actin-myosin interaction (Huxley, 1957). As a result of theoretical considerations to determine isometric force (F) the following equation can be applied:

$$\mathbf{F = n \bullet F \bullet f_{app} / (f_{app} + g_{app})}$$

, where n is the number of the turning-over cross-bridges per half sarcomere, F is the mean force produced by a cross-bridge in the force-generating state, $f_{app}$ and $g_{app}$ are the rate constants characterizing the transitions from the non-force-generating states to the force-generating states and back to the non-force-generating states, respectively (Brenner, 1988;Wolff *et al.*, 1995).

The first possible mechanism to enhance inotropy is the increase of maximal force that the muscle can produce. Three distinct mechanisms could eventuate this end: increase in the number of crossbridges (e.g. in hypertrophy), increase in the force produced by a single crossbridge or prolongation of the period while crossbridges remain in the strongly bound state. A second approach could be the enhancement of cooperativity within the thin filament following calcium activation and the augmentation of the positive feedback by strong actin-myosin interactions on the $Ca^{2+}$-affinity of TnC. A further advantage of this mechanism would be a decreased energy demand of the crossbridge cycle. Unfortunately, no example for such a mechanism has been identified yet.

Finally, $Ca^{2+}$-sensitivity of the myofibrillar proteins could be increased. Distinct – at least five - mechanisms could be involved in this case (Fig. 25):

(1) an increase in the $Ca^{2+}$ affinity of TnC,

(2) an increase in the effectiveness with which $Ca^{2+}$-bound TnC increases the rate of crossbridge formation,

(3) an increased rate of crossbridge attachment (increased $f_{app}$),

(4) a decreased rate of crossbridge detachment (decreased $g_{app}$),

(5) a change in the positive feedback of attached crossbridges on the $Ca^{2+}$ affinity of TnC.

It is to note that most of the compounds that enhance force without altering $[Ca^{2+}]_i$ influence both $Ca^{2+}$ sensitivity and maximal force. Thus their action could be more precisely described as altering $Ca^{2+}$ responsiveness. Despite, these agents are usually termed as $Ca^{2+}$ sensitizers.

Admitting the laxity of this term for the sake of simplicity in the followings we would refer as $Ca^{2+}$ sensitizers to compounds that improve $Ca^{2+}$-responsiveness.

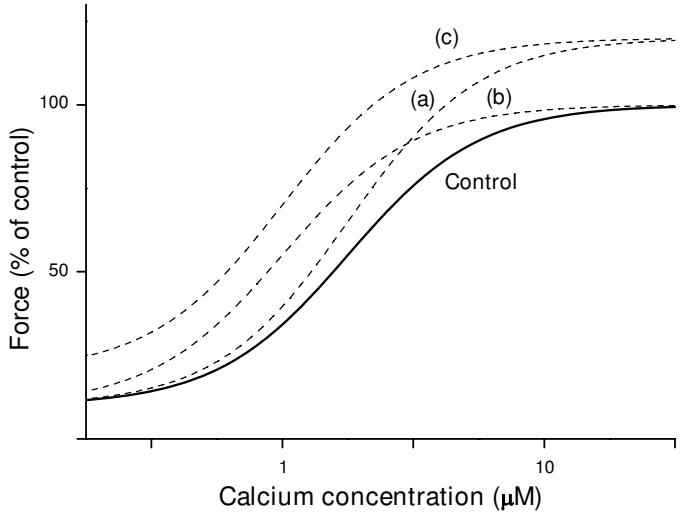



**Figure 25.** Effects of various inotropic mechanisms on the relation between isometric force and $Ca^{2+}$: increasing either the number of crossbridges or the force per crossbridge (curve a); increasing the affinity of troponin for calcium (curve b); increasing $f_{app}$ or decreasing $g_{app}$ (curve c). (Modified from (Lee & Allen, 1997).

$Ca^{2+}$-sensitizing mechanisms may increase the $Ca^{2+}$ affinity of the troponin C (central mechanism; class I) or influence characteristics either of thin filament interaction or of crossbridge cycling (downstream mechanisms) (Endoh, 2001). Experimental evidences revealed that the latter group could be further divided into two classes: compounds that modulate the interaction between the $Ca^{2+}$-bound troponin complex and actin filaments (class II) or compounds that influence crossbridge kinetics directly (class III). Basic properties and examples are presented in Table 1.)

Yet, proper dissection of these functional groups is in part ambiguous. Because of the interactions within the troponin complex certain conditions and compounds (e.g. acidosis and cAMP) affect $Ca^{2+}$-affinity of troponin C acting on another component of the troponin complex (on troponin I, in these cases)(Westfall *et al.*, 2001). It has also to be kept in mind that actin-myosin interaction exerts a positive feedback effect on the $Ca^{2+}$-affinity of troponin C (Kurihara & Komukai, 1996;Lee & Allen, 1997). Thus, $Ca^{2+}$-sensitizing agents cannot be precisely classified without the identification of their binding sites at the molecular level (Endoh, 2001).

| **Mechanism** | **Central mechanism** | **Downstream mechanism** | |
|---|---|---|---|
| *Class* | *I* | *II* | *III* |
| Proteins involved | troponin C | troponin-tropomyosin | actin-myosin |
| $Ca^{2+}$-regulation | + | + | - |
| Mechanism | $Ca^{2+}$-binding affinity | thin filament interaction | crossbridge |
| Example | Pimobendan<br>MCI-154 | Levosimendan<br>EMD 57033 | EMD 57033<br>Org 30029<br>CGP 48506 |

**Table 1.** Classification and basic characteristics of calcium-sensitizing compounds.

Unfortunately, several difficulties set back the development of positive inotropic agents. Compounds that act directly on action-myosin crossbridges and increase maximal force and $Ca^{2+}$-sensitivity (class III) impair relaxation of the myofilaments. The consequent rise of ventricular end-diastolic pressure has a rather detrimental impact on pathological situations characterized by diastolic functional abnormalities (e.g. heart failure). Furthermore, many of the $Ca^{2+}$-sensitizers unite $Ca^{2+}$-sensitizing and PDE inhibitory properties. On the one hand, by the facilitation of calcium reuptake into the sarcoplasmic reticulum, PDE inhibition mediates a beneficial effect. On the other hand, however, activation of protein kinase A may eventually result in an elevated [$Ca^{2+}$] in the cytosol and, through troponin I phosphorylation, in a decreased $Ca^{2+}$-sensitivity.

Which are the properties that characterize the ideal $Ca^{2+}$-sensitizer? An ideal $Ca^{2+}$-sensitizer:

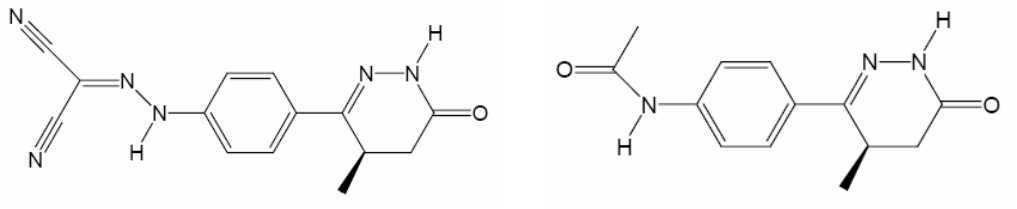
- would have positive inotropic effect and would increase cardiac output,
- would decrease neurohormonal activities (RAAS, sympathetic-activation),
- would not influence heart rate,
- would not enhance oxygen and energy demand of the myocardium,
- would not have arrhythmogenic potential,

so that to raise the survival rate of patients.

**Levosimendan – the first clinically approved $Ca^{2+}$ sensitizer**

Theoretically, a compound that selectively binds to the $Ca^{2+}$-saturated form of troponin C is likely to possess most of the characteristics above. The structure of levosimendan (the (-) enantiomer of {[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]hydrazono} propanedinitrile) was developed as a result of systematic molecular planning (Fig. 26). The carefully designed systematic process resulted in a compound that holds most of the required

beneficial properties of $Ca^{2+}$-sensitizers at the same time being devoid of their detrimental effects. Recently, clinical evidences proved its inotropic potential and favourable impact on patients' survival in acute cardiac decompensation of distinct causes (Follath *et al.*, 2002a;Moiseyev *et al.*, 2002). $Ca^{2+}$-binding to troponin C initiates conformational rearrangement of the molecule (Fig. 27) that results in the formation of a hydrophobic cleft at the N-terminal portion of troponin C. The dihydropiridazinone ring of levosimendan fits into this cleft. The dinitrile functional group binds to electrically charged aminoacids ($Gln^{11}$, $Gln^{16}$, $Asp^{88}$, $Ser^{89}$ and $Lys^{92}$) thus further fastening the molecule. Levosimendan does not increase the $Ca^{2+}$-affinity of the troponin C (Edes *et al.*, 1995) but instead it stabilizes the $Ca^{2+}$-binding induced conformational change of the troponin C enhancing activity of the thin filaments and prolonging contraction this way (Haikala *et al.*, 1995c).



**Levosimendan** **OR-1896**

**Figure 26.** The chemical structures of levosimendan and of its active metabolite OR-1896.

Levosimendan dissociates from the troponin C during the decay of the $Ca^{2+}$-transient that precedes the relaxation phase. As levosimendan binding to the troponin C occurs in a $Ca^{2+}$ dependent manner myofilament relaxation and thus diastolic function remains unaltered during cardiac contraction (Haikala *et al.*, 1995b).

In accordance with these latter observations levosimendan enhanced neither maximal force production nor myosin ATPase activity (Edes *et al.*, 1995;Sato *et al.*, 1998) giving support to the idea that levosimendan exerts its positive inotropic properties without increasing energy demand and oxygen consumption of the myocardium (Haikala *et al.*, 1992). Numerous experimental studies have confirmed concentration-dependent positive inotropic effect of levosimendan (Boknik *et al.*, 1997;Edes *et al.*, 1995;Kristof *et al.*, 1998;Kristof *et al.*, 1999;Lilleberg *et al.*, 1995). Increased contractility at low, therapeutically relevant concentrations (30 nM) was not accompanied by positive chronotropic or lusitropic effects (Edes *et al.*, 1995). Some findings however suggested the involvement of the cAMP-mediated signaling process in the $Ca^{2+}$ sensitization induced by levosimendan in this concentration

range (at and above 30 nM) (Sato *et al.*, 1998). Specific inhibitors of the PKA and PKG (KT5720 and KT5823, respectively) failed to influence the positive inotropy in this concentration range. Therefore, increase in the intracellular cAMP or cGMP levels is unlikely to play a crucial role in the mechanism of positive inotropy (Haikala *et al.*, 1997). Additionally, at low, therapeutic drug concentrations levosimendan does not raise intracellular [$Ca^{2+}$] thus it neither elicit arrhythmias nor impair diastolic relaxation (Cleland & McGowan, 2002).

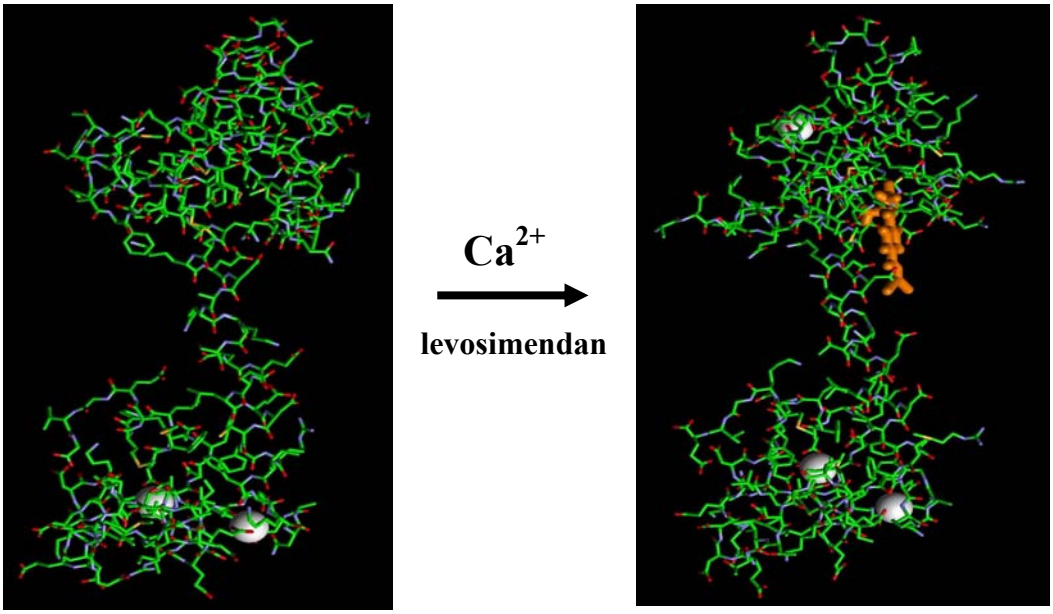



**Figure 27.** Structural changes in cTnI following $Ca^{2+}$ activation and levosimendan binding (an original figure kindly provided by P. Pollesello).

At higher concentrations (over 100 nM), however, levosimendan, similarly to other $Ca^{2+}$ sensitizers, exhibits also PDE inhibitory potential (Edes *et al.*, 1995). The concomitant increase in cAMP results in intracellular alterations like those induced by β-adrenergic stimulation (positive inotropy, chronotropy and luzitropy). This idea is supported by results obtained in experimental animals (Edes *et al.*, 1995;Haikala *et al.*, 1995a) or in human (Hasenfuss *et al.*, 1998). Hence, it is presumable that levosimendan acts preferentially as a $Ca^{2+}$ sensitizer at clinically relevant concentrations, while at higher concentrations PDE inhibition contributes to its positive inotropic potential (Endoh, 2001).

Beside its direct myocardial effects levosimendan efficiently decreases pulmonary capillary wedge pressure and systemic vascular resistance (Follath *et al.*, 2002b;Follath *et al.*, 2002a;Nieminen *et al.*, 2000). This effect is mediated through the opening of ATP-dependent potassium channels (Kaheinen *et al.*, 2001;Nieminen *et al.*, 2000;Pataricza *et al.*,

2000;Yokoshiki *et al.*, 1997) on the vascular smooth muscle cells. The concomitant venous and arteriolar dilation improves oxygen supply of the myocardium. Furthermore, levosimendan decreases myocardial ischemia and reduces infarct size, exerts anti-stunning effect in animals and enhances renal blood flow (Bowman *et al.*, 1999;du Toit *et al.*, 2001;Du Toit *et al.*, 1999;Jamali *et al.*, 1997;Kaheinen *et al.*, 2001;Kersten *et al.*, 2000;Lilleberg *et al.*, 1998;Pagel *et al.*, 1996;Pataricza *et al.*, 2000). Potential unfavorable effects arisen from the combination of levosimendan therapy with other drugs regularly used in cardiovascular medication was also studied. No adverse interactions could be observed with the most commonly used agents (ACEIs, β-blocking agents, nitrates and calcium channel blocking agents)(Mebazaa & Erhardt, 2003).

In contrast to catecholamines, positive hemodynamic effects of levosimendan are sustained for 7 to 10 days following discontinuation of the 24-hour-long intravenous infusion (Slawsky *et al.*, 2000). Since levosimendan has a relatively short half-life (1 hour) sustained hemodynamic effects most probably have to be attributed to one of its metabolites. Indeed, levosimendan is metabolized to OR-1855 and soon further to OR-1896 (Fig. 26), the latter being an active metabolite that has half-life of 80 hours (Antila *et al.*, 1999;Kivikko *et al.*, 2002;Kivikko, 2003;Lehtonen, 2001). The long half-life comparable to the duration of the sustained levosimendan effects predicts inotropic properties of OR-1896 to be similar to those of levosimendan. Indeed, in rabbit and dog ventricular myocardium OR-1896 increased contractile force via $Ca^{2+}$-sensitization and cAMP-mediated signal transduction pathways (Takahashi *et al.*, 2000a;Takahashi *et al.*, 2000b). Therefore, we decided to characterize the $Ca^{2+}$-sensitizing effect of OR-1896 in skinned gunea pig myocytes and its PDE inhibitory potential on purified PDE III and PDE IV isoforms in order to reveal if OR-1986 may play a role in the long-lasting positive inotropy of levosimendan.

# AIMS

Heart failure, a cardiac pathology of outstanding importance, has been a subject for intensive research for decades. Studies that examined the existence and the characteristics of the Frank-Starling mechanism, the main intrinsic regulatory mechanism for force generation of cardiac muscle, in chronic heart failure models brought inconsistent results. Inadequate systolic function despite the dilated cardiac cavities and stretched myofilaments, however, suggested altered stretch-induced calcium-sensitization in the failing heart.

We decided therefore *to examine active and passive myofibrillar properties at the cellular level in a rat model of heart failure* and *to reveal if any relationship exists between these mechanical parameters*. Additionally, previous works proved spatial heterogeneity of electrical and mechanical properties in the myocardium. Our group has already identified a transmural gradient of titin-based passive tension. Such possible gradients of active properties, e.g. stretch-induced calcium-sensitization have not yet been investigated. Hence, *we compared characteristics of myofilaments from the subendocardial and the subepicardial layer in order to reveal possible transmural differences*. Furthermore, for mechanisms that underlie Frank-Starling relationship at the molecular level several mechanical (interfilament lattice spacing, direct myosin-titin interactions, etc.) and biochemical regulatory mechanisms (phosphorylation of troponin complex or regulatory myosin light chains, etc.) were proposed. We also sought *to identify the possible posttranslational changes in myofilaments* under our conditions. Finally, *results obtained in healthy myocardium were compared to those from the diseased heart*. For this purpose we used our well-established rat model of heart failure developed following myocardial infarction. In the first part of the 'Results' chapter our study is reviewed that revealed new and stirring details on the behaviour of stretch-sensitization in chronic heart failure.

Involvement of cTnI and MyBP-C in the stretch-sensitization was previously suggested and examined. Recently, in their study Konhilas et al. found that the length dependence of activation is a variable function of the state of phosphorylation of cTnI (Konhilas *et al.*, 2003). Additionally, they observed a PKA induced increase in sarcomere length dependency of contractile activation that they attributed, solely, to the phosphorylation of cTnI, rejecting any role for cMyBP-C. Their conclusions were based on experiments using myocardial tissue from a transgenic murine model in which the native cTnI isoform was completely replaced by the slow skeletal isoform of TnI. In order to test the correctness of this observation *we*

*decided to determine the effects of SL and PKA-mediated phosphorylation on the myofibrillar $Ca^{2+}$ sensitivity in our murine model completely lacking the cMyBP-C.*

In the third and fourth parts of the results we focus on two studies that concerned heart failure from another, perhaps more practical aspect, namely pharmacological therapy. As discussed previously levosimendan effectively enhances cardiac output. However, for mechanisms of action that mediate positive inotropy in the myocardium basically two possibilities, $Ca^{2+}$-sensitization and the PDE-inhibition, has been proposed. PDE-inhibition may lead to protein kinase A activation and phosphorylation of $Ca^{2+}$-handling proteins and myofibrillar proteins thus eventually increasing intracellular [$Ca^{2+}$] similarly to catecholamines. In contrast, $Ca^{2+}$-sensitization of the contractile machinery is devoid of the deleterious effects of increased intracellular $Ca^{2+}$ load. Hence, the contribution of the two potential mechanisms to the effect of levosimendan at therapeutically relevant concentrations is of primary importance.

In collaboration with the manufacturer of levosimendan (Orion Pharma, Espoo, Finland) we set out a series of experiments in order *to further investigate the mechanism of action by which levosimendan exerts its positive inotropic effect*. Therefore, we *decided to characterize $Ca^{2+}$-sensitizing and PDE-inhibitory potentials* (on the PDE III and PDE IV isozymes) *of levosimendan* in *in vitro* model systems where these putative subcellular mechanisms can be examined independently and under standardized conditions. Comparison of their dose-dependences to that of the inotropic effect established in isolated hearts may reveal the involvement of these distinct mechanisms in the promotion of force generation. In addition, we evaluated if any difference exists between the inotropic properties of levosimendan and the group of compounds with pure PDE inhibitory potential. For this purpose *parameters of levosimendan were compared to those of enoximone*, another clinically introduced positive inotropic agent often cited as an inhibitor of PDE III subtype.

Furthermore, clinical studies revealed prolonged existence of levosimendan-induced positive hemodynamic changes even after the decrease of levosimendan plasma concentration to therapeutically ineffective level. Because of its relatively long half-life **OR-1896**, the main metabolite of levosimendan was suggested to possess positive inotropic potential and thus to maintain enhanced inotropism. We thought that the *characterisation of $Ca^{2+}$-sensitizing and PDE-inhibitory potentials of OR-1896 in parallel with those of levosimendan* would answer this question.

# MATERIALS AND METHODS

## METHODS APPLIED IN FRANCE

### Animals

In order to produce a model of post myocardial infarction heart failure ligature of the left anterior descending (LAD) branch of the left coronary artery was performed in male Wistar rats weighing 180-220 g as described below. To perform experiments rats were euthanised by intraperitoneal injection of pentobarbital (2 g/kg).

Homozygous cardiac MyBP-C null mice were generated as described previously (Carrier *et al.*, 2004). Targeted deletion of the cMyBP-C gene transcription site resulted in mice that lacked the cMyBP-C at both examined ages as confirmed by Western-blot analysis. In our study wild type and knockout female mice were studied at the ages of 5 weeks and 55 weeks.

Experiments were carried out according to the ethical principles laid down by the French Governmental and European Union Council Directives for laboratory animal care.

### Experimental myocardial infarction

To produce post myocardial infarcted hearts rats were subjected to coronary artery ligature (Aimond *et al.*, 1999;Pfeffer *et al.*, 1979). In brief, rats were anesthetized with pentobarbital (60 mg/kg), incubated and ventilated. In median thoracotomy the pericardium was opened and the LAD branch was occluded with a 7.0 silk suture right after its origin from the common left coronary artery. Immediate appearance of pallor and regional dyskinesia signified successful occlusion of the artery. Sham operated animals were subjected to the same treatment without ligation of the LAD coronary artery. Animals were sacrificed 14 weeks after intervention. The size of transmural necrosis was ~25 % of the left ventricular free wall area. Significant changes in morphologic parameters indicated remodeling of the myocardial tissue: the heart weight/body weight ratio (mg/g) increased from 2.9 ± 0.5 in sham (n=7) to 3.8 ± 0.4 in PMI (n=16); the width of isolated cells increased from 25±1 μm (n=40; sham) to 28±1 μm (n=30; PMI). Additionally, hemodynamic measurements revealed decrease in peak systolic pressure and a significant increase in end-diastolic pressure in PMI rats relative to the sham group.

**Experimental solutions**

Following excision hearts were rinsed and initially perfused with a $Ca^{2+}$-free Hanks-HEPES buffered solution containing in mM: NaCl 117.0, KCl 5.7, $NaHCO_3$ 4.4, $KH_2PO_4$ 1.5, $MgCl_2$ 1.7, HEPES 21.0, glucose 11.0, taurine 20.0, pH 7.2, adjusted with NaOH and bubbled with 100% O2. Skinning solutions and activating buffer solutions of intermediated calcium concentrations in single cell force measurements were daily prepared using a relaxing (pCa 9) and a maximal activating (pCa 4.5) solutions (Cazorla *et al.*, 2003). The relaxing and activating buffers contained (in mM): phosphocreatine 12.0, imidazole 30.0, free $Mg^{2+}$ 1.0, EGTA 10.0, $Na_2ATP$ 3.3 and dithiothreitol 0.3 with pCa 9.0 (relaxing solution) and pCa 4.5 (maximal activating solution), pH 7.1 adjusted with acetic acid (osmolarity 280 mOsm). Potassium acetate in appropriate amount was added to adjust ionic strength to 180 mM.

**Myocyte preparation**

Ventricular myocytes were isolated by mechanical dissociation described previously (Cazorla *et al.*, 2000b) with some modifications. Following pentobarbital anesthesia hearts were rapidly excised and rinsed in ice-cold $Ca^{2+}$-free Hanks-HEPES buffer. Then the aorta was cannulated and a 5-min-long retrograde perfusion (3 ml/min) at room temperature with $Ca^{2+}$-free Hanks-HEPES buffer containing 2,3-butanedione monoxime (15 mM) was started. Next, for preskinning the heart was perfused with relaxing solution containing 1% Triton X-100 and protease inhibitors (0.5 mM PMSF, 0.04 mM leupeptin, and 0.01 mM E64) for 7–10 min. The heart was then removed from the perfusion system and the right ventricle as well as the fibrotic tissue from the PMI left ventricular myocardium was discarded. Strips (8 mm long, 2 mm wide, 1 mm thick) from the endocardial and epicardial surfaces of the myocardial wall were dissected and either were frozen in liquid nitrogen for biochemical analysis or further skinned for 10 min at 4°C in relaxing solution containing 1% Triton X-100 for mechanical experiments. Strips were rinsed in ice-cold relaxing solution and mechanically disrupted at 11,000 rpm (Polytron PT45-80 with a PTA 10TS shaft, KINEMATICA AG, Switzerland) for 2–3 s. The resultant suspension of clumps of myocytes, single myocyte-sized preparations and cell fragments was filtered and centrifuged at 1000 rpm for 1 min at 4°C. To remove remaining membranes the pellet was further skinned in relaxing solution with 0.3% Triton X-100 for 6 min at room temperature. The skinned preparations were then extensively washed in relaxing solution containing protease inhibitors as described above. Isolated cells were maintained in relaxing solution on ice and used for 12 hours.

**Force measurements in permeabilized myocyte – sized preparations**

The procedure of cell attachment was identical to that reported previously (Cazorla *et al.*, 1999) with a few modifications. Briefly, the skinned myocyte was mounted at either end to a stepper motor driven micromanipulator (MP-285, Sutter Instrument Company, Novato, CA) and a piezoresistive strain gauge (model AE 801, SensoNor a.s., Horten, Norway; 500 Hz unloaded resonant frequency). To assure high rigidity of the attachment between the myofilaments and the stainless steel needles optical glue (NOA 63, Norland Products, North Brunswick, NJ) polymerized by UV illumination for 3 min was used. The sarcomere length (SL) was determined online at 50 Hz throughout the experiment by using a fast Fourier transform algorithm (FFT). The cell was positioned at the tip of a conical microcapillary that was connected to syringes containing various test solutions. The flow rate was set to approximately 200 μl/min.

Isometric force was normalized to the cell cross-section area using the width of the cell measured directly on the video monitor and an estimated value of 2/3 of cell width for the thickness. This ratio was determined on a population of myocytes loaded with membrane fluorescent indicator di-4-ANEPPS (5 μM for 5 min, Molecular Probes, Eugene, OR) to visualize the surface membrane with a laser scanning confocal microscope (Zeiss LSM510). Cell thickness was measured by Z-axis scanning through the upper and lower surfaces of the cell at a central point. Thus, an averaged value for the ratio of cell - width and the - thickness was determined in sham ENDO (0.67±0.02), sham EPI (0.68±0.01), PMI ENDO (0.66±0.02), and PMI EPI (0.67±0.02) ($n$=40 cells in each group).

Following test activation at pCa 4.5, the preparation was sequentially stretched in relaxing solution to 1.9, 2.1, and 2.3 μm SL at a speed of 0.1 length/sec. Sufficient time was allocated for the development of steady-state force level before the evaluation of the passive tension. After, isometric force-pCa relationships were established at two SL, 1.9 and 2.3 μm (Fig. 28). Active forces at submaximal activations were normalized to maximal tension (pCa 4.5) at the same SL. The relation between force and pCa was fitted to the following equation: force = $[Ca^{2+}]^{nH}/(K + [Ca^{2+}]^{nH})$, where $n_H$ is the Hill coefficient and $pCa_{50}$, pCa for half-maximal activation, equals $-(\log K)/n_H$.

For PKA stimulation, cells were preincubated before attachment for 50 min at room temperature (22°C) with 200 UI of catalytic subunit of PKA (Sigma Chemicals) per ml of relaxing solution.

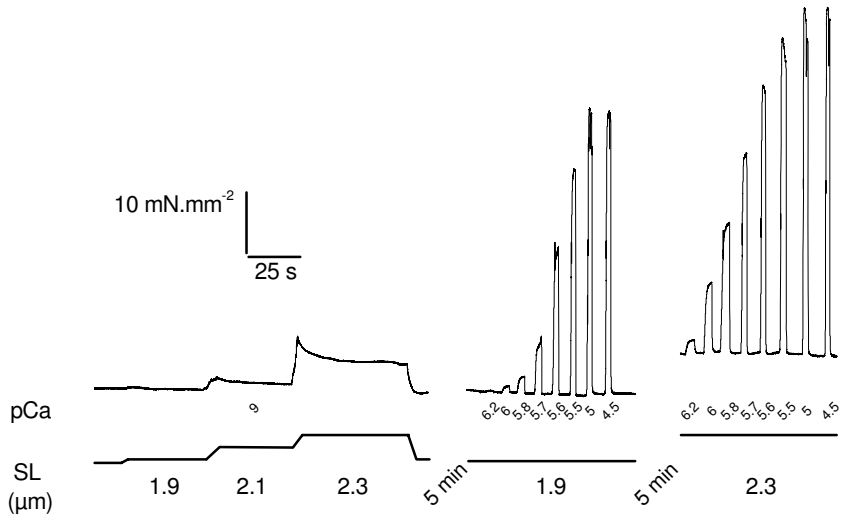



**Figure 28**. Protocole for the measurement of passive tension and for the establishment of stretch-induced calcium-sensitization. Before obtaining the second run of calcium activations myofilaments were stretched to 2.3 µm SL and sufficient time was allowed for stretch-relaxation.

**Biochemical analysis of titin**

Strips dissected from the endocardial and epicardial surface of the myocardium and frozen as described above were used for biochemical analysis. Skinned myocardial strips were pulverized in liquid nitrogen and solubilized in Laemmli buffer for 3 min at 60°C. Additionally, a batch of cells used for mechanical experiments was also solubilized in parallel. The samples were analyzed with SDS-PAGE (2.5–7 % acrylamide gradient gels) (Cazorla *et al.*, 2000a). After electrophoretic separation gels were stained with 0.1% Coomassie blue G 250. Wet gels were acquired with an imaging system (Kodak Image Station 2000R) and the images were analyzed using Kodak 1D image analysis software.

**Biochemical analysis of cTnI**

The total protein homogenates (20 µg) were separated with 15 %SDS-PAGE of cTnI analysis and blotted onto nitrocellulose membrane (Protran®, Schleichen & Schuele). Total cTnI content was determined with an antibody that recognizes the skeletal and cardiac TnI (clone 6F9, Hytest, Finland). PKA treatment was controlled with an antibody directed against the $Ser^{23/24}$ phosphorylated form of cTnI (clone 5E6, Hytest, Finland). Immunodetection was revealed with ECL system (ECL-Plus kit, Amersham Pharmacia). Quantification of signals was performed by densitometry using an imaging system (Kodak Image Station 2000R).

**Immunoblots**

TnI and MLC2 composition of the tissue samples was analyzed with Western immunoblot. Freshly dissected skinned strips either maintained at slack length or stretched by ~20–30% of the initial slack length for 1 min were quick-frozen then homogenized in ice-cold trichloroacetate (10%). This treatment allowed us to keep the phosphorylation status of the proteins. Following three washing steps in acetone /10 mM DTT the pellet was mixed with urea buffer (in M: urea 8.0, DTT 0.5, Tris 0.22, glycin 0.25, saturated glucose, EDTA 0.4, PMSF 0.0005, NaF 0.35) (Hathaway & Haeberle, 1985). Twenty micrograms of extracted proteins were separated either on 15 % SDS-PAGE for TnI analysis or 10 % urea gel for MLC2 isoforms (Hathaway & Haeberle, 1985) and blotted onto nitrocellulose membrane (Protran, Schleichen and Schuele, Dassel, Germany). For both studies, all samples from one animal were loaded on the same gel. MLC2 isoforms were specifically detected with a cardiac MLC2 antibody (Coger SA, Paris, France). Total TnI content and the protein kinase A phosphorylated form of cardiac TnI was determined in parallel with a total cardiac TnI antibody (clone 6F9, Hytest, Turku, Finland) and a PKA phosphorylated cTnI-specific antibody (clone 5E6, Hytest), respectively. Immunodetection was revealed with ECL Plus system (Amersham Pharmacia, Little Chalfont Buckinghamshire, England). Proportions of the phosphorylated proteins were expressed relative to the total amount of the studied protein.

**Real-Time Quantitative RT-PCR**

Real-time quantitative RT-PCR was performed as previously described (Carrier *et al.*, 2004). Total RNA was isolated from 5 mg of mouse ventricles using SV Total RNA Isolation kit (Promega #Z3100). Reverse transcription was performed on 50 ng of total RNA with superscript III reverse transcriptase (Invitrogen #18080-051). cDNA was purified using Microcon MY50 column (Millipore #42416) and the concentration was determined with Nanodrop technology. RT-PCR was performed using the Light cycler system (Roche) with specific primers for ssTnI, cTnI, and Emerin (*EMD)* for internal control. Absolute mRNA levels were calculated from standard curves generated by RT-PCR on serials dilutions of cloned *EMD*, *ssTnI* and *cTnI*. *EMD* was used as endogenous control to normalize the quantification of the mRNA target for differences in the amount of total RNA added to each reaction. Genomic DNA contamination was tested with Agilent bioanalyser and a sterile water sample subjected to RT-PCR in every experiment.

**Data analysis**

Individual pCa – force relationships were fitted to a modified Hill equation in order to determine the $pCa_{50}$ and the Hill coefficient. ΔpCa50 was calculated as the difference in $pCa_{50}$ at 1.9 and 2.3 μm SL for each experiment. One-way or 2-way ANOVA was applied for comparison between groups. When significant interactions were found, a Holm-Sidak t-test was applied (Sigmastat3 software) with $P < 0.05$. The parameters are presented as mean ± s.e.m., 'n' represents the number of the elements.

## METHODS APPLIED IN HUNGARY

**Animals**

To perform isolated perfused heart and isolated myofilament force measurement experiments *Duntley* Hartley guinea pigs of either sex (body weight: 300 - 350 g) were used. In the levosimendan versus OR-1896 study guinea pigs were euthanised with intraperitoneal injection of a cocktail containing ketamine (54 mg/kg), acepromazine (1.8 mg/kg), xylazine (10.9 mg/kg) and heparin (1500 U/kg). In the levosimendan versus enoximone study guinea pigs were euthanised with intraperitoneal injection of sodium pentobarbital (100 mg/kg) and heparin sodium (500 U/kg). Thereafter hearts were excised for isolated heart (Langendorff or working heart) experiments or for single cell force measurements. Left ventricular tissue used for the isolation of skinned myocyte-sized preparations was immediately frozen in liquid nitrogen and stored at –80 °C until further processing.

Animals received human care in accordance with the institutional guidelines for laboratory animal care. The studies were conducted with the permission of the Animal Ethic Committee of Orion Pharma, in accordance with Finnish law and government regulations as well as complying with the rules and institutional guidelines of the University of Debrecen for the use of experimental animals.

**Experimental solutions**

Isolating solution for myofilament preparation contained (in mM): $MgCl_2$ 1.0, KCl 100.0, EGTA 2.0, $Na_2ATP$ 4.0, imidazole 10.0; pH 7.0 adjusted with KOH. The compositions of the relaxing and activating solutions used in force measurements were calculated as described previously (Fabiato & Fabiato, 1979). Both solutions contained (in mM): $Mg^{2+}$ 1.0, MgATP 5.0, phosphocreatine 15.0 and N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid (BES) 100.0. The ionic equivalent was set to 150.0 by KCl at an ionic strength of 186.0. pCa, i.e. -

log [$Ca^{2+}$], values of the activating and relaxing solutions (pH 7.2) were 10.0 and 4.75, respectively, determined by the ratio of free and EGTA-bound [$Ca^{2+}$]. Solutions containing intermediate levels of free $Ca^{2+}$ were obtained by mixing appropriate amounts of relaxing and activating solutions.

Concentrated stock solutions of levosimendan (1 mM; 10 mM), OR-1896 (10 mM) and enoximone (100 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at 4 °C. Test concentrations of the compounds were prepared right previous to application on each experimental day by dissolving appropriate amount of stock solution in different test solutions. Final concentration of DMSO in experimental solutions did not exceed 0.1 % in isolated heart studies and 0.2 % in single cell force-measurements.

**Myocyte preparation**

Sufficient amount of frozen left ventricular myocardial tissue of the guinea pig was thawed in isolating solution and disrupted at 4°C by means of a tissue homogenizer. The suspension of myocyte-sized preparations was treated for 5 min with 0.5 % Triton X-100 at room temperature, centrifuged and rinsed twice with ice-cold isolating solution. The detergent Triton X-100 permeabilized membranous structures of the myocytes thus allowing precise control of the medium composition around the myofibrillar proteins and the study of the contractile properties under standardized conditions (i.e. defined $[Ca^{2+}]_i$ and sarcomere length). Skinned preparations were maintained in isolating solution on ice and used within 12 hours.

**Force measurements in permeabilized myocyte – sized preparations**

The skinned preparations were visualized by means of an inverted microscope (Fig. 30). For force measurement single myocyte-sized preparations were selected and attached with silicone adhesive (100% silicone, Aquarium sealant, Dow Corning, Midland, USA) at both ends to micromanipulator controlled stainless steel needles (Fig. 29). One needle was connected to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada) while the other to a force transducer (SensoNor, Horten, Norway). The average SL of the cell was determined using a spatial Fourier transform as previously described (Fan *et al.*, 1997) and set to 2.2 μm. Cross-sectional area of the preparations were calculated assuming elliptical cross-section and using two perpendicular diameters measured microscopically. All experiments were carried out at 15°C.

**Figure 29.** Arrangement of the experimental equipment in Debrecen (left). Skinned preparations were connected to an electromagnetic motor and a force transducer (right).

The contractile machinery was activated by transferring the myocyte from a droplet of relaxing to activating solution by moving the microscope stage laterally. Once isometric force reached its maximum a slack test (a rapid shortening – 20 % of the original length - followed by stretch to the original length) was applied to determine peak force level (Fig. 30).

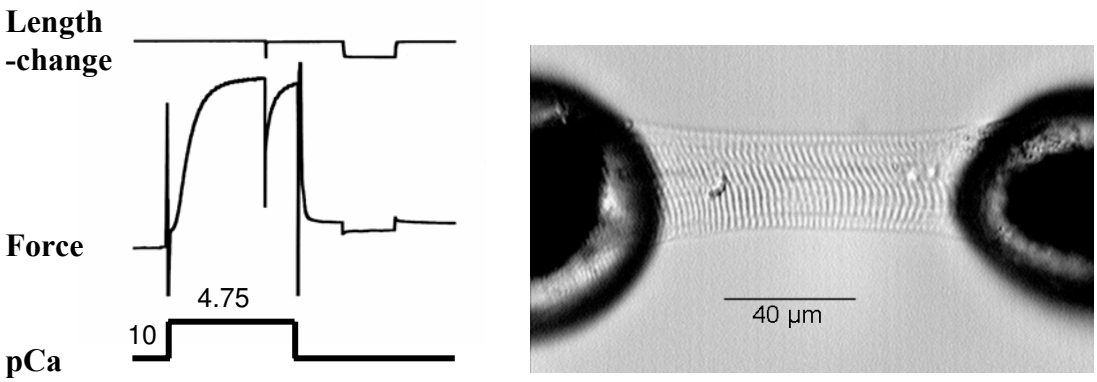



**Figure 30.** Experimental protocol used in Debrecen to measure passive and active mechanical proprties of the skinned myofilaments (left). Image of a myocyte attached to stainless steel needles with silicone adhesive (right).

The cell was then transferred back to the relaxing solution where a longer slack test of the same amplitude was used to establish passive force. Active force values at each [$Ca^{2+}$] were calculated as the difference between peak and passive force levels. To test the stability of the preparations reference maximal (pCa 4.75) activations were performed at the beginning and at the end of each experimental run. To correct for the run-down of the preparation active force values of test activations were normalized to an interpolated reference maximal force value calculated on the basis of a hypothetical linear decay. Force signals were recorded by a pen-

recorder, and following digitalization at a sampling rate at 20 Hz stored in a personal computer. During rapid slack tests the sampling rate was 1 kHz.

**Inhibition of phosphodiesterase-isozymes**

In order to obtain high level of enzyme purity PDE type III and IV isozymes were isolated from guinea pig hearts (levosimendan vs. OR-1896)/human platelets (levosimendan vs. enoximone) and a human myeloid leukemia promonocytic cell line (U-937), respectively. The procedures of isozyme isolation and those of the determination of phosphodiesterase activity were reported by others (Frodsham & Jones, 1992;Torphy *et al.*, 1992;Weishaar *et al.*, 1986b). In brief, the supernatant fraction of tissue homogenate was applied to DEAE-Sepharose column and PDE-activity was subsequently eluted with a linear sodium acetate gradient buffer. Collected fractions possessing peak PDE activities were further purified and assayed for cAMP phosphodiesterase activity. PDE isozymes were incubated at 30 °C for 30 min in a reaction mixture containing [$^3$H]cAMP (0.1 μM) and cAMP (0.1 μM) with or without levosimendan or enoximone. Liquid scintillation detection method was employed to measure [$^3$H]5'AMP product. Milrinone and rolipram were used as reference compounds to verify the precision of the assays. Phosphodiesterase-inhibitory potentials were given as the mean of the results from the duplicated inhibitory tests.

**Langendorff-perfused isolated hearts**

The heart was rapidly excised and rinsed at room temperature in oxygenated perfusion buffer. The aorta was cannulated and retrograde perfusion of the heart was started in the thermostatically controlled moist chamber of the Langendorff apparatus (Hugo Sachs Elektronik, Germany). Modified Tyrode solution (37 °C) equilibrated with carbogen (95% $O_2$ and 5% $CO_2$) was used as perfusion buffer. The composition of the Tyrode solution was (in mM): NaCl 135.0, $MgCl_2$ 1.0, KCl 5.0, $CaCl_2$ 2.0, $NaHCO_3$ 15.0, $Na_2HPO_4$ 1.0, glucose 10.0, pH 7.3–7.4. Following a short prestabilization period (10 min), a latex balloon (size 4) was introduced into the left ventricle through the left pulmonary vein and the left atrium. The balloon was coupled to a pressure transducer by which the isovolumetric left ventricular pressure was recorded. The pressure signal was amplified (two-channel bridge amplifier Type 301, Hugo Sachs Elektronik) and the digitized (frequency 1 kHz) signals were collected by means of the Acquisition v3.1 program (Orion Pharma). At the beginning of the experiment, the volume of the balloon was adjusted so as to obtain a diastolic pressure of approximately 5 mm Hg. Samples of the pressure signal recorded for a period of 3 s were digitized and

stored every 20 s. For each measure, the samples recorded over 3 min were averaged. The left ventricular pressure signals were used to calculate the positive peak of the first derivative of left ventricular pressure as a function of time ($+ dP/dt_{max}$). The hearts were allowed to stabilize for 30–50 min in order to reach a steady state in the coronary flow, which was thereafter fixed to its maximal value (in average 10.6 ml/min). After 15 min further stabilization, the hearts were paced at 280 beat/min and allowed to stabilize for additional 15 min. Then, either levosimendan or OR-1896 was added to the perfusion buffer in cumulative manner increasing drug concentration at 15-min intervals in the concentration range of 0.01-1 μM.

**Isolated guinea pig heart perfused in working heart mode**

Following rapid excision hearts were immediately rinsed in ice-cold perfusion buffer. The aorta was cannulated and the heart was suspended in a temperature controlled moist chamber. Retrograde perfusion was started in Langendorff mode at a perfusion pressure of 100 cm $H_2O$. The modified Krebs-Henseleit buffer (KHB) applied for all perfusion protocols in the working heart experiments consisted (in mM): 118.4 NaCl, 4.1 KCl, 2.5 $CaCl_2$, 25 $NaHCO_3$, 1.17 $KH_2PO_4$, 1.46 $MgCl_2$ and 11.1 glucose. The perfusion buffer was saturated with a mixture of 95% $O_2$ and 5% $CO_2$, the pH was set to 7.4 at 37°C. During the 10-min-long washout period a pulmonary vein was cannulated. Subsequently perfusion was switched to anterograde working heart mode (Weishaar *et al.*, 1986a) and the left atrial filling pressure and the left ventricular afterload were set to 15 cm $H_2O$ and 100 cm $H_2O$, respectively, both maintained by hydrostatic columns. The hearts were allowed to stabilize for 30 min before the first data sampling. Heart rate was registered by Haemosys computer acquisition system from Experimetria (Budapest, Hungary). Aortic and coronary flow rates were measured by timed collection of the perfusion buffer pumped through the aortic cannula and of the coronary perfusate dripping from the heart, respectively.

**Data analysis**

Values are given as mean ± S.E.M. The number of elements in the experimental group is indicated by ‘n’. The dependences of the isometric force and PDE inhibition on the levosimendan and enoximone concentrations were characterized by fitting the experimental data to a modified Hill equation. Statistical significance was calculated by analysis of variance (ANOVA, repeated measures) followed by Dunnet’s two-tailed test. In the working-heart experiments and the isolated myocyte force measurements Student unpaired t test was

used to compare drug-induced effects. The $Ca^{2+}$-sensitizing effect of levosimendan was analyzed by applying the Wilcoxon signed-rank test. Statistical significance was accepted at $P < 0.05$.

# RESULTS

## I. TRANSMURAL STRETCH-DEPENDENT REGULATION OF CONTRACTILE PROPERTIES IN RAT HEART AND ITS ALTERATION AFTER MYOCARDIAL INFARCTION.

### Problem

An increase in the end-diastolic volume of the cardiac cavities results in enhanced inotropism of the myocardium. Despite the achievements done in the previous years numerous details of the cellular mechanisms involved in the regulation of the Frank-Starling law are still to be elucidated. The role of several components has been presumed. Length dependent increase in the calcium affinity of troponin C may be such a factor (Babu *et al.*, 1988;Tavi *et al.*, 1998). Decreased lattice spacing following stretch increased probability of the actin-myosin interaction that may also enhance contractile force (Fuchs & Wang, 1996). Additionally, alterations in the passive tension may also have an indirect effect on the actomyosin interaction by influencing the interfilament lattice spacing (Fukuda *et al.*, 2003). It is to note that according to previous observations (Cazorla *et al.*, 1999;Cazorla *et al.*, 2001) $Ca^{2+}$-sensitivity of the myofilaments is rather correlated to the changes in titin-based passive tension than to SL changes.

Former studies proved differences in terms of electrical properties (Sicouri & Antzelevitch, 1991) and cellular stiffness (Cazorla *et al.*, 2000b) among the myocytes originated from different regions and layers (sub-endocardium and sub-epicardium) of the myocardium. It is known that cardiac performance and function are dramatically altered at the organ level in heart failure. In spite of the numerous studies changes of the Frank-Starling mechanism in heart failure are still controversial. The Frank-Starling relationship was found maintained (Holubarsch *et al.*, 1996) or lost (Schwinger *et al.*, 1994) in human heart failure. So far, using however different models, variable effects of heart failure on myofilament $Ca^{2+}$-sensitivity were reported (de Tombe, 1998). Alterations in the content, distribution and phosphorylation of the contractile proteins were also extensively examined. A decrease in myosin light chain 2 (MLC2) content was found in human CHF (Margossian *et al.*, 1992). Various changes of MLC2 phosphorylation in heart failure were observed (Liu *et al.*, 1995a;Morano *et al.*, 1988;van der Velden *et al.*, 2003a). MLC2 phosphorylation is known to be associated with increased $Ca^{2+}$-sensitivity (Clement *et al.*, 1992) therefore its phosphorylation state in heart

failure is of particular importance in terms of contractile force determination. The aim of this study was to provide new insights into the physiological and pathological stretch-dependent regulation of cardiac contractility. For this purpose we used rat isolated skinned cardiomyocytes from normal and diseased hearts. We hypothesized that:

- myofibrillar $Ca^{2+}$-sensitivity was not uniformly regulated across the ventricular wall, and
- acute stretch modulates phosphorylation of contractile and/or regulatory proteins.

**Results**

Stretch-dependent contractile properties of isolated skinned myocytes from the EPI and ENDO layers were analysed as described previously (Cazorla *et al.*, 1999) and compared in sham and PMI hearts. Myofilament $Ca^{2+}$-sensitivity was characterized under isometric conditions at 1.9 μm and 2.3 μm SLs. At 1.9 μm SL no significant difference in $Ca^{2+}$-sensitivity ($pCa_{50}$) between wall layers of sham and PMI hearts could be detected. In contrast, the stretch to 2.3 μm resulted in larger calcium-sensitisation in sham ENDO cells than in sham EPI ones. Interestingly, stretch induced $Ca^{2+}$-sensitisation was identical in the two corresponding myocardial layers of PMI hearts. Additionally, stiffness of the myofilaments was also characterized by stretching the cells from slack length to 2.3 μm SL in relaxing solution. Both in sham and PMI hearts ENDO cells proved to be stiffer than EPI cells. Detailed analysis of titin proved no isoform difference between the two layers. Instead, it revealed a gradient in the number of titin molecules per half-sarcomere (characterized by the titin/MHC ratio) within the ventricular wall.

Stretch-induced $Ca^{2+}$-sensitisation ($\Delta pCa_{50}$) and passive tension developed at 2.3 μm SL showed a remarkable relationship. In sham hearts a positive correlation between passive tension and the extent of stretch-induced $Ca^{2+}$-sensitisation could be established. In contrast, diminished stretch-sensitisation in the subendocardial layer of the PMI hearts blunted the positive correlation. Furthermore, phosphorylation states of various sarcomeric proteins were explored in order to explain the gradient in stretch sensitization across the left ventricular free wall in sham and its alteration in PMI hearts. Phosphorylation states of myofilament regulatory molecules were examined before and after 1-min stretch (corresponding to ~1.9 μm SL and ~2.3 μm SL, respectively). PKA phosphorylation of troponin I decreases $Ca^{2+}$ sensitivity and is thought to enhance length-dependent activation of the myofilaments (Konhilas *et al.*, 2003). We observed no difference in TnI phosphorylation among the examined groups. Furthermore, by means of urea gel separation four different forms of the myosin light chain 2 (the two ventricular isoforms, VLC2a and VLC2b and their

phosphorylated forms, P-VLC2a and P-VLC2b) could be separated. VLC2a was less phosphorylated in the ENDO layer of PMI than in that of sham hearts and the degree of phosphorylation was not affected by stretch in any of the samples. On the other hand, stretch resulted in a marked increase of the VLC2b phosphorylation in the ENDO-SHAM cells but not in ENDO-PMI ones.

To sum up, in our work we demonstrated for the first time a gradient of passive and active mechanical properties across the left ventricular free wall that is considerably altered in the failing, remodelled heart. The increasing number of titin molecules per half-sarcomere (characterized by the titin/MHC ratio) correlated well with the gradient in myofilament stiffness observed along the myocardial wall. Interestingly, disappearance of the positive correlation between passive tension and the extent of stretch-induced $Ca^{2+}$-sensitisation presumed functional alterations in the ENDO-PMI myofilaments. This proposal was supported by the observed decrease in myosin light chain 2 isoform VLC2b phosphorylation in the ENDO-PMI filaments. The relationship observed between the phosphorylation of the VLC2b and the extent of stretch-induced $Ca^{2+}$-sensitisation suggests an important role of VLC2b in the Frank-Starling mechanism. Additionally, it is presumable that post-infarction remodeling of the myocardium affects stretch-dependent regulation of myofilament contractility predominantly in the sub-endocardial layer of the myocardial wall.

The study is presented in the form of a scientific article:

Cazorla O., **Szilagyi S.**, Le Guennec J. Y., Vassort G. & Lacampagne, A. (2004).

Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. *The Faseb Journal* 19**:**88-90.

**Significance and perspectives**

Our study proved positive correlation between titin based passive tension and stretch-induced increase in calcium sensitivity. Stretch sensitisation was accompanied with an increase in VLC2 phosphorylation in the sub-endocardial layer. These observations, however, raise several questions that require further investigations:

- Which signaling pathway is involved in the stretch induced phosphorylation of VLC2?
- What are the structural consequences of the phosphorylation and the mechanism of force enhancement at the molecular level?
- What is the impact of such a regulation on the overall contraction pattern throughout the ventricular wall?

Furthermore, this transmural modulation was shown to be blunted during the remodeling process that takes place in the failing left ventricular myocardium. The gradients of stretch sensitisation and that of VLC2-phosphorylation disappeared in parallel. Changes in myofibrillar functional characteristics necessarily entail alterations of the left ventricular transmural contraction pattern. It still remains to be elucidated, however, whether these alterations are compensatory, thus beneficial changes or on the contrary, signs for decompensation of the myocardium that is no more able to cope with the increased hemodynamic demands.

## II. ACTIVATION OF MYOFILAMENTS IN A HYPERTROPHIC MOUSE MODEL LACKING THE CARDIAC MYOSIN BINDING PROTEIN C: EFFECT OF LENGTH AND PKA PHOSPHORYLATION.

### Problem

The thick filament component cardiac myosin binding protein C (cMyBP-C) is expressed exclusively in the heart and differs in three specific regions from the two skeletal isoforms (reviewed in (Winegrad, 1999)). Particularly, in the MyBP-C motif it contains phosphorylation sites and the MyBP-C has binding sites also for actin, titin, and myosin (reviewed in (Flashman *et al.*, 2004)). The C-terminal C10 domain binds to the light meromyosin and the C1C2 region binds to the S2 region of the myosin. The observations that mutations in the human gene of the cMyBP-C may be associated with familial hypertrophic cardiomyopathy intensified the interest in this protein (Bonne *et al.*, 1995;Watkins *et al.*, 1995).

The regulatory role of cMyBP-C on cardiac contractility is still controversial. Studies applying extraction, truncation or knock out model of cMyBP-C resulted in different effects on myofibrillar $Ca^{2+}$ sensitivity. Hampered MyBP-C function resulted in increased (Witt *et al.*, 2001), unchanged (Palmer *et al.*, 2004b) or decreased (Harris *et al.*, 2002) $Ca^{2+}$ responsiveness. Furthermore, the role of cMyBP-C in the length-dependent activation is still uncertain. The 4 potential phosphorylation sites per protein predict regulatory function of the cMyBP-C. One of the sites is regulated by a $Ca^{2+}$-calmodulin kinase bound to the thick filaments and three sites by the cAMP-regulated protein kinase (PKA) (Hartzell & Glass, 1984). The PKA-dependent phosphorylation of cMyBP-C was shown to modify both the protein's interaction with actin and myosin and the activity of the actin-activated myosin ATPase (Gruen *et al.*, 1999;Kulikovskaya *et al.*, 2003).

β-adrenergic stimulation is a well-known mechanism to modulate cardiac contractility and hemodynamics through the activation of PKA, which predominantly targets proteins involved in $Ca^{2+}$ handling (reviewed in (Solaro & Van Eyk, 1996)). In addition, PKA phosphorylates also contractile proteins such as cardiac troponin I (cTnI), titin, and cMyBP-C resulting in decreased myofilament $Ca^{2+}$ sensitivity (Solaro *et al.*, 1976). Capability of the PKA to phosphorylate several myofibrillar proteins sets back the establishment of the precise role of cMyBP-C in cardiac contraction regulation. A most challenging problem is the dissociation of PKA-induced effects mediated by cTnI or cMyBP-C phosphorylation. The slow skeletal isoform of TnI (ssTnI) cannot be phosphorylated by PKA. The replacement of native cTnI by

ssTnI was shown to eliminate the effect of PKA on $Ca^{2+}$ sensitivity (Konhilas *et al.*, 2003). These observations suggest that it is the cTnI that modulates $Ca^{2+}$ sensitivity of activation and it mediates the PKA effect on myofilament activation. In contrast, in a mouse model recently developed by Carrier and colleagues complete lack of cMyBP-C (KO) resulted in a striking impairment of LV relaxation (i.e. a 2-fold increase in relaxation time and a blunted relaxation phase under β-adrenergic stimulation) (Carrier *et al.*, 2004). This finding strongly suggests an altered $Ca^{2+}$ sensitization of the myofilaments lacking cMyBP-C.

The aim of this study was therefore to characterize the effects of sarcomere length (SL) and PKA-mediated phosphorylation on the myofibrillar $Ca^{2+}$ sensitivity and to compare the obtained parameters in KO and wild type mice (WT). To this end KO mice showing mild and severe degree of ventricular hypertrophy at 5 and 55 weeks of age, respectively, were compared with WT littermates.

**Results**

First, passive mechanical properties of myocytes isolated from young (~5 weeks, KO-5) and adult (~55 weeks, KO-55) transgenic mice were analyzed. Passive tension did not differ between myocytes isolated from WT and KO mice of the same age. In both WT and KO myofilaments a decrease in passive tension was noticed after PKA treatment, most probably due to the PKA-dependent phosphorylation of titin I-band segment similarly to that reported previously. The level of PKA-induced cTnI phosphorylation was also evaluated by Western blot analysis. Basal phosphorylation levels of cTnI were similar in WT and KO cells and PKA phosphorylated cTnI to the same extent in both WT and KO.

Then, the length-dependent properties of myofilament activation were examined in WT and KO myocytes. At both ages myocytes isolated from KO mice had a higher $Ca^{2+}$-sensitivity at 1.9 μm SL than those from WT mice. Increasing SL to 2.3 μm produced a leftward shift of the $Ca^{2+}$-tension relationship ($\Delta pCa_{50}$). At 2.3 μm SL, there was no significant difference in the $pCa_{50}$ between WT and KO at either age. Indeed, the length-dependent activation, illustrated by the $\Delta pCa_{50}$ significantly decreased in KO myofilaments compared with WT. This alteration was, however, more pronounced in adult animals than in young mice. Deficiency of cMyBP-C was also associated with reduced slopes of the $Ca^{2+}$-force relationships at both SLs as indicated by the lower Hill coefficients even if significance in KO-5 mice at short SL was not reached. $T_{max}$ was not altered significantly in either of the studied groups.

The $Ca^{2+}$ sensitivity of the myofilaments preincubated with PKA was determined at both SLs and compared with previously obtained control values. PKA treatment of WT myocytes induced desensitization of the myofilaments at both SLs and ages without any effects on the sensitivity of $Ca^{2+}$ activation to length. In contrast, in KO mice barely significant rightward shift of the tension–pCa relationship was observed only at 1.9 μm SL in young animals. In both KO-5 and KO-55 mice stretch-induced increase in $Ca^{2+}$ sensitivity similarly to the maximal active tension remained unchanged following PKA treatment.

As a next step TnI expression in WT and KO myocytes was analyzed. In KO myocardium, preferentially in young animals a lower band of TnI appeared at the molecular weight similar to that of the ssTnI isoform. Electrophoretic motility of this band was different from that of the degradation products of cTnI obtained from a WT myocardium. Expression of ssTnI in the heart during development was previously reported (Siedner *et al.*, 2003). In our conditions, ssTnI was hardly detectable in WT myocardium while represented 24% and 7% of the total TnI in the KO-5 and KO-55 myocardium, respectively. The amount of mRNA for ssTnI and cTnI, determined by quantitative RT-PCR was similar in WT and KO animals and independent of the age. The crossing point cycle number (CP), used as an index of mRNA expression, did not vary in KO mice when expressed relative to WT at both ages. We observed, however, that CP for cTnI exceeded the CP for ssTnI, indicating that the amount of the cTnI mRNA was approximately 125-fold higher than that of the ssTnI mRNA. Since ssTnI does not contain the PKA phosphorylation sites its expression could be in part responsible for the altered PKA effect observed in KO mice.

To test this hypothesis, contractile parameters, $EC_{50}$ (the $Ca^{2+}$ concentration generating half maximal force) and the PKA-induced desensitization of myofilaments relative to the basal level were expressed as functions of the ssTnI content. This analysis proved no correlation between changes in contractile parameters following PKA treatment and the amount of ssTnI. Rather, the alteration in myofilament $Ca^{2+}$ sensitivity at both SLs was dependent on the presence of cMyBP-C. Thus, the difference in the response of $Ca^{2+}$ sensitivity to PKA-induced phosphorylation depending on whether cMyBP-C was present (WT) or not (KO) suggests that the presence and the phosphorylation status of cMyBP-C are decisive factors in the determination and regulation of the myocardial contractility.

The study is presented in the form of a manuscript submitted for publication:

Cazorla O.[†], **Szilagyi S.**[†], Vignier N., Salazar G., Krämer E., Vassort G., Carrier L., Lacampagne A.

Activation of myofilaments in a hypertrophic mouse model lacking the cardiac myosin binding protein C: effect of length and PKA phosphorylation.

([†] These authors contributed equally to this work.)

**Significance and perspectives**

β-adrenergic stimulation modulates cardiac contractility through protein kinase A (PKA), which phosphorylates also cTnI and cMyBP-C. The relative contribution of these proteins to the regulation of myofilament $Ca^{2+}$ sensitivity is still controversial because of difficulty in targeting specific protein phosphorylation.

The goal of this study was to determine the envolvement of MyBP-C in PKA-phosphorylation and SL-mediated changes in the myofibrillar $Ca^{2+}$ sensitivity. These properties were studied in MyBP-C knockout mice at 5 and 55 weeks of age showing mild and severe degree of hypertrophy, respectively, in order to reveal also possible age dependent alterations. We demonstrated that the lack of cMyBP-C 1/ did not influence passive tension or maximal active tension, 2/ reduced the SL-dependent change in $Ca^{2+}$ sensitivity and 3/ almost abolished the PKA-induced decrease in $Ca^{2+}$ sensitivity compared to wild type myofilaments. These effects were independent of both age and the severity of heart failure strongly suggesting that they are due to cMyBP-C deficiency rather than to hypertrophy-associated remodeling.

The $Ca^{2+}$ sensitization of myofilaments in cMyBP-C KO mice observed in the present study at short SL is in agreement with the partial removal of cMyBP-C (Hofmann *et al.*, 1991). In our hands the increase in $Ca^{2+}$ sensitivity at 1.9 μm SL in KO mice was accompanied by a decrease in cooperativity in myosin head attachment. Indeed, increase in $Ca^{2+}$ sensitivity and decrease in cooperativity occur together with increased flexibility and decreased order of myosin heads (Sweeney & Stull, 1990). Additionally, the presence of cMyBP-C was shown to be necessary for the regulation of myosin head distribution between ordered and disordered states (Kulikovskaya *et al.*, 2003).

The mechanisms by which changes in SL affect $Ca^{2+}$ sensitivity are not clear yet. In the absence of normal cMyBP-C, $Ca^{2+}$ sensitivity is already enhanced, and therefore further increase in SL has substantially less effect. In published papers on cMyBP-C predominantly one single SL was used that varied from 1.8 μm (Calaghan *et al.*, 2000), to 2.1 μm (Hofmann *et al.*, 1991;Yang *et al.*, 1999), 2.2 μm (Palmer *et al.*, 2004a), and finally to 2.25–2.3 μm (Harris *et al.*, 2002;Korte *et al.*, 2003). In line with our results, these studies at the shortest SL report large effects of cMyBP-C deficiency on $Ca^{2+}$ sensitization. Additionally, we would predict that the effects of cMyBP-C manipulation is reduced or abolished with stretch, i.e. at higher SLs. Interfilament distance could be an evident mediator of both PKA and stretch-induced effects especially in view of the facts that both cMyBP-C phosphorylation (by itself) and sarcomere lengthening decrease interfilament distance (Konhilas *et al.*, 2003) It was also proposed that length-dependence of activation could be a variable function of the

phosphorylation state of cTnI (Konhilas *et al.*, 2003), and MLC-2 (Cazorla *et al.*, 2005). From the present study, it is suggested that cMyBP-C should be also considered as a myofilament regulatory protein involved in stretch-sensitization. Another consequence of stretch is the increase in titin-based passive tension. Titin affects cardiac contractility by altering the likelihood of actomyosin interaction *via* either modulating interfilament spacing or straining the thick filament (Cazorla *et al.*, 1999;Fukuda *et al.*, 2003). Since cMyBP-C interacts with both titin and myosin, it could transmit part of the tension to the myosin head. Such a mechanism was observed in skeletal muscle (Wakabayashi *et al.*, 1994).

Konhilas *et al* reported a PKA-effect on length-dependent activation, not observed in the present study (Konhilas *et al.*, 2003). Methodological differences (application of β-blockers and carbamyl choline before manipulation to decrease the overall level of phosphorylation) may explain this contradiction. Various basal levels of phosphorylation before the PKA treatment could also explain the diverse effects of PKA on stretch-sensitization published by others: reduced (Kajiwara *et al.*, 2000), unchanged (van der Velden *et al.*, 2000) or increased (Konhilas *et al.*, 2003). PKA-induced phosphorylation had almost no effect on $Ca^{2+}$ sensitivity in KO myocytes at long or short SL, despite equal increases in cTnI phosphorylation of WT and KO. This is in line with the work by Yang *et al.* showing an impaired response during isoproterenol infusion of mice expressing a mutated cMyBP-C lacking both the titin and myosin binding sites at the C-terminus (Yang *et al.*, 2001). ssTnI is transiently expressed in the heart during development and almost disappears at 6-8 weeks of age (Siedner *et al.*, 2003). Re-expression of ssTnI was shown in various pathological models such as respiratory hypercapnic acidosis (Urboniene *et al.*, 2005), and endotoxemia (Layland *et al.*, 2005). The protective effect of ssTnI against the β-adrenergic $Ca^{2+}$-desensitization of myofilaments has been reinforced using a transgenic model in which cTnI was completely replaced by ssTnI (Fentzke *et al.*, 1999;Konhilas *et al.*, 2003).

In the present study, a maintained expression or a re-expression of ssTnI, most probably result of the post-transcriptional regulation, was observed solely in young KO animals. ssTnI was shown to induce a desensitization of myofilaments (Fentzke *et al.*, 1999;Konhilas *et al.*, 2003). We suggested therefore that its expression could in part influence the $Ca^{2+}$-sensitivity of myofilaments in our KO mice. Our finding that the desensitization of myofilaments did not correlate with the level of ssTnI expression, however, does not support this idea. Rather, the observed effects seem to be the consequences of the lack of cMyBP-C and not those of ssTnI expression. For minor impact of increased ssTnI expression on myofilament $Ca^{2+}$-sensitivity

various hypotheses can be proposed: 1/ the inadequate level of ssTnI to play a functional role, 2/ only partial incorporation of ssTnI into the sarcomeres resulting in negligible effects.

Our results further support the view that at least some of PKA-phosphorylation mediated effects on myofibrillar $Ca^{2+}$-sensitivity require cMyBP-C. Nevertheless, one cannot distinguish from the present work whether it is the protein itself or its phosphorylated form that is involved in the regulation of cardiac contraction. This question remains to be clarified.

## III. THE EFFECTS OF LEVOSIMENDAN AND OR-1896 ON ISOLATED HEARTS, SKINNED MYOCYTES AND PHOSPHODIESTERASE ENZYMES OF THE GUINEA PIG.

### Problem

Clinical trials provided evidence for the efficacy of the recently developed positive inotropic agent levosimendan in the treatment of the acute decompensation of chronic heart failure and acute myocardial infarction (Follath *et al.*, 2002a;Moiseyev *et al.*, 2002). In severe low-output heart failure (LIDO study) levosimendan effectively increased mean cardiac output and decreased the mean pulmonary wedge pressure thus supporting cardiac function and lowering mortality. Opening of ATP-dependent $K^+$-channels in vascular smooth muscle cells results in coronary and peripheral vasodilation (Kaheinen *et al.*, 2001;Pataricza *et al.*, 2000;Yokoshiki *et al.*, 1997). In contrast, in point of positive inotropic potential in the myocardium several distinct mechanisms of action have been evolved. Increasing body of evidence confirms the stereoselective interaction of levosimendan with the $Ca^{2+}$-saturated form of the cardiac troponin C (Pollesello *et al.*, 1994;Sorsa *et al.*, 2003;Sorsa *et al.*, 2001). Stabilizing the conformational change in the structure of the troponin C evoked by $Ca^{2+}$-binding levosimendan prolongs the interaction of actin and myosin hence promoting contractile force in a $Ca^{2+}$-dependent manner. In addition, PDE inhibition has also been raised as a possible mechanism of action due to the structural similarity of levosimendan to a group of PDE inhibitor molecules (Ajiro *et al.*, 2002;Boknik *et al.*, 1997;Takahashi *et al.*, 2000b;Zimmermann *et al.*, 1998) In this case, augmented protein kinase A activation and the consequent increase in the amplitude of the intracellular $Ca^{2+}$ transient is presumed to be the mechanism for positive inotropy. Results obtained in studies addressing the relationship between positive inotropy and changes of the intracellular $Ca^{2+}$ level following levosimendan application are, however, still controversial (Boknik *et al.*, 1997;Brixius *et al.*, 2002;Edes *et al.*, 1995;Hasenfuss *et al.*, 1998).

Interestingly, the positive effects of levosimendan remain demonstrable for several days following drug administration. A possible explanation for the persistence of the levosimendan effects in contempt of its relatively short half-life ($t_{1/2}$= ~1 hour) is the accumulation of an active metabolite possessing similar functional characteristics to those of levosimendan. This metabolite, OR-1896, has a longer half-life (~80 hours) and previous pharmacokinetic studies (Kivikko *et al.*, 2002;Kivikko, 2003) assumed its role in the prolongation of positive inotropic

effects. This concept was further supported by *in vitro* and *in vivo* studies on cardiac preparations of the dog (Takahashi *et al.*, 2000b). Therefore, we decided to test also OR-1896 to characterize its $Ca^{2+}$-sensitizing effect and inhibitory potential on PDE III and PDE IV isozymes.

On the basis of the above described controversies and observations we performed a set of experiments with the following aims:

- to further elucidate the mechanism of action by which levosimendan induces positive inotropy in the myocardium
- to confirm positive inotropic effect and to study the possible mechanisms of action of the OR-1896.

**Results**

Positive inotropic effects of levosimendan and OR-1896 were tested in Langendorff-perfused isolated guinea pig hearts. The two compounds exhibited similar cardiotonic potential although levosimendan was slightly more potent than OR-1896. In order to characterise $Ca^{2+}$-sensitizing and PDE inhibitory potentials of levosimendan and OR-1896 concentration-dependences of the presumed intracellular effects were established in *in vitro* model systems (isolated myofibrillar force measurements and biochemical assays on purified PDE isoforms, respectively). These approaches allowed the investigation of $Ca^{2+}$-sensitizing and PDE inhibitory properties independently, under standardized conditions. Both levosimendan and OR-1896 proved to be potent $Ca^{2+}$-sensitizers increasing isometric force development to approximately the same extent at similar, clinically relevant concentrations. PDE inhibitory effect was tested on two PDE isoforms being dominant in the left ventricular myocardium, namely PDE III and PDE IV. Although the concentration range in which levosimendan inhibited PDE III overlapped with that of $Ca^{2+}$-sensitization, inhibition of the PDE IV isozyme required 10000-folds higher levosimendan concentration. Similarly, OR-1896 inhibited only PDE III at concentrations where it exhibited $Ca^{2+}$-sensitizing properties.

These results in context of previous observations provided explanation for several uncertainties regarding the mechanism of action of levosimendan within the myocardium. First, we confirmed in skinned myocyte-sized preparations direct $Ca^{2+}$-sensitizing effect of levosimendan on myofibrillar proteins. Second, levosimendan proved to be a highly selective inhibitor of PDE III isozyme compared to PDE IV (selectivity factor ≈ 10000). This ratio far exceeds the corresponding values of other PDE inhibitors generally regarded as selective PDE III inhibitors (e.g. milrinone, selectivity factor ≈ 14)(de Cheffoy de Courcelles *et al.*, 1992). In

fact, to our knowledge PDE selectivity of levosimendan found in our study is the highest among all known PDE inhibitors. Considering this finding, interpretation of observations attained using other, presumably PDE III selective compounds needs prudence. Third, it was previously demonstrated in rat and rabbit cardiomyocytes that positive inotropism can be achieved only through the simultaneous inhibition of more PDE isozymes (Shahid & Nicholson, 1990;Verde *et al.*, 1999). Furthermore, PDE inhibition decreased the predicted intracellular compartmentation of cAMP thus allowing its degradation by other PDE isoforms. At clinically relevant free plasma concentrations (6 or 12 nM, respectively) levosimendan and OR-1896 show $Ca^{2+}$-sensitizing properties and inhibit PDE III but not PDE IV. Therefore, activation of the protein kinase A signalling pathway as the mediator of levosimendan and OR-1896 induced positive inotropism at these concentrations is rather unlikely. Fourth, our results are in accordance with those of Lancaster and Cook (Lancaster & Cook, 1997) who demonstrated increased cell shortening without changes in intracellular calcium concentration in isolated guinea pig cardiomyocytes following levosimendan application. Finally, direct $Ca^{2+}$-sensitizing properties of the slowly-degrading active metabolite, OR-1896 may well explain sustained positive inotropic effects observed after the suspension of intravenous levosimendan infusion.

The study is presented in the form of a scientific article:

**Szilagyi S.**, Pollesello P., Levijoki J., Kaheinen P., Haikala H., Edes I. & Papp Z. (2004).
The effects of levosimendan and OR-1896 on isolated hearts, myocyte-sized preparations and phosphodiesterase enzymes of the guinea pig. *European Journal of Pharmacology* 486:67-74.

**Significance and perspectives**

Levosimendan is the first clinically approved representative of $Ca^{2+}$-sensitizers, a novel group of compounds that were developed not only for a short-term, symptomatic treatment of heart failure but also for a long-term therapy aimed at preventing the occurrence or worsening of heart failure (Cleland *et al.*, 2003;Greenberg *et al.*, 2003;Lehmann *et al.*, 2003). Recent clinical evidences proved advantages of levosimendan compared with dobutamine treatment in severe low-output heart failure. The LIDO study (Follath *et al.*, 2002a) demonstrated that the intravenous administration of levosimendan induced more pronounced hemodynamic improvements in patients with decompensated heart failure, exhibited a better tolerability and safety profile and in a retrospective analysis, provided a long-term improvement in survival (Follath, 2003). The RUSSLAN study was conducted to examine the efficacy of levosimendan treatment in left ventricular heart failure as a result of an acute myocardial infarction. It demonstrated that administration of levosimendan reduced the risk of death and worsening heart failure both at 14 and at 180 days. Our result that the levosimendan metabolite OR-1896 also exhibits $Ca^{2+}$-sensitizing potential explains, at least in part the beneficial impact of levosimendan on the long-term survival. Due to the evidences learned from clinical studies and positive clinical experiences additional clinical trials have been initiated to broaden therapeutic indications of levosimendan (CASINO, SURVIVE, REVIVE-1 and REVIVE-2) (Papp *et al.*, 2005). Intravenous levosimendan therapy is now fully supported for most common causes of acute heart failure (following AMI, heart surgery and transplantation; progressive severe heart failure in spite of optimal therapy, etc.). Additionally, clinical observations raised the rationale and presumptive benefit of levosimendan administration in chronic heart failure beyond the acute decompensated episodes. This idea, however, necessitates development of a more comfortable way of drug delivery (oral or transdermal way in contrast to intravenous administration). In addition, chronic administration of levosimendan requires additional studies on its pharmacokinetics, safety and tolerability as well as detailed characterisation of chronic levosimendan effect on both the cardiovascular system and other organs. Furthermore, long-term drug interactions between levosimendan and conventional drugs of chronic heart failure management have yet to be precisely established. Finally, a complex, accurate and conscious molecular planning process resulted in the chemical structure of levosimendan. Further improvements on the basis of the levosimendan structure or the discovery of other active substances in the future will hopefully provide additional advantages on this promising way of $Ca^{2+}$ sensitization.

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

## Problem

Conventional cardiotonic drugs used for the management of heart failure exhibit well-known adverse effects or have pharmacokinetic characteristics that often limit their application. For instance the onset of arrhythmias and the narrow therapeutic concentration window (cardiac glycosides) or the lack of oral activity (dopamine and related compounds) are such characteristics. Hence, in the last decades efforts have been made to develop positive inotropic drugs without these unfavourable properties. Levosimendan and enoximone are two representatives of newly developed and launched positive inotropic agents. Both drugs have been proved to promote cardiac output, however, in the background of their inotropic effects distinct mechanisms of action are predicted. In the myocardium levosimendan was shown to promote actin-myosin interaction through its direct effect on the $Ca^{2+}$-saturated form of TnC. Additionally, activation of ATP-dependent $K^{+}$-channels and the consecutive vasodilation also contributes to the improved cardiac performance. Data about PDE inhibitory potential of levosimendan are sparse. It is to note, however, that in our previous study the selectivity factor of levosimendan for PDE III over PDE IV isozyme was 10000. This value far exceeds that of milrinone (14) often cited as a PDE III selective PDE inhibitor. In contrast to levosimendan, enoximone is thought to improve cardiac performance (i.e. it increases intracellular $Ca^{2+}$-transient and exerts vasodilation) through PDE III inhibition. The degree of its selectivity (e.g. over PDE IV) is still uncertain (e.g. 4 in (Bethke *et al.*, 1992) and 60 in (de Cheffoy de Courcelles *et al.*, 1992)). To our best knowledge, direct effect of enoximone on myofibrillar $Ca^{2+}$-sensitvity has not yet been tested. Therefore, in a set of experiments we decided to compare the pharmacological potentials of levosimendan and enoximone in three different experimental systems. We attempted to determine the contribution of $Ca^{2+}$-sensitization and PDE-inhibition to the positive inotropic effects of the drugs in a therapeutically relevant concentration range. For the sake of the cause:

- we established positive inotropic potential of both drugs in isolated heart preparation perfused in working-heart mode,

- tested and compared the possible direct $Ca^{2+}$-sensitizing potential of enoximone to that of levosimendan in permeabilized, myocyte-sized myofibrillar preparations of the guinea pig , and
- in *in vitro* PDE inhibitory assays applying purified enzymes we determined the selectivity profiles of levosimendan and enoximone on PDE III and PDE IV isoforms.

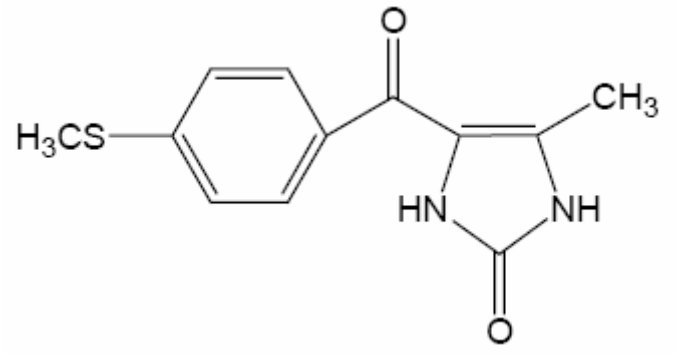



**Figure 31.** The chemical structure of enoximone.

**Results**

Consistent with clinical data (Caldicott *et al.*, 1993) in isolated working heart preparation of the guinea pig both levosimendan and enoximone improved cardiac performance (characterised by cardiac output and stroke volume). In the studied concentration range (between 1 nM and 1 μM), however, levosimendan was more potent than enoximone. In addition, both drugs increased coronary flow but in contrast to levosimendan enoximone failed to improve aortic output. This difference suggests a more pronounced contribution of coronary vasodilation and increase in microcirculation to the positive inotropy induced by enoximone than in the case of levosimendan.

In order to reveal pharmacological background of their distinct inotropic profiles putative intracellular effects of levosimendan and enoximone were further investigated. Similarly to our previous observations levosimendan proved to be a $Ca^{2+}$-sensitizer ($EC_{50}$ = 8.4 nM). In contrast, up to the concentration of 10 μM enoximone failed to increase the isometric force in permeabilized cardiomyocytes. Furthermore, PDE inhibitory potentials of levosimendan and enoximone showed marked differences. Levosimendan ($IC_{50}$ = 1.4 nM) is a 1300-fold more potent inhibitor of PDE III than enoximone. PDE III selectivity of levosimendan over PDE IV isozyme (selectivity factor ≈ 8000) was consistent to that found in our previous study (Szilagyi *et al.*, 2004). Corresponding values for enoximone found by others vary between 4

and 60 (Bethke *et al.*, 1992;de Cheffoy de Courcelles *et al.*, 1992). In our experiments selectivity factor of enoximone proved to be approximately 90, thus indicating enoximone a 90-fold less selective inhibitor of PDE III than levosimendan. In previous studies enoximone was characterized in different cardiac preparations of guinea pig (Loffelholz *et al.*, 1989) and of cat (Dage *et al.*, 1987). Positive inotropic effects increased continuously even up to drug concentration (1 mM) far exceeding that of clinical relevance (1 μM). Because of the maximal inhibition of PDE III isozyme and the complete lack of direct myofibrillar effects in the low micromolar concentration range other mechanisms causing further increase in the inotropic effects at concentrations higher than 1 μM are to presume. As a possible mechanism PDE IV inhibition should be considered by all means since the inhibitory potential of enoximone develops in the corresponding concentration range ($IC_{50}$ = 160 μM). Importantly, decreased intracellular compartmentation of cAMP caused by PDE inhibition (Jurevicius & Fischmeister, 1996) and the observation that simultaneous inhibition of at least two PDE isozymes (in particular those of low $K_m$, i.e. PDE III and PDE IV subtypes) are required for the elevation of intracellular cAMP concentration (Shahid & Nicholson, 1990;Verde *et al.*, 1999) well support this idea. On the other hand, levosimendan showed maximal inotropic effect at concentrations far below the range where PDE IV inhibition could act suggesting no role for dual PDE isozyme inhibition and elevated intracellular cAMP level in the mechanism of levosimendan-induced positive inotropism.

To sum up our results it can be concluded that:

- in clinically relevant concentrations both levosimendan and enoximone exert positive inotropic effect in isolated working heart, however, with distinct characteristics,
- an increase in the myofibrillar $Ca^{2+}$-sensitivity plays decisive role in the development of levosimendan-induced positive inotropy, while in contrast, effect on coronary microcirculation is a fundamental mechanism that contributes to the enoximone-effect,
- levosimendan is a much more potent and selective inhibitor of PDE III than enoximone,
- in view of the therapeutic plasma level of levosimendan simultaneous PDE III and PDE IV inhibition and subsequent cAMP elevation are not likely to be the mechanism of levosimendan action during clinical application.

The study is presented in the form of a scientific article:

**Szilágyi S.**, Pollesello P., Levijoki J., Haikala H., Bak I., Tósaki A., Borbély A., Édes I. & Papp Z. (2005).
Two inotropes with different mechanisms of action: contractile, PDE-inhibitory and direct myofibrillar effects of levosimendan and enoximone. *Journal of Cardiovascular Pharmacology* 46(3):369-376.

**Significance and perspectives**

Comparison of levosimendan with enoximone revealed that up to 1 μM concentration levosimendan induced more potent positive inotropic response in isolated guinea pig hearts. Positive inotropic effect of levosimendan in this concentration range is primarily attributable to its $Ca^{2+}$-sensitizing property in contrast that of enoximone is based solely on PDE-inhibition.

The intravenous (i.v.) formulation of enoximone (Perfan® I.V., Myogen) was first approved in Europe in 1989. It is indicated in hospital settings for the treatment of patients with acute heart failure and for weaning patients from cardiopulmonary bypass following open-heart surgery. Entering the advanced stages of CHF cardiac function of CHF patients progressively deteriorates leading to pulmonary congestion and acute heart failure. At this critical point patients must be hospitalized and aggressively treated with a combination of intravenous diuretics, positive inotropes and natriuretic peptide in order to increase the efficiency of the cardiovascular system providing symptomatic relief. After stabilization and discharge from the hospital, unfortunately, patients often decompensate again within months and must be readmitted to the hospital. With the progression of their disease, frequency of hospitalization increases until patients must be maintained on both confining and costly continuous or intermittent treatment with i.v. agents. Therefore, patients with advanced CHF could greatly benefit from the chronic use of an oral inotropic agent that would provide all desired benefits of the intravenously applicable drugs (symptomatic relief, improved quality of life, etc.). At the same time reduced frequency of hospitalization would considerably decrease the overall costs associated with the treatment of CHF.

The recognition of the need for orally applicable positive inotropes resulted in the development of enoximone capsules. In the 1980's phase I and II clinical trials were conducted to evaluate efficacy of enoximone at doses that we now consider high. At doses from 100 to 300 mg administered three times a day patents treated with oral enoximone demonstrated clinically significant increase in quality of life scores and maximal exercise capacity. Promising results were, however, overshadowed by a phase II placebo-controlled trial involving 151 patients in which 100 mg enoximone three times a day significantly increased mortality rate compared to placebo. Observations from these trials suggested that enoximone capsules administered at lower doses might retain inotropic efficacy without increasing mortality. Thus new clinical studies were designed recently to test oral enoximone therapy at lower doses.

In parallel with our experiments results of three phase III clinical trials with enoximone capsules were published. ESSENTIAL I and II were randomized, double-blind, placebo-controlled trials with identical designs enrolling patients from America and Europe, respectively. These two trials were designed to evaluate the safety and efficacy of low dose oral enoximone treatment (25 and 50 mg, administered three times a day) in patients with NYHA Class III and IV CHF. Unfortunately, both trials failed to demonstrate a statistically sifgnificant benefit for any of the three co-primary end-points (time to first cardiovascular hospitalization or all-cause mortality, exercise capacity at 6 months and patient self assessment of well being at 6 months). The third clinical trial was based on the hypothesis that low-dose enoximone could replace i.v. inotropic therapy in a substantial portion of patients with ultra-advanced CHF. The EMOTE trial was also a randomized, placebo-controlled, double-blind trial investigating whether oral enoximone was an effective agent for weaning patients off of i.v. inotropes. Although at 60 and 90 days patients fared significantly better on enoximone, at 30 days the difference between the enoximone and the placebo group was not significant. Hence, the primary end point of the study, i.e. weaning success from i.v. inotropes, was not reached.

Based on the results of the phase III clinical trials the producer of enoximone stopped further clinical evaluation and terminated the development of enoximone capsules in 2005. In light of the disappointing outcome of phase III clinical trials with enoximone capsules more and more attention is turned now toward the potential oral formulations of other positive inotropic agents. Increasing number of chronic heart failure patients with NYHA Class IV CHF justifies the importance of oral positive inotropic therapy and urges further development.

# CONCLUSIONS AND GENERAL DISCUSSION

Increasing number of patients in the developed countries makes chronic heart failure a public health problem of prominent importance. Hence it urges both basic and pharmacological research to reveal underlying pathological alterations in order to identify new possible targets and strategies for medical therapy. (**Chapter - Introduction**)

Enormous efforts done in the last decades explored the extremely diversified and compound nature for the development of CHF. Numerous alterations in the whole organism (e.g. complex circulatory and neurohormonal regulatory changes) as well as in the heart (e.g. hypertrophy and remodeling) were identified. Advances in molecular biological techniques allowed the determination of diverse changes at the molecular level in e.g. contractile protein expression and function, energy metabolism, electrophysiological properties, calcium handling, EC-coupling, cytoskeleton, etc. On the other hand, recent evidences from extensive international clinical trials have brought dramatic changes in the therapeutic management of CHF patients. Introduction of the neurohormonal blockades as well as attempts with novel pharmacological approaches (e.g. calcium-sensitisation) improved the efficacy of handling. Recent findings on the pathophysiology of CHF as well as current state of medical treatment and clinical calcium-sensitisation are briefly reviewed (**Chapter- Heart Failure)**.

In this thesis problems raised from both pathophysiological and pharmacological aspects were investigated applying up-to-date functional, cellular and biochemical experimental methods. Isometric force measurements in isolated permeabilized cardiomyocytes were used to characterize active and passive mechanical myofilament properties in a rat model of CHF following myocardial infarction (**Results-I**) and in a cMyBP-C deficient knock out mouse model (**Results-II**) of cardiac hypertrophy and failure. Furthermore, $Ca^{2+}$-sensitizing potentials of positive inotropes levosimendan and OR-1896 (**Results-III)** as well as enoximone (**Results-IV)** were studied directly on myofilaments. Single cell force measurements were combined with both classical physiological experiments and several molecular biological methods in order to explore relationships among contractile properties at the organ or cellular level and possible alterations in myofibrillar protein composition or posttranslational modification.

In a study summarized in **Results-I** an attempt was made to elucidate possible molecular changes that may at least in part underlie altered Frank-Starling mechanism in heart failure. Contractile parameters were determined at 1.9 μm and 2.3 μm SLs in sham and diseased

hearts taking into account also the localization of the myocytes. We demonstrated for the first time a gradient of passive and active mechanical properties across the left ventricular free wall the latter being considerably altered in the failing, remodelled heart. For molecular background of the increasing stiffness from the sub-epicardium towards the sub-endocardium a gradient in the number of titin molecules per half-sarcomere was proved. At 1.9 μm SL no significant difference in $Ca^{2+}$-sensitivity among the examined myocyte groups was detected. In sham animals stretching the sarcomeres to 2.3 μm SL induced larger calcium-sensitization in ENDO cells than in EPI ones. Interestingly, this difference between the two wall layers disappeared in the PMI hearts. A decrease in myosin light chain 2 isoform VLC2b phosphorylation in the ENDO-PMI filaments was observed in parallel with the disappearance of the positive correlation between passive tension and the extent of stretch-induced $Ca^{2+}$-sensitisation. Hence, these results suggest a regulatory role for VLC2b in the Frank-Starling mechanism.

It is interesting to speculate on the possible molecular regulatory mechanism(s) that may be involved in the phosphorylation of VLC2b following acute stretch. In vivo phosphorylation of the regulatory light chain in the myocardium was first proved in 1972 (Perrie *et al.*, 1972). Furthermore, the cardiac isoform of MLCK was cloned in 2001 (Davis *et al.*, 2001). In addition, treatment of skinned myocytes with MLCK resulted in increased $Ca^{2+}$-sensitivity (Clement *et al.*, 1992) and this effect was associated with an increase in VLC2 phosphorylation (Sanbe *et al.*, 1999). Hence cardiac MLCK (MYLK2) is a possible mediator of VLC2 phosphorylation in the myocardium. In non-contractile systems MLCK can be activated by several signalling pathways including $Ca^{2+}$-calmodulin complex and $p^{42/44}$MAPK. The role of these kinases in the regulation is rather unclear but worth for consideration. On the other hand, the degree of myosin light chain phosphorylation is also determined by the myosin phosphatase activity. Inhibition of the myosin phosphatase activity e.g. by Rho kinase or integrin-linked kinase phosphorylation (Muranyi *et al.*, 2002) may also result in increased myosin light chain phosphorylation and enhanced calcium activation.

The cardiac myosin binding protein C is expressed exclusively in the heart as a component of the thick filament. Multiple phosphorylation- and potential binding sites for actin, titin, and myosin predict a regulatory role for this molecule. Indeed, several mutations in the human gene of the cMyBP-C were associated with familial hypertrophic cardiomyopathy. Previous attempts using different models to define the impact of cMyBP-C on the myofilament calcium responsiveness led to controversial results. The PKA-dependent phosphorylation of cMyBP-C was shown to modify both the protein's interaction with actin and myosin and the activity of

the myosin ATPase. Unfortunately, precise determination of the effect of cMyBP-C-phosphorylation at the cellular level is hampered by the fact that PKA phosphorylates also several other $Ca^{2+}$-handling and contractile proteins. Recently, *Carrier at al.* (Carrier *et al.*, 2004) developed a knock out mouse model lacking completely the cMyBP-C It is noteworthy that in this model complete lack of cMyBP-C resulted in a striking impairment of LV relaxation (i.e. a 2-fold increase in relaxation time and a blunted relaxation phase under α-adrenergic stimulation) suggesting alteration in myofilament contractile properties. Examination of the KO model allowed the dissociation of PKA-induced myofibrillar effects mediated by cTnI or cMyBP-C phosphorylation (**Results-II**). No difference in the passive tension, basal cTnI phosphorylation levels and the extent of PKA-induced cTnI phosphorylation between WT and KO mice was observed. Despite similar $Ca^{2+}$-responsiveness at 2.3 μm SL the length-dependent activation, illustrated by the $\Delta pCa_{50}$ significantly decreased in KO myofilaments compared with WT due to the increased $Ca^{2+}$-sensitivity at 1.9 μm SL. $T_{max}$ was not altered in either of the studied groups but the absence of cMyBP-C was associated with reduced slopes of the force-pCa relationships at both SLs indicating decreased cooperativity within the contractile machinery. In addition, the PKA-induced decrease in $Ca^{2+}$-sensitivity observed in WT mice was almost completely abolished in the KO animals. The stretch-induced increase in $Ca^{2+}$ sensitivity was not affected by the PKA treatment in either of the studied groups. Examination of troponin expression in the WT and KO tissue samples revealed the presence of the ssTnI isoform in the KO but it was hardly detectable in WT mice. Since the ssTnI isoform cannot be phosphorylated by PKA its increased expression in the KO mice could be in part responsible for the altered PKA effect. Detailed analysis of PKA-induced desensitization as a function of ssTnI content, however, showed no correlation between these parameters. Taken together, these data support the hypothesis that $Ca^{2+}$-sensitivity and PKA responsiveness at both SLs depend on the presence and phosphorylation status of cMyBP-C, rather than those of cTnI indicating a decisive role for cMyBP-C in the regulation of myofilament contractility. Our results, however, challenge the conclusion of *Konhilas at al.* (Konhilas *et al.*, 2003) stating that the reduction in $Ca^{2+}$ sensitivity and the increase in the SL dependency of contractile activation induced by PKA treatment was due, solely to the phosphorylation of cTnI, not cMyBP-C.

Positive inotropic therapy represents a valuable and indispensable tool in the management of heart failure. In clinical studies levosimendan, the only one clinically approved $Ca^{2+}$-sensitizer so far, was shown to exert long lasting positive inotropic effect following application in acute heart failure. For mechanism of action beside $Ca^{2+}$-sensitization also PDE inhibition has been

assumed. Therefore, we decided to characterize its myocardial inotropic effect in isolated guinea pig heart. Additionally, using single cell force measurements and PDE-inhibition assays as simplified model systems an attempt was made to dissect its two predicted mechanisms of action in order to reveal their contributions to cardiac inotropy (**Results-III**). First, in our hands levosimendan exerted a potent positive inotropic effect in isolated hearts. Second, in the therapeutically relevant concentration range levosimendan increased active force in permeabilized myofilaments. Third, PDE inhibition assays proved notably high PDE III over PDE IV isoenzyme selectivity of levosimendan suggesting that levosimendan fails to evoke dual inhibition of the two PDE isoforms at its therapeutic concentration. In addition, previous investigations concluded that dual inhibition of low $K_m$ cAMP PDEs (PDE III and PDE IV) is the prerequisite for the PDE-dependent elevation in $[cAMP]_i$. Furthermore, PDE inhibition decreases the predicted intracellular compartmentation of cAMP, thus decreasing its local concentration and allowing its degradation by other PDE isoenzymes. In conclusion, positive inotropic effect of levosimendan is to be attributed to its $Ca^{2+}$-sensitizing property. On the contrary, PKA induced changes (e.g. increase in $[Ca^{2+}]_i$) due to $[cAMP]_i$ elevation are rather unlikely to contribute to force enhancement in the myocardium. The levosimendan metabolite OR-1896 exhibited similar inotropic effect to that of levosimendan with somewhat lower $Ca^{2+}$-sensitizing potential and PDE selectivity. These characteristics identified OR-1896 as a possible mediator for long-term beneficial effects of levosimendan on cardiac contractility.

In another study levosimendan and enoximone, a PDE inhibitor, were examined in parallel (**Results-IV**). In guinea pig working hearts levosimendan induced a more potent increase in cardiac output and systolic volume than enoximone did. Complete lack of $Ca^{2+}$-sensitization as well as markedly less PDE selectivity and consequent possibility for dual PDE III and PDE IV inhibition at therapeutically relevant concentrations suggested that enoximone induced positive inotropy is based solely on PDE inhibition. Hence, our results propose that in clinically relevant concentrations levosimendan and enoximone exert their positive inotropic effects through distinct mechanisms, $Ca^{2+}$-sensitization and PDE inhibition, respectively.

A main finding of this work is the observed high PDE III selectivity of levosimendan compared to corresponding values of other PDE inhibitors reported thus far. In accordance with previous issues our results suggest that higher PDE selectivity is associated with no or less pronounced involvement of cAMP induced signaling events in the regulation of contractility. Hence, PDE selectivity is likely to be an important parameter when screening potential positive inotropic compounds of the future. It is also to note that interactions

between PDE inhibitors in differing compartments, as well as concentration dependent dynamic alterations in the compartmentation of cAMP may also complicate precise description of intracellular events. Clearly, a satisfactory characterization of the role of compartmentation in the myocardial cAMP homeostasis needs further investigations. Finally, when evaluating cardiovascular effects of the different compounds both their cardiac and hemodynamic effects have to be taken into consideration. For instance, PDE inhibition in the vessels causes vasodilation that in turn decreases the preload of the cardiac cavities. This may have a favorable impact on the overloaded myocardium in dilated cardiomyopathy supporting contractile function and providing symptomatic relief for heart failure patients.

# REFERENCES

Agarkova, I., Ehler, E., Lange, S., Schoenauer, R., & Perriard, JC. (2003). M-band: a safeguard for sarcomere stability? *J Muscle Res Cell Motil.* **24**, 191-203.

Aimond, F., Alvarez, J. L., Rauzier, J.-M., Lorente, P., & Vassort, G. (1999). Ionic basis of ventricular arrhythmias in remodeled rat heart during long-term myocardial infarction. *Cardiovascular Research* **42**, 402-415.

Ajiro, Y., Hagiwara, N., Katsube, Y., Sperelakis, N., & Kasanuki, H. (2002). Levosimendan increases L-type Ca2+ current via phosphodiesterase-3 inhibition in human cardiac myocytes. *European Journal of Pharmacology* **435**, 27-33.

Akar, F. G. & Rosenbaum, D. S. (2003). Transmural electrophysiological heterogeneities underlying arrhythmogenesis in heart failure. *Circ Res* **93**, 638-45.

Allard, M. F., Schonekess, B. O., Henning, S. L., English, D. R., & Lopaschuk, G. D. (1994). Contribution of oxidative metabolism and glycolysis to ATP production in hypertrophied hearts. *Am J Physiol* **267**, 742-50.

Allen, D. G., Eisner, D. A., Pirolo, J. S., & Smith, G. L. (1985). The relationship between intracellular calcium and contraction in calcium-overloaded ferret papillary muscles. *J Physiol* **364**, 169-182.

Allen, D. G. & Kurihara, S. (1982). The effects of muscle length on intracellular calcium transients in mammalian cardiac muscle. *J Physiol* **327**, 79-94.

Alpert, N. R., Mulieri, L. A., & Warshaw, D. (2002). The failing human heart. *Cardiovascular Research* **54**, 1-10.

Alvarez, J., Hamplova, J., Hohaus, A., Morano, I., Haase, H., & Vassort, G. (2004). Calcium current in rat cardiomyocytes is modulated by the carboxyl-terminal ahnak domain. *J Biol.Chem.* **279**, 12456-12461.

Anderson, P. A., Greig, A., Mark, T. M., Malouf, N. N., Oakeley, A. E., Ungerleider, R. M., Allen, P. D., & Kay, B. K. (1995). Molecular basis of human cardiac troponin T isoforms expressed in the developing, adult, and failing heart. *Circ.Res.* **76**, 681-686.

Anderson, P. A., Malouf, N. N., Oakeley, A. E., Pagani, E. D., & Allen, P. D. (1991). Troponin T isoform expression in humans. A comparison among normal and failing adult heart, fetal heart, and adult and fetal skeletal muscle. *Circ.Res.* **69**, 1226-1233.

Antila, S., Huuskonen, H., Nevalainen, T., Kanerva, H., Vanninen, P., & Lehtonen, L. (1999). Site dependent bioavailability and metabolism of levosimendan in dogs. *Eur.J Pharm.Sci.* **9**, 85-91.

Arai, M., Alpert, N. R., MacLennan, D. H., Barton, P., & Periasamy, M. (1993). Alterations in sarcoplasmic reticulum gene expression in human heart failure. A possible mechanism for alterations in systolic and diastolic properties of the failing myocardium. *Circ.Res.* **72**, 463-469.

Arber, S., Halder, G., & Caroni, P. (1994). Muscle LIM protein, a novel essential regulator of myogenesis, promotes myogenic differentiation. *Cell* **79**, 221-231.

Babu, A., Sonnenblick, E., & Gulati, J. (1988). Molecular basis for the influence of muscle length on myocardial performance. *Science* **240**, 74-76.

Bache, R. J., Zhang, J., Murakami, Y., Zhang, Y., Cho, Y. K., Merkle, H., Gong, G., From, A. H., & Ugurbil, K. (1999). Myocardial oxygenation at high workstates in hearts with left ventricular hypertrophy. *Cardiovasc Res* **42**, 616-26.

Bagnato, P., Barone, V., Giacomello, E., Rossi, D., & Sorrentino, V. (2003). Binding of an ankyrin-1 isoform to obscurin suggests a molecular link between the sarcoplasmic reticulum and myofibrils in striated muscles. *J Cell Biol.* **160**, 245-253.

Bang, M. L., Centner, T., Fornoff, F., Geach, A. J., Gotthardt, M., McNabb, M., Witt, C. C., Labeit, D., Gregorio, C. C., Granzier, H., & Labeit, S. (2001). The complete gene sequence of titin, expression of an unusual approximately 700-kDa titin isoform, and its interaction with obscurin identify a novel Z-line to I-band linking system. *Circ.Res.* **89**, 1065-1072.

Barresi, R., Di Blasi, C., Negri, T., Brugnoni, R., Vitali, A., Felisari, G., Salandi, A., Daniel, S., Cornelio, F., Morandi, L., & Mora, M. (2000). Disruption of heart sarcoglycan complex and severe cardiomyopathy caused by beta sarcoglycan mutations. *J Med.Genet.* **37**, 102-107.

Barta, J., Toth, A., Jaquet, K., Redlich, A., Edes, I., & Papp, Z. (2003). Calpain-1-dependent degradation of troponin I mutants found in familial hypertrophic cardiomyopathy. *Mol Cell Biochem* **251**, 83-8.

Beer, M., Seyfarth, T., Sandstede, J., Landschutz, W., Lipke, C., Kostler, H., von Kienlin, M., Harre, K., Hahn, D., & Neubauer, S. (2002). Absolute concentrations of high-energy phosphate metabolites in normal, hypertrophied, and failing human myocardium measured noninvasively with (31)P-SLOOP magnetic resonance spectroscopy. *J Am Coll Cardiol* **40**, 1267-74.

Bell, S. P., Nyland, L., Tischler, M. D., McNabb, M., Granzier, H., & LeWinter, M. M. (2000). Alterations in the determinants of diastolic suction during pacing tachycardia. *Circ.Res.* **87**, 235-240.

Benaud, C., Gentil, B. J., Assard, N., Court, M., Garin, J., Delphin, C., & Baudier, J. (2004). AHNAK interaction with the annexin 2/S100A10 complex regulates cell membrane cytoarchitecture. *J Cell Biol.* **164**, 133-144.

Benitah, J. P., Gomez, A. M., Bailly, P., Da Ponte, J. P., Berson, G., Delgado, C., & Lorente, P. (1993). Heterogeneity of the early outward current in ventricular cells isolated from normal and hypertrophied rat hearts. *J Physiol* **469**, 111-138.

Benitah, J. P., Perrier, E., Gomez, A. M., & Vassort, G. (2001). Effects of aldosterone on transient outward K+ current density in rat ventricular myocytes. *J Physiol* **537**, 151-60.

Berne, R. M. & Lewy, M. N. (1998). *Physiology*, 4th edition ed., pp. 343. Mosby, Inc., St. Louis.

Bers, D. M., Eisner, D. A., & Valdivia, H. H. (2003). Sarcoplasmic reticulum Ca2+ and heart failure: roles of diastolic leak and Ca2+ transport. *Circ.Res.* **93**, 487-490.

Bethke, T., Eschenhagen, T., Klimkiewicz, A., Kohl, C., von der, L. H., Mehl, H., Mende, U., Meyer, W., Neumann, J., Rosswag, S., & . (1992). Phosphodiesterase inhibition by enoximone in preparations from nonfailing and failing human hearts. *Arzneimittelforschung.* **42**, 437-445.

Beuckelmann, D. J. & Erdmann, E. (1992). $Ca^{2+}$-currents and intracellular $[Ca^{2+}]_i$-transients in single ventricular myocytes isolated from terminally failing human myocardium. *Basic Res Cardiol* **87 Suppl 1**, 235-43.

Beuckelmann, D. J., Nabauer, M., & Erdmann, E. (1991). Characteristics of calcium-current in isolated human ventricular myocytes from patients with terminal heart failure. *J Mol Cell Cardiol* **23**, 929-937.

Beuckelmann, D. J., Nabauer, M., & Erdmann, E. (1992). Intracellular calcium handling in isolated ventricular myocytes from patients with terminal heart failure. *Circulation* **85**, 1046-1055.

Beuckelmann, D. J., Nabauer, M., & Erdmann, E. (1993). Alterations of $K^+$ currents in isolated human ventricular myocytes from patients with terminal heart failure. *Circ Res* **73**, 379-85.

Beuckelmann, D. J., Nabauer, M., Kruger, C., & Erdmann, E. (1995). Altered diastolic [Ca2+]i handling in human ventricular myocytes from patients with terminal heart failure. *Am.Heart J* **129**, 684-689.

Bishop, S. P. & Altschuld, R. A. (1970). Increased glycolytic metabolism in cardiac hypertrophy and congestive failure. *Am J Physiol* **218**, 153-9.

Boknik, P., Neumann, J., Kaspareit, G., Schmitz, W., Scholz, H., Vahlensieck, U., & Zimmermann, N. (1997). Mechanisms of the Contractile Effects of Levosimendan in the Mammalian Heart. *J Pharmacol Exp Ther* **280**, 277-283.

Bonne, G., Carrier, L., Bercovici, J., Cruaud, C., Richard, P., Hainque, B., Gautel, M., Labeit, S., James, M., Beckmann, J., Weissenbach, J., Vosberg, H. P., Fiszman, M., Komajda, M., & Schwartz, K. (1995). Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. *Nat.Genet.* **11**, 438-440.

Bowman, P., Haikala, H., & Paul, R. J. (1999). Levosimendan, a calcium sensitizer in cardiac muscle, induces relaxation in coronary smooth muscle through calcium desensitization. *J Pharmacol.Exp.Ther.* **288**, 316-325.

Braunwald, E. & Bristow, M. R. (2000). Congestive Heart Failure: Fifty Years of Progress. *Circulation* **102**, IV-14-IV-23.

Bremel, R. D. & Weber, A. (1972). Cooperation within actin filament in vertebrate skeletal muscle. *Nat.New Biol.* **238**, 97-101.

Brenner, B. (1988). Effect of $Ca^{2+}$ on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: implications for regulation of muscle contraction. *Proc.Natl.Acad.Sci.U.S.A* **85**, 3265-3269.

Brilla, C. G., Pick, R., Tan, L. B., Janicki, J. S., & Weber, K. T. (1990). Remodeling of the rat right and left ventricles in experimental hypertension. *Circ Res* **67**, 1355-64.

Brilla, C. G., Zhou, G., Matsubara, L., & Weber, K. T. (1994). Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. *J Mol Cell Cardiol* **26**, 809-20.

Bristow, M. P. M. R. (1997). Mechanism of Action of Beta-Blocking Agents in Heart Failure. *The American Journal of Cardiology* **80**, 26L-40L.

Brixius, K., Reicke, S., & Schwinger, R. H. G. (2002). Beneficial effects of the Ca2+ sensitizer levosimendan in human myocardium. *Am J Physiol Heart Circ Physiol* **282**, 131-137.

Brodsky, G. L., Muntoni, F., Miocic, S., Sinagra, G., Sewry, C., & Mestroni, L. (2000). Lamin A/C gene mutation associated with dilated cardiomyopathy with variable skeletal muscle involvement. *Circulation* **101**, 473-476.

Bryant, S. M., Shipsey, S. J., & Hart, G. (1997). Regional differences in electrical and mechanical properties of myocytes from guinea-pig hearts with mild left ventricular hypertrophy. *Cardiovasc.Res.* **35**, 315-323.

Bullard, B., Ferguson, C., Minajeva, A., Leake, M. C., Gautel, M., Labeit, D., Ding, L., Labeit, S., Horwitz, J., Leonard, K. R., & Linke, W. A. (2004). Association of the chaperone alphaB-crystallin with titin in heart muscle. *J Biol.Chem.* **279**, 7917-7924.

Calaghan, S. C., Trinick, J., Knight, P. J., & White, E. (2000). A role for C-protein in the regulation of contraction and intracellular Ca2+ in intact rat ventricular myocytes. *J Physiol (Lond)* **528**, 151-156.

Caldicott, L. D., Hawley, K., Heppell, R., Woodmansey, P. A., & Channer, K. S. (1993). Intravenous enoximone or dobutamine for severe heart failure after acute myocardial infarction: a randomized double-blind trial. *Eur.Heart J* **14**, 696-700.

Campbell, K. S., Patel, J. R., & Moss, R. L. (2003). Cycling cross-bridges increase myocardial stiffness at submaximal levels of Ca2+ activation. *Biophys.J* **84**, 3807-3815.

Capasso, J. M., Palackal, T., Olivetti, G., & Anversa, P. (1990). Left ventricular failure induced by long-term hypertension in rats. *Circ Res* **66**, 1400-12.

Capogrossi, M. C., Stern, M. D., Spurgeon, H. A., & Lakatta, E. G. (1988). Spontaneous Ca2+ release from the sarcoplasmic reticulum limits Ca2+-dependent twitch potentiation in individual cardiac myocytes. A mechanism for maximum inotropy in the myocardium. *J Gen.Physiol* **91**, 133-155.

Carr, A. N., Schmidt, A. G., Suzuki, Y., del Monte, F., Sato, Y., Lanner, C., Breeden, K., Jing, S. L., Allen, P. B., Greengard, P., Yatani, A., Hoit, B. D., Grupp, I. L., Hajjar, R. J., DePaoli-Roach, A. A., & Kranias, E. G. (2002). Type 1 phosphatase, a negative regulator of cardiac function. *Mol Cell Biol* **22**, 4124-35.

Carrier, L., Bonne, G., Bahrend, E., Yu, B., Richard, P., Niel, F., Hainque, B., Cruaud, C., Gary, F., Labeit, S., Bouhour, J. B., Dubourg, O., Desnos, M., Hagege, A. A., Trent, R. J., Komajda, M., Fiszman, M., & Schwartz, K. (1997). Organization and sequence of human

cardiac myosin binding protein C gene (MYBPC3) and identification of mutations predicted to produce truncated proteins in familial hypertrophic cardiomyopathy. *Circ.Res.* **80**, 427-434.

Carrier, L., Knoll, R., Vignier, N., Keller, D. I., Bausero, P., Prudhon, B., Isnard, R., Ambroisine, M.-L., & Fiszman, M. (2004). Asymmetric septal hypertrophy in heterozygous cMyBP-C null mice. *Cardiovascular Research* **63**, 293-304.

Cazorla, O., Freiburg, A., Helmes, M., Centner, T., McNabb, M., Wu, Y., Trombitas, K., Labeit, S., & Granzier, H. (2000a). Differential Expression of Cardiac Titin Isoforms and Modulation of Cellular Stiffness. *Circ Res* **86**, 59-67.

Cazorla, O., Szilagyi, S., Le Guennec, J. Y., Vassort, G., & Lacampagne, A. (2005). Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. *Faseb J* **19**, 88-90.

Cazorla, O., Lacampagne, A., Fauconnier, J., & Vassort, G. (2003). SR33805, a Ca2+ antagonist with length-dependent Ca2+-sensitizing properties in cardiac myocytes. *Br J Pharmacol* **139**, 99-108.

Cazorla, O., Le Guennec, J.-Y., & White, E. (2000b). Length-Tension Relationships of Sub-epicardial and Sub-endocardial Single Ventricular Myocytes from Rat and Ferret Hearts. *Journal of Molecular and Cellular Cardiology* **32**, 735-744.

Cazorla, O., Pascarel, C., Garnier, D., & Le Guennec, J.-Y. (1997). Resting Tension Participates in the Modulation of Active Tension in Isolated Guinea Pig Ventricular Myocytes. *Journal of Molecular and Cellular Cardiology* **29**, 1629-1637.

Cazorla, O., Vassort, G., Garnier, D., & Le Guennec, J.-Y. (1999). Length Modulation of Active Force in Rat Cardiac Myocytes: is Titin the Sensor? *Journal of Molecular and Cellular Cardiology* **31**, 1215-1227.

Cazorla, O., Wu, Y., Irving, T. C., & Granzier, H. (2001). Titin-Based Modulation of Calcium Sensitivity of Active Tension in Mouse Skinned Cardiac Myocytes. *Circ Res* **88**, 1028-1035.

Chen, X., Piacentino, V. 3., Furukawa, S., Goldman, B., Margulies, K. B., & Houser, S. R. (2002). L-type Ca2+ channel density and regulation are altered in failing human ventricular myocytes and recover after support with mechanical assist devices. *Circ Res* **91**, 517-24.

Cheng, W., Li, B., Kajstura, J., Li, P., Wolin, M. S., Sonnenblick, E. H., Hintze, T. H., Olivetti, G., & Anversa, P. (1995). Stretch-induced programmed myocyte cell death. *J Clin Invest* **96**, 2247-59.

Cleland, J. G. & McGowan, J. (2002). Levosimendan: a new era for inodilator therapy for heart failure? *Curr.Opin.Cardiol* **17**, 257-265.

Cleland, J. G. F., Takala, A., Apajasalo, M., Zethraeus, N., & Kobelt, G. (2003). Intravenous levosimendan treatment is cost-effective compared with dobutamine in severe low-output heart failure: an analysis based on the international LIDO trial. *European Journal of Heart Failure* **5**, 101-108.

Clement, O., Puceat, M., Walsh, M. P., & Vassort G. (1992). Protein kinase C enhances myosin light-chain kinase effects on force development and ATPase activity in rat single skinned cardiac cells. *Biochem J.* **285**, 311-7.

Collins, J. F., Pawloski-Dahm, C., Davis, M. G., Ball, N., Dorn, G. W., & Walsh, R. A. (1996). The role of the cytoskeleton in left ventricular pressure overload hypertrophy and failure. *J Mol Cell Cardiol* **28**, 1435-1443.

Communal, C., Singh, K., Pimentel, D. R., & Colucci, W. S. (1998). Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. *Circulation* **98**, 1329-34.

Communal, C., Singh, K., Sawyer, D. B., & Colucci, W. S. (1999). Opposing effects of beta(1)- and beta(2)-adrenergic receptors on cardiac myocyte apoptosis : role of a pertussis toxin-sensitive G protein. *Circulation* **100**, 2210-2.

Cone, J., Wang, S., Tandon, N., Fong, M., Sun, B., Sakurai, K., Yoshitake, M., Kambayashi, J., & Liu, Y. (1999). Comparison of the effects of cilostazol and milrinone on intracellular cAMP levels and cellular function in platelets and cardiac cells. *J Cardiovasc.Pharmacol.* **34**, 497-504.

Conway, M. A., Allis, J., Ouwerkerk, R., Niioka, T., Rajagopalan, B., & Radda, G. K. (1991). Detection of low phosphocreatine to ATP ratio in failing hypertrophied human myocardium by 31P magnetic resonance spectroscopy. *Lancet* **338**, 973-6.

Cowie, M. R., Mosterd, A., Wood, D. A., Deckers, J. W., Poole-Wilson, P. A., Sutton, G. C., & Grobbee, D. E. (1997). The epidemiology of heart failure. *Eur Heart J* **18**, 208-25.

Craig, R. & Lehman, W. (2001). Crossbridge and tropomyosin positions observed in native, interacting thick and thin filaments. *J Mol Biol.* **311**, 1027-1036.

Craig, R., Padron, R., & Kendrick-Jones, J. (1987). Structural changes accompanying phosphorylation of tarantula muscle myosin filaments. *J Cell Biol.* **105**, 1319-1327.

Dage, R. C., Kariya, T., Hsieh, C. P., Roebel, L. E., Cheng, H. C., Schnettler, R. A., & Grisar, J. M. (1987). Pharmacology of enoximone. *Am.J Cardiol* **60**, 10C-14C.

Dantzig, J. A., Goldman, Y. E., Millar, N. C., Lacktis, J., & Homsher, E. (1992). Reversal of the cross-bridge force-generating transition by photogeneration of phosphate in rabbit psoas muscle fibres. *J Physiol* **451**, 247-278.

Dantzig, J. A., Hibberd, M. G., Trentham, D. R., & Goldman, Y. E. (1991). Cross-bridge kinetics in the presence of MgADP investigated by photolysis of caged ATP in rabbit psoas muscle fibres. *J Physiol* **432**, 639-680.

Danziger, R. S., Raffaeli, S., Moreno-Sanchez, R., Sakai, M., Capogrossi, M. C., Spurgeon, H. A., Hansford, R. G., & Lakatta, E. G. (1988). Extracellular ATP has a potent effect to enhance cytosolic calcium and contractility in single ventricular myocytes. *Cell Calcium* **9**, 193-199.

Davis, J. S., Hassanzadeh, S., Winitsky, S., Lin, H., Satorius, C., Vemuri, R., Aletras, A. H., Wen, H., & Epstein, N. D. (2001). The overall pattern of cardiac contraction depends on a spatial gradient of myosin regulatory light chain phosphorylation. *Cell* **107(5)**, 631-41.

de Cheffoy de Courcelles, de Loore, K., Freyne, E., & Janssen, P. A. (1992). Inhibition of human cardiac cyclic AMP-phosphodiesterases by R 80122, a new selective cyclic AMP-phosphodiesterase III inhibitor: a comparison with other cardiotonic compounds. *J Pharmacol.Exp.Ther.* **263**, 6-14.

De Mello, W. C. & Monterrubio, J. (2004). Intracellular and extracellular angiotensin II enhance the L-type calcium current in the failing heart. *Hypertension* **44**, 360-364.

de Roos, A., Doornbos, J., Luyten, P. R., Oosterwaal, L. J., van der Wall, E. E., & den Hollander, J. A. (1992). Cardiac metabolism in patients with dilated and hypertrophic cardiomyopathy: assessment with proton-decoupled P-31 MR spectroscopy. *J Magn Reson Imaging* **2**, 711-9.

de Tombe, P. P. (1998). Altered contractile function in heart failure. *Cardiovascular Research* **37**, 367-380.

De Young, M. B. & Scarpa, A. (1987). Extracellular ATP induces $Ca^{2+}$ transients in cardiac myocytes which are potentiated by norepinephrine. *FEBS Lett.* **223**, 53-58.

del Monte, F., Harding, S. E., Dec, G. W., Gwathmey, J. K., & Hajjar, R. J. (2002). Targeting phospholamban by gene transfer in human heart failure. *Circulation* **105**, 904-7.

Deten, A., Holzl, A., Leicht, M., Barth, W., & Zimmer, H. G. (2001). Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. *J Mol Cell Cardiol* **33**, 1191-207.

Dhalla, N. S., Dixon, I. M., Rupp, H., & Barwinsky, J. (1991). Experimental congestive heart failure due to myocardial infarction: sarcolemmal receptors and cation transporters. *Basic Res Cardiol* **86 Suppl 3**, 13-23.

Dong, W., Rosenfeld, S. S., Wang, C. K., Gordon, A. M., & Cheung, H. C. (1996). Kinetic studies of calcium binding to the regulatory site of troponin C from cardiac muscle. *J Biol.Chem.* **271**, 688-694.

Dong, W. J., Wang, C. K., Gordon, A. M., Rosenfeld, S. S., & Cheung, H. C. (1997). A kinetic model for the binding of Ca2+ to the regulatory site of troponin from cardiac muscle. *J Biol.Chem.* **272**, 19229-19235.

Drexler, H., Hanze, J., Finckh, M., Lu, W., Just, H., & Lang, R. E. (1989). Atrial natriuretic peptide in a rat model of cardiac failure. Atrial and ventricular mRNA, atrial content, plasma levels, and effect of volume loading. *Circulation* **79**, 620-33.

du Toit, E., Hofmann, D., McCarthy, J., & Pineda, C. (2001). Effect of levosimendan on myocardial contractility, coronary and peripheral blood flow, and arrhythmias during coronary artery ligation and reperfusion in the in vivo pig model. *Heart* **86**, 81-87.

Du Toit, E. F., Muller, C. A., McCarthy, J., & Opie, L. H. (1999). Levosimendan: Effects of a Calcium Sensitizer on Function and Arrhythmias and Cyclic Nucleotide Levels during Ischemia/Reperfusion in the Langendorff-Perfused Guinea Pig Heart. *J Pharmacol Exp Ther* **290**, 505-514.

Dzeja, P. P., Vitkevicius, K. T., Redfield, M. M., Burnett, J. C., & Terzic, A. (1999). Adenylate kinase-catalyzed phosphotransfer in the myocardium : increased contribution in heart failure. *Circ Res* **84**, 1137-43.

Edes, I., Kiss, E., Kitada, Y., Powers, F. M., Papp, J. G., Kranias, E. G., & Solaro, R. J. (1995). Effects of Levosimendan, a Cardiotonic Agent Targeted to Troponin C, on Cardiac Function and on Phosphorylation and $Ca^{2+}$ Sensitivity of Cardiac Myofibrils and Sarcoplasmic Reticulum in Guinea Pig Heart. *Circ Res* **77**, 107-113.

Eisenberg, E. & Hill, T. L. (1985). Muscle contraction and free energy transduction in biological systems. *Science* **227**, 999-1006.

El-Armouche, A., Pamminger, T., Ditz, D., Zolk, O., & Eschenhagen, T. (2004). Decreased protein and phosphorylation level of the protein phosphatase inhibitor-1 in failing human hearts. *Cardiovasc Res* **61**, 87-93.

Endoh, M. (2001). Mechanism of action of $Ca^{2+}$ sensitizers--update 2001. *Cardiovasc Drugs Ther* **15**, 397-403.

Fabiato, A. (1980). Sarcomere length dependence of calcium release from the sarcoplasmic reticulum of skinned cardiac cells demonstrated by differential microspectrophotometry with Arsenazo III (abstract). *J Gen Physiol.* **76**, 15a.

Fabiato, A. & Fabiato, F. (1975). Dependence of the contractile activation of skinned cardiac cells on the sarcomere length. *Nature* **256**, 54-56.

Fabiato, A. & Fabiato, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *J Physiol (Paris)* **75**, 463-505.

Fan, D., Wannenburg, T., & de Tombe, P. P. (1997). Decreased myocyte tension development and calcium responsiveness in rat right ventricular pressure overload. *Circulation* **95**, 2312-2317.

Farhadian, F., Contard, F., Sabri, A., Samuel, J. L., & Rappaport, L. (1996). Fibronectin and basement membrane in cardiovascular organogenesis and disease pathogenesis. *Cardiovasc.Res.* **32**, 433-442.

Fatkin, D., MacRae, C., Sasaki, T., Wolff, M. R., Porcu, M., Frenneaux, M., Atherton, J., Vidaillet, H. J., Jr., Spudich, S., De Girolami, U., Seidman, J. G., Seidman, C., Muntoni, F., Muehle, G., Johnson, W., & McDonough, B. (1999). Missense mutations in the rod domain of the lamin A/C gene as causes of dilated cardiomyopathy and conduction-system disease. *N.Engl.J Med.* **341**, 1715-1724.

Fedida, D. & Giles, W. R. (1991). Regional variations in action potentials and transient outward current in myocytes isolated from rabbit left ventricle. *J Physiol* **442**, 191-209.

Fentzke, R. C., Buck, S. H., Patel, J. R., Lin, H., Wolska, B. M., Stojanovic, M. O., Martin, A. F., Solaro, R. J., Moss, R. L., & Leiden, J. M. (1999). Impaired cardiomyocyte relaxation and diastolic function in transgenic mice expressing slow skeletal troponin I in the heart. *J Physiol (Lond)* **517**, 143-157.

Fischer, H. & Frohlicher, R. (1948). Wiederherstellung des normalen Herzschlages nach elektrisch erteugtem Kammerflimmern durch Adenosintriphosphorsaure. *Helv Physiol Pharmacol Acta* **3**, 155-156.

Fischer, Y., Becker, C., & Loken, C. (1999). Purinergic inhibition of glucose transport in cardiomyocytes. *J Biol.Chem.* **274**, 755-761.

Flashman, E., Redwood, C., Moolman-Smook, J., & Watkins, H. (2004). Cardiac Myosin Binding Protein C: Its Role in Physiology and Disease. *Circ Res* **94**, 1279-1289.

Flesch, M., Schwinger, R. H., Schnabel, P., Schiffer, F., van, G., I, Bavendiek, U., Sudkamp, M., Kuhn-Regnier, F., & Bohm, M. (1996). Sarcoplasmic reticulum Ca2+ATPase and phospholamban mRNA and protein levels in end-stage heart failure due to ischemic or dilated cardiomyopathy. *J Mol Med.* **74**, 321-332.

Follath, F. (2003). Levosimendan in patients with low-output heart failure: lessons from the LIDO trial. *Ital.Heart J* **4 Suppl 2**, 34S-38S.

Follath, F., Cleland, J. G. F., Just, H., Papp, J. G. Y., Scholz, H., Peuhkurinen, K., Harjola, V. P., Mitrovic, V., Abdalla, M., & Sandell, E.-P. (2002b). Efficacy and safety of intravenous levosimendan compared with dobutamine in severe low-output heart failure (the LIDO study): a randomised double-blind trial. *The Lancet* **360**, 196-202.

Follath, F., Cleland, J. G. F., Just, H., Papp, J. G. Y., Scholz, H., Peuhkurinen, K., Harjola, V. P., Mitrovic, V., Abdalla, M., & Sandell, E.-P. (2002a). Efficacy and safety of intravenous levosimendan compared with dobutamine in severe low-output heart failure (the LIDO study): a randomised double-blind trial. *The Lancet* **360**, 196-202.

Fowler, M. B., Laser, J. A., Hopkins, G. L., Minobe, W., & Bristow, M. R. (1986). Assessment of the beta-adrenergic receptor pathway in the intact failing human heart: progressive receptor down-regulation and subsensitivity to agonist response. *Circulation* **74**, 1290-302.

Francis, G. S., Cohn, J. N., Johnson, G., Rector, T. S., Goldman, S., & Simon, A. (1993). Plasma norepinephrine, plasma renin activity, and congestive heart failure. Relations to survival and the effects of therapy in V-HeFT II. The V-HeFT VA Cooperative Studies Group. *Circulation* **87**, 40-8.

Freiburg, A., Trombitas, K., Hell, W., Cazorla, O., Fougerousse, F., Centner, T., Kolmerer, B., Witt, C., Beckmann, J. S., Gregorio, C. C., Granzier, H., & Labeit, S. (2000). Series of exon-skipping events in the elastic spring region of titin as the structural basis for myofibrillar elastic diversity. *Circ.Res.* **86**, 1114-1121.

Frey, N. & Olson, E. N. (2002). Calsarcin-3, a novel skeletal muscle-specific member of the calsarcin family, interacts with multiple Z-disc proteins. *J Biol.Chem.* **277**, 13998-14004.

Frodsham, G. & Jones, R. B. (1992). Effect of flosequinan upon isoenzymes of phosphodiesterase from guinea-pig cardiac and vascular smooth muscle. *Eur.J Pharmacol.* **211**, 383-391.

Fuchs, F. & Wang, Y.-P. (1996). Sarcomere Length Versus Interfilament Spacing as Determinants of Cardiac Myofilament $Ca^{2+}$ Sensitivity and $Ca^{2+}$ Binding. *Journal of Molecular and Cellular Cardiology* **28**, 1375-1383.

Fukuda, N., Fujita, H., Fujita, T., & Ishiwata, S. (1998). Regulatory roles of MgADP and calcium in tension development of skinned cardiac muscle. *J Muscle Res.Cell Motil.* **19**, 909-921.

Fukuda, N. & Granzier, H. (2004). Role of the giant elastic protein titin in the Frank-Starling mechanism of the heart. *Curr.Vasc.Pharmacol.* **2**, 135-139.

Fukuda, N., Kajiwara, H., Ishiwata, S., & Kurihara, S. (2000). Effects of MgADP on length dependence of tension generation in skinned rat cardiac muscle. *Circ.Res.* **86**, E1-E6.

Fukuda, N., Sasaki, D., Ishiwata, S., & Kurihara, S. (2001). Length Dependence of Tension Generation in Rat Skinned Cardiac Muscle: Role of Titin in the Frank-Starling Mechanism of the Heart. *Circulation* **104**, 1639-1645.

Fukuda, N., Wu, Y., Farman, G., Irving, T. C., & Granzier, H. (2003). Titin isoform variance and length dependence of activation in skinned bovine cardiac muscle. *J Physiol (Lond)* **553**, 147-154.

Furukawa, T., Bassett, A. L., Furukawa, N., Kimura, S., & Myerburg, R. J. (1993). The ionic mechanism of reperfusion-induced early afterdepolarizations in feline left ventricular hypertrophy. *J Clin Invest* **91**, 1521-31.

Furukawa, T., Ono, Y., Tsuchiya, H., Katayama, Y., Bang, M. L., Labeit, D., Labeit, S., Inagaki, N., & Gregorio, C. C. (2001). Specific interaction of the potassium channel beta-subunit minK with the sarcomeric protein T-cap suggests a T-tubule-myofibril linking system. *J Mol Biol.* **313**, 775-784.

Garnier, A., Fortin, D., Delomenie, C., Momken, I., Veksler, V., & Ventura-Clapier, R. (2003). Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. *J Physiol* **551**, 491-501.

Geeves, M. A. & Holmes, K. C. (1999). Structural mechanism of muscle contraction. *Annu.Rev.Biochem.* **68**, 687-728.

Gerull, B., Gramlich, M., Atherton, J., McNabb, M., Trombitas, K., Sasse-Klaassen, S., Seidman, J. G., Seidman, C., Granzier, H., Labeit, S., Frenneaux, M., & Thierfelder, L. (2002). Mutations of TTN, encoding the giant muscle filament titin, cause familial dilated cardiomyopathy. *Nat.Genet.* **30**, 201-204.

Gilbert, E. M., Sandoval, A., Larrabee, P., Renlund, D. G., O'Connell, J. B., & Bristow, M. R. (1993). Lisinopril lowers cardiac adrenergic drive and increases beta-receptor density in the failing human heart. *Circulation* **88**, 472-80.

Goldberg, A. T., Bond, B. R., Mukherjee, R., New, R. B., Zellner, J. L., Crawford, F. A., Jr., & Spinale, F. G. (2000). Endothelin receptor pathway in human left ventricular myocytes: relation to contractility. *Ann.Thorac.Surg.* **69**, 711-715.

Golstein, P. (1997). Controlling Cell Death. *Science* **275**, 1081-1082.

Gordon, A. M., Homsher, E., & Regnier, M. (2000). Regulation of contraction in striated muscle. *Physiol Rev.* **80**, 853-924.

Gordon, A. M., Regnier, M., & Homsher, E. (2001). Skeletal and cardiac muscle contractile activation: tropomyosin "rocks and rolls". *News Physiol Sci.* **16**, 49-55.

Gotthardt, M., Hammer, R. E., Hubner, N., Monti, J., Witt, C. C., McNabb, M., Richardson, J. A., Granzier, H., Labeit, S., & Herz, J. (2003). Conditional expression of mutant M-line titins results in cardiomyopathy with altered sarcomere structure. *J Biol.Chem.* **278**, 6059-6065.

Gottlieb, R. A., Burleson, K. O., Kloner, R. A., Babior, B. M., & Engler, R. L. (1994). Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* **94**, 1621-8.

Goulding, D., Bullard, B., & Gautel, M. (1997). A survey of in situ sarcomere extension in mouse skeletal muscle. *J Muscle Res.Cell Motil.* **18**, 465-472.

Granzier, H. L. & Irving T.C. (1995). Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. *Biophys J.* **68**, 1027-44.

Granzier, H. L. & Labeit, S. (2004). The Giant Protein Titin: A Major Player in Myocardial Mechanics, Signaling, and Disease. *Circ Res* **94**, 284-295.

Greaser, M. L., Berri, M., Warren, C. M., & Mozdziak, P. E. (2002). Species variations in cDNA sequence and exon splicing patterns in the extensible I-band region of cardiac titin: relation to passive tension. *J Muscle Res.Cell Motil.* **23**, 473-482.

Greenberg, B., Borghi, C., & Perrone, S. (2003). Pharmacotherapeutic approaches for decompensated heart failure: a role for the calcium sensitiser, levosimendan? *European Journal of Heart Failure* **5**, 13-21.

Gregorio, C. C., Trombitas, K., Centner, T., Kolmerer, B., Stier, G., Kunke, K., Suzuki, K., Obermayr, F., Herrmann, B., Granzier, H., Sorimachi, H., & Labeit, S. (1998). The NH2 terminus of titin spans the Z-disc: its interaction with a novel 19-kD ligand (T-cap) is required for sarcomeric integrity. *J Cell Biol.* **143**, 1013-1027.

Grossman, W., Jones, D., & McLaurin, L. P. (1975). Wall stress and patterns of hypertrophy in the human left ventricle. *J Clin Invest* **56**, 56-64.

Gruen, M., Prinz, H., & Gautel, M. (1999). cAPK-phosphorylation controls the interaction of the regulatory domain of cardiac myosin binding protein C with myosin-S2 in an on-off fashion. *FEBS Letters* **453**, 254-259.

Guarda, E., Katwa, L. C., Myers, P. R., Tyagi, S. C., & Weber, K. T. (1993). Effects of endothelins on collagen turnover in cardiac fibroblasts. *Cardiovasc Res* **27**, 2130-4.

Gulati, J., Sonnenblick, E., & Babu, A. (1991). The role of troponin C in the length dependence of Ca(2+)-sensitive force of mammalian skeletal and cardiac muscles. *J Physiol* **441**, 305-324.

Gurlek, A., Kilickap, M., Dincer, I., Dandachi, R., Tutkak, H., & Oral, D. (2001). Effect of losartan on circulating TNFalpha levels and left ventricular systolic performance in patients with heart failure. *J Cardiovasc Risk* **8**, 279-82.

Gustafsson, A. B. & Gottlieb, R. A. (2003). Mechanisms of apoptosis in the heart. *J Clin Immunol* **23**, 447-59.

Gwathmey, J. K., Copelas, L., MacKinnon, R., Schoen, F. J., Feldman, M. D., Grossman, W., & Morgan, J. P. (1987). Abnormal intracellular calcium handling in myocardium from patients with end-stage heart failure. *Circ.Res.* **61**, 70-76.

Haase, H., Pagel, I., Khalina, Y., Zacharzowsky, U., Person, V., Lutsch, G., Petzhold, D., Kott, M., Schaper, J., & Morano, I. (2004). The carboxyl-terminal ahnak domain induces actin bundling and stabilizes muscle contraction. *Faseb J* **18**, 839-841.

Haase, H., Podzuweit, T., Lutsch, G., Hohaus, A., Kostka, S., Lindschau, C., Kott, M., Kraft, R., & Morano, I. (1999). Signaling from beta-adrenoceptor to L-type calcium channel: identification of a novel cardiac protein kinase A target possessing similarities to AHNAK. *Faseb J* **13**, 2161-2172.

Haghighi, K., Kolokathis, F., Pater, L., Lynch, R. A., Asahi, M., Gramolini, A. O., Fan, G. C., Tsiapras, D., Hahn, H. S., Adamopoulos, S., Liggett, S. B., Dorn, G. W. 2., MacLennan, D. H., Kremastinos, D. T., & Kranias, E. G. (2003). Human phospholamban null results in lethal dilated cardiomyopathy revealing a critical difference between mouse and human. *J Clin Invest* **111**, 869-76.

Haikala, H., Levijoki, J., & Linden, I. B. (1995a). Troponin C-mediated calcium sensitization by levosimendan accelerates the proportional development of isometric tension. *J Mol Cell Cardiol* **27**, 2155-65.

Haikala, H., Nissinen, E., Etemadzadeh, E., Levijoki, J., & Linden, I. B. (1995b). Troponin C-mediated calcium sensitization induced by levosimendan does not impair relaxation. *J Cardiovasc.Pharmacol.* **25**, 794-801.

Haikala, H., Nissinen, E., Etemadzadeh, E., Linden, IB., & Pohto, P. (1992). Levosimendan increases Ca2+-sensitivity without enhancing myosin ATPase activity and impairing relaxation. *J Mol Cell Cardiol.* **24**, S.97.

Haikala, H., Kaheinen, P., Levijoki, J., & Linden, I.-B. (1997). The role of cAMP- and cGMP-dependent protein kinases in the cardiac actions of the new calcium sensitizer, levosimendan. *Cardiovascular Research* **34**, 536-546.

Haikala, H., Kaivola, J., Nissinen, E., Wall, P., Levijoki, J., & Linden, I.-B. (1995c). Cardiac troponin C as a target protein for a novel calcium sensitizing drug, levosimendan. *Journal of Molecular and Cellular Cardiology* **27**, 1859-1866.

Hannon, J. D., Martyn, D. A., & Gordon, A. M. (1992). Effects of cycling and rigor crossbridges on the conformation of cardiac troponin C. *Circ.Res.* **71**, 984-991.

Hardie, D. G. & Carling, D. (1997). The AMP-activated protein kinase--fuel gauge of the mammalian cell? *Eur J Biochem* **246**, 259-73.

Harris, S. P., Bartley, C. R., Hacker, T. A., McDonald, K. S., Douglas, P. S., Greaser, M. L., Powers, P. A., & Moss, R. L. (2002). Hypertrophic Cardiomyopathy in Cardiac Myosin Binding Protein-C Knockout Mice. *Circ Res* **90**, 594-601.

Hartzell, H. & Glass, D. (1984). Phosphorylation of purified cardiac muscle C-protein by purified cAMP- dependent and endogenous Ca2+-calmodulin-dependent protein kinases. *J.Biol.Chem.* **259**, 15587-15596.

Hasenfuss, G., Mulieri, L. A., Leavitt, B. J., Allen, P. D., Haeberle, J. R., & Alpert, N. R. (1992). Alteration of contractile function and excitation-contraction coupling in dilated cardiomyopathy. *Circ.Res.* **70**, 1225-1232.

Hasenfuss, G., Pieske, B., Castell, M., Kretschmann, B., Maier, L. S., & Just, H. (1998). Influence of the Novel Inotropic Agent Levosimendan on Isometric Tension and Calcium Cycling in Failing Human Myocardium. *Circulation* **98**, 2141-2147.

Hathaway, D. R. & Haeberle, J. R. (1985). A radioimmunoblotting method for measuring myosin light chain phosphorylation levels in smooth muscle. *Am.J Physiol* **249**, C345-C351.

Hazard, A. L., Kohout, S. C., Stricker, N. L., Putkey, J. A., & Falke, J. J. (1998). The kinetic cycle of cardiac troponin C: calcium binding and dissociation at site II trigger slow conformational rearrangements. *Protein Sci.* **7**, 2451-2459.

Hein, S., Kostin, S., Heling, A., Maeno, Y., & Schaper, J. (2000). The role of the cytoskeleton in heart failure. *Cardiovasc.Res.* **45**, 273-278.

Heinke, M. Y., Yao, M., Chang, D., Einstein, R., & dos Remedios, C. G. (2001). Apoptosis of ventricular and atrial myocytes from pacing-induced canine heart failure. *Cardiovasc Res* **49**, 127-34.

Helmes, M., Lim, C. C., Liao, R., Bharti, A., Cui, L., & Sawyer, D. B. (2003). Titin determines the Frank-Starling relation in early diastole. *J Gen Physiol.* **121(2)**, 97-110.

Herron, T. J., Korte, F. S., & McDonald, K. S. (2001). Loaded shortening and power output in cardiac myocytes are dependent on myosin heavy chain isoform expression. *Am.J Physiol Heart Circ.Physiol* **281**, H1217-H1222.

Hibberd, M. G. & Jewell, B. R. (1982). Calcium- and length-dependent force production in rat ventricular muscle. *J Physiol* **329**, 527-540.

Hirano, Y., Abe, S., Sawanobori, T., & Hiraoka, M. (1991). External ATP-induced changes in [Ca2+]i and membrane currents in mammalian atrial myocytes. *Am.J Physiol* **260**, C673-C680.

Hirzel, H. O., Tuchschmid, C. R., Schneider, J., Krayenbuehl, H. P., & Schaub, M. C. (1985). Relationship between myosin isoenzyme composition, hemodynamics, and myocardial structure in various forms of human cardiac hypertrophy. *Circ.Res.* **57**, 729-740.

Hittinger, L., Shannon, R. P., Kohin, S., Manders, W. T., Kelly, P., & Vatner, S. F. (1990). Exercise-induced subendocardial dysfunction in dogs with left ventricular hypertrophy. *Circ Res* **66**, 329-43.

Hofmann, P., Hartzell, H., & Moss, R. (1991). Alterations in Ca2+ sensitive tension due to partial extraction of C- protein from rat skinned cardiac myocytes and rabbit skeletal muscle fibers. *J.Gen.Physiol.* **97**, 1141-1163.

Hohaus, A., Person, V., Behlke, J., Schaper, J., Morano, I., & Haase, H. (2002). The carboxyl-terminal region of ahnak provides a link between cardiac L-type Ca2+ channels and the actin-based cytoskeleton. *Faseb J* **16**, 1205-1216.

Holmes, K. C. (1997). The swinging lever-arm hypothesis of muscle contraction. *Curr.Biol.* **7**, R112-R118.

Holubarsch, C., Ruf, T., Goldstein, D. J., Ashton, R. C., Nickl, W., Pieske, B., Pioch, K., Ludemann, J., Wiesner, S., Hasenfuss, G., Posival, H., Just, H., & Burkhoff, D. (1996). Existence of the Frank-Starling Mechanism in the Failing Human Heart: Investigations on the Organ, Tissue, and Sarcomere Levels. *Circulation* **94**, 683-689.

Hopkins, S. C., Sabido-David, C., Corrie, J. E., Irving, M., & Goldman, Y. E. (1998). Fluorescence polarization transients from rhodamine isomers on the myosin regulatory light chain in skeletal muscle fibers. *Biophys.J* **74**, 3093-3110.

Hoshijima, M., Ikeda, Y., Iwanaga, Y., Minamisawa, S., Date, M. O., Gu, Y., Iwatate, M., Li, M., Wang, L., Wilson, J. M., Wang, Y., Ross, J., Jr., & Chien, K. R. (2002). Chronic suppression of heart-failure progression by a pseudophosphorylated mutant of phospholamban via in vivo cardiac rAAV gene delivery. *Nat Med* **8**, 864-71.

Houser, S. R., Freeman, A. R., Jaeger, J. M., Breisch, E. A., Coulson, R. L., Carey, R., & Spann, J. F. (1981). Resting potential changes associated with Na-K pump in failing heart muscle. *Am J Physiol* **240**, 168-76.

Howard, J. & Spudich, J. A. (1996). Is the lever arm of myosin a molecular elastic element? *Proc.Natl.Acad.Sci.U.S.A* **93**, 4462-4464.

Howell, W. H. & Donaldson, F. Jr. (1884). Experiments upon the heart of the dog with reference to the maximal volume of blood sent out by the left ventricle in a single beat, and the influence of variations in venous pressure, arterial pressure, and pulse rate upon the work done by the heart. *Philos Trans Roy Soc Lond Part I.* **175**, 139-160.

Huxley, A. F. (1957). Muscle structure and theories of contraction. *Prog.Biophys.Biophys.Chem.* **7**, 255-318.

Huxley, A. F. & Simmons, R. M. (1971). Proposed mechanism of force generation in striated muscle. *Nature* **233**, 533-538.

Huxley, H. E., Stewart, A., Sosa, H., & Irving, T. (1994). X-ray diffraction measurements of the extensibility of actin and myosin filaments in contracting muscle. *Biophys.J* **67**, 2411-2421.

Ingwall, J. S. & Weiss, R. G. (2004). Is the Failing Heart Energy Starved?: On Using Chemical Energy to Support Cardiac Function. *Circ Res* **95**, 135-145.

Irving, M., St Claire, A. T., Sabido-David, C., Craik, J. S., Brandmeier, B., Kendrick-Jones, J., Corrie, J. E., Trentham, D. R., & Goldman, Y. E. (1995). Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle. *Nature* **375**, 688-691.

Irving, T. C., Konhilas, J., Perry, D., Fischetti, R., & de Tombe, P. P. (2000). Myofilament lattice spacing as a function of sarcomere length in isolated rat myocardium. *Am.J Physiol Heart Circ.Physiol* **279**, H2568-H2573.

Isaacs, W. B., Kim, I. S., Struve, A., & Fulton, A. B. (1992). Association of titin and myosin heavy chain in developing skeletal muscle. *Proc.Natl.Acad.Sci.U.S.A* **89**, 7496-7500.

Ishiwata, S. & Oosawa, F. (1974). A regulatory mechanism of muscle contraction based on the flexibility change of the thin filaments. *J Mechanochem.Cell Motil.* **3**, 9-17.

Itoh-Satoh, M., Hayashi, T., Nishi, H., Koga, Y., Arimura, T., Koyanagi, T., Takahashi, M., Hohda, S., Ueda, K., Nouchi, T., Hiroe, M., Marumo, F., Imaizumi, T., Yasunami, M., & Kimura, A. (2002). Titin mutations as the molecular basis for dilated cardiomyopathy. *Biochem.Biophys.Res.Commun.* **291**, 385-393.

Jamali, I. N., Kersten, J. R., Pagel, P. S., Hettrick, D. A., & Warltier, D. C. (1997). Intracoronary levosimendan enhances contractile function of stunned myocardium. *Anesth.Analg.* **85**, 23-29.

Janicki, J. S., Brower, G. L., Gardner, J. D., Chancey, A. L., & Stewart, J. A., Jr. (2004). The dynamic interaction between matrix metalloproteinase activity and adverse myocardial remodeling. *Heart Fail Rev* **9**, 33-42.

Jurevicius, J. & Fischmeister, R. (1996). cAMP compartmentation is responsible for a local activation of cardiac $Ca^{2+}$ channels by beta -adrenergic agonists. *PNAS* **93**, 295-299.

Kaab, S., Nuss, H. B., Chiamvimonvat, N., O'Rourke, B., Pak, P. H., Kass, D. A., Marban, E., & Tomaselli, G. F. (1996). Ionic mechanism of action potential prolongation in ventricular myocytes from dogs with pacing-induced heart failure. *Circ Res* **78**, 262-73.

Kaheinen, P., Pollesello, P., Levijoki, J., & Haikala, H. (2001). Levosimendan increases diastolic coronary flow in isolated guinea-pig heart by opening ATP-sensitive potassium channels. *J Cardiovasc.Pharmacol.* **37**, 367-374.

Kajiwara, H., Morimoto, S., Fukuda, N., Ohtsuki, I., & Kurihara, S. (2000). Effect of troponin I phosphorylation by protein kinase A on length-dependence of tension activation in skinned cardiac muscle fibers. *Biochem.Biophys.Res.Commun.* **272**, 104-110.

Kajstura, J., Cigola, E., Malhotra, A., Li, P., Cheng, W., Meggs, L. G., & Anversa, P. (1997). Angiotensin II induces apoptosis of adult ventricular myocytes in vitro. *J Mol Cell Cardiol* **29**, 859-70.

Kang, T., Humphrey, J. D., & Yin, F. C. (1996). Comparison of biaxial mechanical properties of excised endocardium and epicardium. *Am.J Physiol* **270**, H2169-H2176.

Kapadia, S. R., Oral, H., Lee, J., Nakano, M., Taffet, G. E., & Mann, D. L. (1997). Hemodynamic regulation of tumor necrosis factor-alpha gene and protein expression in adult feline myocardium. *Circ Res* **81**, 187-95.

Kass, R. S. & Blair, M. L. (1981). Effects of angiotensin II on membrane current in cardiac Purkinje fibers. *J Mol Cell Cardiol* **13**, 797-809.

Kaumann, A., Bartel, S., Molenaar, P., Sanders, L., Burrell, K., Vetter, D., Hempel, P., Karczewski, P., & Krause, E. G. (1999). Activation of beta2-adrenergic receptors hastens relaxation and mediates phosphorylation of phospholamban, troponin I, and C-protein in ventricular myocardium from patients with terminal heart failure. *Circulation* **99**, 65-72.

Keller, A., Demeurie, J., Merkulova, T., Geraud, G., Cywiner-Golenzer, C., Lucas, M., & Chatelet, F. P. (2000). Fibre-type distribution and subcellular localisation of alpha and beta enolase in mouse striated muscle. *Biol.Cell* **92**, 527-535.

Kellermayer, M. S., Smith, S. B., Bustamante, C., & Granzier, H. L. (2001). Mechanical fatigue in repetitively stretched single molecules of titin. *Biophys.J* **80**, 852-863.

Kelso, E. J., McDermott, B. J., & Silke, B. (1995). Actions of the novel vasodilator, flosequinan, in isolated ventricular cardiomyocytes. *J Cardiovasc.Pharmacol.* **25**, 376-386.

Kentish, J. C. & Wrzosek, A. (1998). Changes in force and cytosolic $Ca^{2+}$ concentration after length changes in isolated rat ventricular trabeculae. *J Physiol* **506 ( Pt 2)**, 431-444.

Kersten, J. R., Montgomery, M. W., Pagel, P. S., & Warltier, D. C. (2000). Levosimendan, a new positive inotropic drug, decreases myocardial infarct size via activation of $K(_{ATP})$ channels. *Anesth.Analg.* **90**, 5-11.

Kinbara, K., Sorimachi, H., Ishiura, S., & Suzuki, K. (1997). Muscle-specific calpain, p94, interacts with the extreme C-terminal region of connectin, a unique region flanked by two immunoglobulin C2 motifs. *Arch.Biochem.Biophys.* **342**, 99-107.

Kirchengast, M. & Munter, K. (1999). Endothelin-1 and endothelin receptor antagonists in cardiovascular remodeling. *Proc Soc Exp Biol Med* **221**, 312-25.

Kitsis, R. N. & Scheuer, J. (1996). Functional significance of alterations in cardiac contractile protein isoforms. *Clin.Cardiol* **19**, 9-18.

Kivikko, M., Antila, S., Eha, J., Lehtonen, L., & Pentikainen, P. (2002). Pharmacodynamics and safety of a new calcium sensitizer, levosimendan, and its metabolites during an extended infusion in patients with severe heart failure. *J Clin Pharmacol* **42**, 43-51.

Kivikko, M. L. L. C. WS. (2003). Sustained hemodynamic effects of intravenous levosimendan. *Circulation.* **107**, 81-6.

Knoll, R., Hoshijima, M., Hoffman, H. M., Person, V., Lorenzen-Schmidt, I., Bang, M. L., Hayashi, T., Shiga, N., Yasukawa, H., Schaper, W., McKenna, W., Yokoyama, M., Schork, N. J., Omens, J. H., McCulloch, A. D., Kimura, A., Gregorio, C. C., Poller, W., Schaper, J., Schultheiss, H. P., & Chien, K. R. (2002). The cardiac mechanical stretch sensor machinery involves a Z disc complex that is defective in a subset of human dilated cardiomyopathy. *Cell* **111**, 943-55.

Komiyama, M., Kouchi, K., Maruyama, K., & Shimada, Y. (1993). Dynamics of actin and assembly of connectin (titin) during myofibrillogenesis in embryonic chick cardiac muscle cells in vitro. *Dev.Dyn.* **196**, 291-299.

Konhilas, J. P., Irving, T. C., & de Tombe, P. P. (2002a). Length-dependent activation in three striated muscle types of the rat. *J Physiol (Lond)* **544**, 225-236.

Konhilas, J. P., Irving, T. C., & de Tombe, P. P. (2002b). Myofilament Calcium Sensitivity in Skinned Rat Cardiac Trabeculae: Role of Interfilament Spacing. *Circ Res* **90**, 59-65.

Konhilas, J. P., Irving, T. C., Wolska, B. M., Jweied, E. E., Martin, A. F., Solaro, R. J., & de Tombe, P. P. (2003). Troponin I in the murine myocardium: influence on length-dependent activation and interfilament spacing. *J Physiol (Lond)* **547**, 951-961.

Kontrogianni-Konstantopoulos, A. & Bloch, R. J. (2003). The hydrophilic domain of small ankyrin-1 interacts with the two N-terminal immunoglobulin domains of titin. *J Biol.Chem.* **278**, 3985-3991.

Korte, F. S., McDonald, K. S., Harris, S. P., & Moss, R. L. (2003). Loaded Shortening, Power Output, and Rate of Force Redevelopment Are Increased With Knockout of Cardiac Myosin Binding Protein-C. *Circ Res* 01.RES.0000096363.85588.9A.

Krayenbuehl, H. P., Hess, O. M., Schneider, J., & Turina, M. (1983). Physiologic or pathologic hypertrophy. *Eur Heart J* **4 Suppl A**, 29-34.

Kristof, E., Szigeti, G., Papp, Z., Bodi, A., Ball, N. A., Walsh, R. A., & Edes, I. (1999). The effects of levosimendan on the left ventricular function and protein phosphorylation in post-ischemic guinea pig hearts. *Basic Res.Cardiol* **94**, 223-230.

Kristof, E., Szigeti, G., Papp, Z., Bodi, A., Facsko, A., Kovacs, L., Papp, J. G., Kranias, E. G., & Edes, I. (1998). Cardiac responses to calcium sensitizers and isoproterenol in intact guinea pig hearts. Effects on cyclic AMP levels, protein phosphorylation, myoplasmic calcium concentration, and left ventricular function. *Ann.N.Y.Acad.Sci.* **853**, 316-319.

Krizanova, O., Orlicky, J., Masanova, C., Juhaszova, M., & Hudecova, S. (1997). Angiotensin I modulates Ca-transport systems in the rat heart through angiotensin II. *J Mol Cell Cardiol* **29**, 1739-46.

Krown, K. A., Page, M. T., Nguyen, C., Zechner, D., Gutierrez, V., Comstock, K. L., Glembotski, C. C., Quintana, P. J., & Sabbadini, R. A. (1996). Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest* **98**, 2854-65.

Kulikovskaya, I., McClellan, G., Flavigny, J., Carrier, L., & Winegrad, S. (2003). Effect of MyBP-C Binding to Actin on Contractility in Heart Muscle. *J.Gen.Physiol.* **122**, 761-774.

Kulke, M., Fujita-Becker, S., Rostkova, E., Neagoe, C., Labeit, D., Manstein, D. J., Gautel, M., & Linke, W. A. (2001). Interaction between PEVK-titin and actin filaments: origin of a viscous force component in cardiac myofibrils. *Circ.Res.* **89**, 874-881.

Kunst, G., Kress, K. R., Gruen, M., Uttenweiler, D., Gautel, M., & Fink, R. H. A. (2000). Myosin Binding Protein C, a Phosphorylation-Dependent Force Regulator in Muscle That Controls the Attachment of Myosin Heads by Its Interaction With Myosin S2. *Circ Res* **86**, 51-58.

Kurihara, S. & Komukai, K. (1996). Cross-bridge-dependent changes in the intracellular $Ca^{2+}$ concentration in mammalian cardiac muscles. *Jpn.Heart J* **37**, 143-152.

Kuzmin, A. I., Lakomkin, V. L., Kapelko, V. I., & Vassort, G. (1998). Interstitial ATP level and degradation in control and postmyocardial infarcted rats. *Am.J Physiol* **275**, C766-C771.

Labeit, D., Watanabe, K., Witt, C., Fujita, H., Wu, Y., Lahmers, S., Funck, T., Labeit, S., & Granzier, H. (2003). Calcium-dependent molecular spring elements in the giant protein titin. *PNAS* 2235652100.

Labeit, S., Gautel, M., Lakey, A., & Trinick, J. (1992). Towards a molecular understanding of titin. *EMBO J* **11**, 1711-1716.

Labeit, S. & Kolmerer, B. (1995). Titins: giant proteins in charge of muscle ultrastructure and elasticity. *Science* **270**, 293-296.

Lahmers, S., Wu, Y., Call, D. R., Labeit, S., & Granzier, H. (2004). Developmental control of titin isoform expression and passive stiffness in fetal and neonatal myocardium. *Circ.Res.* **94**, 505-513.

Lancaster, M. K. & Cook, S. J. (1997). The effects of levosimendan on [Ca2+]i in guinea-pig isolated ventricular myocytes. *European Journal of Pharmacology* **339**, 97-100.

Lange, S., Auerbach, D., McLoughlin, P., Perriard, E., Schafer, B. W., Perriard, J. C., & Ehler, E. (2002). Subcellular targeting of metabolic enzymes to titin in heart muscle may be mediated by DRAL/FHL-2. *J Cell Sci.* **115**, 4925-4936.

Laugwitz, K. L., Moretti, A., Weig, H. J., Gillitzer, A., Pinkernell, K., Ott, T., Pragst, I., Stadele, C., Seyfarth, M., Schomig, A., & Ungerer, M. (2001). Blocking caspase-activated apoptosis improves contractility in failing myocardium. *Hum Gene Ther* **12**, 2051-63.

Laurita, K. R., Katra, R., Wible, B., Wan, X., & Koo, M. H. (2003). Transmural heterogeneity of calcium handling in canine. *Circ.Res.* **92**, 668-675.

Layland, J., Cave, A. C., Warren, C., Grieve, D. J., Sparks, E., Kentish, J. C., Solaro, R. J., & Shah, A. M. (2005). Protection against endotoxemia-induced contractile dysfunction in mice with cardiac-specific expression of slow skeletal troponin I. *Faseb J* **19**, 1137-1139.

Leclercq, J. F., Swynghedauw, B., Bouveret, P., Faucomprez, C., & Piguet, V. (1976). Myofibrillar ATPase, DNA and hydroxyproline content of human hypertrophied heart. *Eur.J Clin.Invest* **6**, 27-33.

Lee, J. A. & Allen, D. G. (1997). Calcium sensitisers: mechanisms of action and potential usefulness as inotropes. *Cardiovascular Research* **36**, 10-20.

Legssyer, A., Poggioli, J., Renard, D., & Vassort, G. (1988). ATP and other adenine compounds increase mechanical activity and inositol trisphosphate production in rat heart. *J Physiol* **401**, 185-199.

Lehmann, A., Boldt, J., & Kirchner, J. (2003). The role of Ca++-sensitizers for the treatment of heart failure. *Curr.Opin.Crit Care* **9**, 337-344.

Lehnart, S. E., Wehrens, X. H., & Marks, A. R. (2004). Calstabin deficiency, ryanodine receptors, and sudden cardiac death. *Biochem.Biophys.Res.Commun.* **322**, 1267-1279.

Lehtonen, L. A. (2001). Levosimendan: a parenteral calcium-sensitising drug with additional vasodilatory properties. *Expert Opin.Investig.Drugs* **10**, 955-970.

Levine, R. J., Kensler, R. W., Yang, Z., Stull, J. T., & Sweeney, H. L. (1996). Myosin light chain phosphorylation affects the structure of rabbit skeletal muscle thick filaments. *Biophys.J* **71**, 898-907.

Levine, R. J., Kensler, R. W., Yang, Z., & Sweeney, H. L. (1995). Myosin regulatory light chain phosphorylation and the production of functionally significant changes in myosin head arrangement on striated muscle thick filaments. *Biophys.J* **68**, 224S.

Li, D., Tapscoft, T., Gonzalez, O., Burch, P. E., Quinones, M. A., Zoghbi, W. A., Hill, R., Bachinski, L. L., Mann, D. L., & Roberts, R. (1999). Desmin mutation responsible for idiopathic dilated cardiomyopathy. *Circulation* **100**, 461-464.

Li, Y. Y., Feldman, A. M., Sun, Y., & McTiernan, C. F. (1998). Differential expression of tissue inhibitors of metalloproteinases in the failing human heart. *Circulation* **98**, 1728-34.

Liao, R., Jain, M., Cui, L., D'Agostino, J., Aiello, F., Luptak, I., Ngoy, S., Mortensen, R. M., & Tian, R. (2002). Cardiac-specific overexpression of GLUT1 prevents the development of heart failure attributable to pressure overload in mice. *Circulation* **106**, 2125-31.

Liao, R., Nascimben, L., Friedrich, J., Gwathmey, J. K., & Ingwall, J. S. (1996). Decreased energy reserve in an animal model of dilated cardiomyopathy. Relationship to contractile performance. *Circ Res* **78**, 893-902.

Lilleberg, J., Nieminen, M. S., Akkila, J., Heikkila, L., Kuitunen, A., Lehtonen, L., Verkkala, K., Mattila, S., & Salmenpera, M. (1998). Effects of a new calcium sensitizer, levosimendan, on haemodynamics, coronary blood flow and myocardial substrate utilization early after coronary artery bypass grafting. *Eur.Heart J* **19**, 660-668.

Lilleberg, J., Sundberg, S., & Nieminen, M. S. (1995). Dose-range study of a new calcium sensitizer, levosimendan, in patients with left ventricular dysfunction. *J Cardiovasc.Pharmacol.* **26 Suppl 1**, S63-S69.

Linck, B., Boknik, P., Eschenhagen, T., Muller, F. U., Neumann, J., Nose, M., Jones, L. R., Schmitz, W., & Scholz, H. (1996). Messenger RNA expression and immunological quantification of phospholamban and SR-Ca(2+)-ATPase in failing and nonfailing human hearts. *Cardiovasc.Res.* **31**, 625-632.

Lindner, M., Erdmann, E., & Beuckelmann, D. J. (1998). Calcium content of the sarcoplasmic reticulum in isolated ventricular myocytes from patients with terminal heart failure. *J Mol Cell Cardiol* **30**, 743-749.

Linke, W. A., Ivemeyer, M., Mundel, P., Stockmeier, M. R., & Kolmerer, B. (1998a). Nature of PEVK-titin elasticity in skeletal muscle. *Proc.Natl.Acad.Sci.U.S.A* **95**, 8052-8057.

Linke, W. A., Stockmeier, M. R., Ivemeyer, M., Hosser, H., & Mundel, P. (1998b). Characterizing titin's I-band Ig domain region as an entropic spring. *J Cell Sci.* **111**, 1567-1574.

Linzbach, A. J. (1960). Heart failure from the point of view of quantitative anatomy. *Am J Cardiol* **5**, 370-82.

Litovsky, S. H. & Antzelevitch, C. (1988). Transient outward current prominent in canine ventricular epicardium but not endocardium. *Circ.Res.* **62**, 116-126.

Liu, X., Shao, Q., & Dhalla, N. S. (1995a). Myosin Light Chain Phosphorylation in Cardiac Hypertrophy and Failure due to Myocardial Infarction. *Journal of Molecular and Cellular Cardiology* **27**, 2613-2621.

Liu, Y., Cigola, E., Cheng, W., Kajstura, J., Olivetti, G., Hintze, T. H., & Anversa, P. (1995b). Myocyte nuclear mitotic division and programmed myocyte cell death characterize the cardiac myopathy induced by rapid ventricular pacing in dogs. *Lab Invest* **73**, 771-87.

Liversage, A. D., Holmes, D., Knight, P. J., Tskhovrebova, L., & Trinick, J. (2001). Titin and the Sarcomere Symmetry Paradox. *Journal of Molecular Biology* **305**, 401-409.

Loffelholz, K., Dressler, H., & Lindmar, R. (1989). Effects of the phosphodiesterase inhibitor enoximone on the autonomic innervation of the isolated heart. *J Cardiovasc.Pharmacol.* **14**, S33-S39.

Lohse, M. J., Engelhardt, S., & Eschenhagen, T. (2003). What Is the Role of {beta}-Adrenergic Signaling in Heart Failure? *Circ Res* **93**, 896-906.

Lombardi, V., Piazzesi, G., Ferenczi, M. A., Thirlwell, H., Dobbie, I., & Irving, M. (1995). Elastic distortion of myosin heads and repriming of the working stroke in muscle. *Nature* **374**, 553-555.

Long, X., Boluyt, M. O., Hipolito, M. L., Lundberg, M. S., Zheng, J. S., O'Neill, L., Cirielli, C., Lakatta, E. G., & Crow, M. T. (1997). p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. *J Clin Invest* **99**, 2635-43.

Lowes, B. D., Minobe, W., Abraham, W. T., Rizeq, M. N., Bohlmeyer, T. J., Quaife, R. A., Roden, R. L., Dutcher, D. L., Robertson, A. D., Voelkel, N. F., Badesch, D. B., Groves, B. M., Gilbert, E. M., & Bristow, M. R. (1997). Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium. *J Clin.Invest* **100**, 2315-2324.

Lu, Z., Moss, R. L., & Walker, J. W. (1993). Tension transients initiated by photogeneration of MgADP in skinned skeletal muscle fibers. *J Gen Physiol* **101**, 867-888.

MacKenna, D. A., Omens, J. H., & Covell, J. W. (1996). Left ventricular perimysial collagen fibers uncoil rather than stretch during diastolic filling. *Basic Res.Cardiol* **91**, 111-122.

MacKenna, D. A., Omens, J. H., McCulloch, A. D., & Covell, J. W. (1994). Contribution of collagen matrix to passive left ventricular mechanics in isolated rat hearts. *Am.J Physiol* **266**, H1007-H1018.

Maeda, M., Holder, E., Lowes, B., Valent, S., & Bies, R. D. (1997). Dilated cardiomyopathy associated with deficiency of the cytoskeletal protein metavinculin. *Circulation* **95**, 17-20.

Malinin, N. L., Boldin, M. P., Kovalenko, A. V., & Wallach, D. (1997). MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* **385**, 540-544.

Mann, D. L., Kent, R. L., Parsons, B., & Cooper, G. t. (1992). Adrenergic effects on the biology of the adult mammalian cardiocyte. *Circulation* **85**, 790-804.

Mann, D. L. (1999). Mechanisms and Models in Heart Failure : A Combinatorial Approach. *Circulation* **100**, 999-1008.

Mann, D. L. (2002). Inflammatory Mediators and the Failing Heart: Past, Present, and the Foreseeable Future. *Circ Res* **91**, 988-998.

Margossian, S. S., White, H. D., Caulfield, J. B., Norton, P., Taylor, S., & Slayter, H. S. (1992). Light chain 2 profile and activity of human ventricular myosin during dilated cardiomyopathy. Identification of a causal agent for impaired myocardial function. *Circulation* **85**, 1720-1733.

Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H., & Kikuchi, M. (1984). Molecular size and shape of beta-connectin, an elastic protein of striated muscle. *J Biochem.(Tokyo)* **95**, 1423-1433.

Marx, S. O., Reiken, S., Hisamatsu, Y., Jayaraman, T., Burkhoff, D., Rosemblit, N., & Marks, A. R. (2000). PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell* **101**, 365-376.

Mayans, O., van der Ven, P. F., Wilm, M., Mues, A., Young, P., Furst, D. O., Wilmanns, M., & Gautel, M. (1998a). Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature* **395(6705)**, 863-9.

Mayans, O., van der Ven, P. F., Wilm, M., Mues, A., Young, P., Furst, D. O., Wilmanns, M., & Gautel, M. (1998b). Structural basis for activation of the titin kinase domain during myofibrillogenesis. *Nature* **395(6705)**, 863-9.

McDonald, K. S., Field, L. J., Parmacek, M. S., Soonpaa, M., Leiden, J. M., & Moss, R. L. (1995). Length dependence of Ca2+ sensitivity of tension in mouse cardiac myocytes expressing skeletal troponin C. *J Physiol* **483 ( Pt 1)**, 131-139.

McDonald, K. S. & Moss, R. L. (1995). Osmotic compression of single cardiac myocytes eliminates the reduction in Ca2+ sensitivity of tension at short sarcomere length. *Circ.Res.* **77**, 199-205.

McElhinny, A. S., Kakinuma, K., Sorimachi, H., Labeit, S., & Gregorio, C. C. (2002). Muscle-specific RING finger-1 interacts with titin to regulate sarcomeric M-line and thick filament structure and may have nuclear functions via its interaction with glucocorticoid modulatory element binding protein-1. *J Cell Biol.* **157**, 125-136.

McIntosh, M. A., Cobbe, S. M., & Smith, G. L. (2000). Heterogeneous changes in action potential and intracellular Ca2+ in left ventricular myocyte sub-types from rabbits with heart failure. *Cardiovascular Research* **45**, 397-409.

McKillop, D. F. & Geeves, M. A. (1993). Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys.J* **65**, 693-701.

Mebazaa, A. & Erhardt, L. (2003). Levosimendan: a new dual-action drug in the treatment of acute heart failure. *Int.J Clin.Pract.* **57**, 410-416.

Metzger, J. M., Greaser, M. L., & Moss, R. L. (1989). Variations in cross-bridge attachment rate and tension with phosphorylation of myosin in mammalian skinned skeletal muscle fibers. Implications for twitch potentiation in intact muscle. *J Gen Physiol* **93**, 855-883.

Mewes, T. & Ravens, U. (1994). L-type calcium currents of human myocytes from ventricle of non-failing and failing hearts and from atrium. *J Mol Cell Cardiol* **26**, 1307-20.

Meyer, M., Schillinger, W., Pieske, B., Holubarsch, C., Heilmann, C., Posival, H., Kuwajima, G., Mikoshiba, K., Just, H., & Hasenfuss, G. (1995). Alterations of Sarcoplasmic Reticulum Proteins in Failing Human Dilated Cardiomyopathy. *Circulation* **92**, 778-784.

Miller, L. W. (2003). Limitations of current medical therapies for the treatment of heart failure. *Rev.Cardiovasc.Med.* **4 Suppl 2**, S21-S29.

Miller, M. K., Bang, M. L., Witt, C. C., Labeit, D., Trombitas, C., Watanabe, K., Granzier, H., McElhinny, A. S., Gregorio, C. C., & Labeit, S. (2003). The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. *J Mol Biol.* **333**, 951-964.

Millevoi, S., Trombitas, K., Kolmerer, B., Kostin, S., Schaper, J., Pelin, K., Granzier, H., & Labeit, S. (1998). Characterization of nebulette and nebulin and emerging concepts of their roles for vertebrate Z-discs. *J Mol Biol.* **282**, 111-123.

Milner, D. J., Weitzer, G., Tran, D., Bradley, A., & Capetanaki, Y. (1996b). Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *J Cell Biol.* **134**, 1255-1270.

Milner, D. J., Weitzer, G., Tran, D., Bradley, A., & Capetanaki, Y. (1996a). Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *J Cell Biol.* **134**, 1255-1270.

Minajeva, A., Kulke, M., Fernandez, J. M., & Linke, W. A. (2001). Unfolding of titin domains explains the viscoelastic behavior of skeletal myofibrils. *Biophys.J* **80**, 1442-1451.

Miyata, S., Minobe, W., Bristow, M. R., & Leinwand, L. A. (2000). Myosin heavy chain isoform expression in the failing and nonfailing human heart. *Circ.Res.* **86**, 386-390.

Moiseyev, V. S., Poder, P., Andrejevs, N., Ruda, M. Y., Golikov, A. P., Lazebnik, L. B., Kobalava, Z. D., Lehtonen, L. A., Laine, T., & Nieminen, M. S. (2002). Safety and efficacy of a novel calcium sensitizer, levosimendan, in patients with left ventricular failure due to an acute myocardial infarction. A randomized, placebo-controlled, double-blind study (RUSSLAN). *European Heart Journal* **23**, 1422-1432.

Mongillo, M., McSorley, T., Evellin, S., Sood, A., Lissandron, V., Terrin, A., Huston, E., Hannawacker, A., Lohse, M. J., Pozzan, T., Houslay, M. D., & Zaccolo, M. (2004). Fluorescence resonance energy transfer-based analysis of cAMP dynamics in live neonatal rat cardiac myocytes reveals distinct functions of compartmentalized phosphodiesterases. *Circ.Res.* **95**, 67-75.

Morano, I., Lengsfeld, M., Ganten, U., Ganten, D., & Ruegg, J. C. (1988). Chronic hypertension changes myosin isoenzyme pattern and decreases myosin phosphorylation in the rat heart. *J Mol Cell Cardiol.* **20**, 875-86.

Morano, I. (1999). Tuning the human heart molecular motors by myosin light chains. *J Mol Med.* **77**, 544-555.

Morano, I., Hadicke, K., Haase, H., Bohm, M., Erdmann, E., & Schaub, M. C. (1997). Changes in essential myosin light chain isoform expression provide a molecular basis for isometric force regulation in the failing human heart. *J Mol Cell Cardiol* **29**, 1177-1187.

Morano, I., Hadicke, K., Grom, S., Koch, A., Schwinger, R. H. G., Bohm, M., Bartel, S., Erdmann, E., & Krause, E.-G. (1994). Titin, Myosin Light Chains and C-Protein in the Developing and Failing Human Heart. *Journal of Molecular and Cellular Cardiology* **26**, 361-368.

Moss, R. L., Nwoye, L. O., & Greaser, M. L. (1991). Substitution of cardiac troponin C into rabbit muscle does not alter the length dependence of Ca2+ sensitivity of tension. *J Physiol* **440**, 273-289.

Moss, R. L., Razumova, M., & Fitzsimons, D. P. (2004). Myosin Crossbridge Activation of Cardiac Thin Filaments: Implications for Myocardial Function in Health and Disease. *Circ Res* **94**, 1290-1300.

Muranyi, A., MacDonald, J. A., Deng, J. T., Wilson, D. P., Haystead, T. A., Walsh, M. P., Erdodi, F., Kiss, E., Wu, Y., & Hartshorne, D. J. (2002). Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. *Biochem J.* **366**, 211-6.

Nagashima, H. & Asakura, S. (1982). Studies on co-operative properties of tropomyosin-actin and tropomyosin-troponin-actin complexes by the use of N-ethylmaleimide-treated and untreated species of myosin subfragment 1. *J Mol Biol.* **155**, 409-428.

Naito, H., Arisawa, J., Harada, K., Yamagami, H., Kozuka, T., & Tamura, S. (1996). Normal nonuniformity of left ventricular contraction. Assessment by cine MR imaging with presaturation myocardial tagging. *Acta Radiol.* **37**, 767-774.

Nakae, I., Mitsunami, K., Omura, T., Yabe, T., Tsutamoto, T., Matsuo, S., Takahashi, M., Morikawa, S., Inubushi, T., Nakamura, Y., Kinoshita, M., & Horie, M. (2003). Proton magnetic resonance spectroscopy can detect creatine depletion associated with the progression of heart failure in cardiomyopathy. *J Am Coll Cardiol* **42**, 1587-93.

Nakao, K., Minobe, W., Roden, R., Bristow, M. R., & Leinwand, L. A. (1997). Myosin heavy chain gene expression in human heart failure. *J Clin.Invest* **100**, 2362-2370.

Narolska, N. A., Eiras, S., van Loon, R. B., Boontje, N. M., Zaremba, R., Spiegelen, B., Sr., Stooker, W., Huybregts, M. A., Visser, F. C., van, d., V, & Stienen, G. J. (2005). Myosin heavy chain composition and the economy of contraction in healthy and diseased human myocardium. *J Muscle Res.Cell Motil.* 1-10.

Narula, J., Haider, N., Virmani, R., DiSalvo, T. G., Kolodgie, F. D., Hajjar, R. J., Schmidt, U., Semigran, M. J., Dec, G. W., & Khaw, B. A. (1996). Apoptosis in myocytes in end-stage heart failure. *N Engl J Med* **335**, 1182-9.

Nascimben, L., Ingwall, J. S., Pauletto, P., Friedrich, J., Gwathmey, J. K., Saks, V., Pessina, A. C., & Allen, P. D. (1996). Creatine kinase system in failing and nonfailing human myocardium. *Circulation* **94**, 1894-901.

Neagoe, C., Kulke, M., del Monte, F., Gwathmey, J. K., de Tombe, P. P., Hajjar, R. J., & Linke, W. A. (2002). Titin Isoform Switch in Ischemic Human Heart Disease. *Circulation* **106**, 1333-1341.

Neubauer, S., Horn, M., Cramer, M., Harre, K., Newell, J. B., Peters, W., Pabst, T., Ertl, G., Hahn, D., Ingwall, J. S., & Kochsiek, K. (1997). Myocardial phosphocreatine-to-ATP ratio is a predictor of mortality in patients with dilated cardiomyopathy. *Circulation* **96**, 2190-6.

Neubauer, S., Remkes, H., Spindler, M., Horn, M., Wiesmann, F., Prestle, J., Walzel, B., Ertl, G., Hasenfuss, G., & Wallimann, T. (1999). Downregulation of the Na(+)-creatine cotransporter in failing human myocardium and in experimental heart failure. *Circulation* **100**, 1847-50.

Neumann, J. (2002). Altered phosphatase activity in heart failure, influence on $Ca^{2+}$ movement. *Basic Res Cardiol* **97 Suppl 1**, 91-5.

Nian, M., Lee, P., Khaper, N., & Liu, P. (2004). Inflammatory Cytokines and Postmyocardial Infarction Remodeling. *Circ Res* **94**, 1543-1553.

Nicholas, G., Thomas, M., Langley, B., Somers, W., Patel, K., Kemp, C. F., Sharma, M., & Kambadur, R. (2002). Titin-cap associates with, and regulates secretion of, Myostatin. *J Cell Physiol* **193**, 120-131.

Nieminen, M. S., Akkila, J., Hasenfuss, G., Kleber, F. X., Lehtonen, L. A., Mitrovic, V., Nyquist, O., & Remme, W. J. (2000). Hemodynamic and neurohumoral effects of continuous infusion of levosimendan in patients with congestive heart failure. *J Am.Coll.Cardiol* **36**, 1903-1912.

Nuss, H. B. & Houser, S. R. (1993). T-type Ca2+ current is expressed in hypertrophied adult feline left ventricular myocytes. *Circ.Res.* **73**, 777-782.

O'Rourke, B., Kass, D. A., Tomaselli, G. F., Kaab, S., Tunin, R., & Marban, E. (1999). Mechanisms of altered excitation-contraction coupling in canine tachycardia-induced heart failure, I: experimental studies. *Circ.Res.* **84**, 562-570.

Oakley, C. E., Hambly, B. D., Curmi, P. M., & Brown, L. J. (2004). Myosin binding protein C: structural abnormalities in familial hypertrophic cardiomyopathy. *Cell Res.* **14**, 95-110.

Ohtsuki, I. & Shiraishi, F. (2002). Periodic binding of troponin C.I and troponin I to tropomyosin-actin filaments. *J Biochem.(Tokyo)* **131**, 739-743.

Olivetti, G., Abbi, R., Quaini, F., Kajstura, J., Cheng, W., Nitahara, J. A., Quaini, E., Di Loreto, C., Beltrami, C. A., Krajewski, S., Reed, J. C., & Anversa, P. (1997). Apoptosis in the failing human heart. *N Engl J Med* **336**, 1131-41.

Ono, Y., Kakinuma, K., Torii, F., Irie, A., Nakagawa, K., Labeit, S., Abe, K., Suzuki, K., & Sorimachi, H. (2004). Possible regulation of the conventional calpain system by skeletal muscle-specific calpain, p94/calpain 3. *J Biol.Chem.* **279**, 2761-2771.

Opie, L. H. (1998). *The heart: physiology, from cell to circulation*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, U.S.A.

Orrenius, S., McConkey, D. J., Bellomo, G., & Nicotera, P. (1989). Role of Ca2+ in toxic cell killing. *Trends Pharmacol Sci* **10**, 281-5.

Osorio, J. C., Stanley, W. C., Linke, A., Castellari, M., Diep, Q. N., Panchal, A. R., Hintze, T. H., Lopaschuk, G. D., & Recchia, F. A. (2002). Impaired myocardial fatty acid oxidation and reduced protein expression of retinoid X receptor-alpha in pacing-induced heart failure. *Circulation* **106**, 606-12.

Ouadid, H., Albat, B., & Nargeot, J. (1995). Calcium currents in diseased human cardiac cells. *J Cardiovasc Pharmacol* **25**, 282-91.

Pagani, E. D., Alousi, A. A., Grant, A. M., Older, T. M., Dziuban, S. W., Jr., & Allen, P. D. (1988). Changes in myofibrillar content and Mg-ATPase activity in ventricular tissues from

patients with heart failure caused by coronary artery disease, cardiomyopathy, or mitral valve insufficiency. *Circ.Res.* **63**, 380-385.

Pagel, P. S., Hettrick, D. A., & Warltier, D. C. (1996). Influence of levosimendan, pimobendan, and milrinone on the regional distribution of cardiac output in anaesthetized dogs. *Br J Pharmacol.* **119**, 609-615.

Palmer, B. M., Georgakopoulos, D., Janssen, P. M., Wang, Y., Alpert, N. R., Belardi, D. F., Harris, S. P., Moss, R. L., Burgon, P. G., Seidman, C. E., Seidman, J. G., Maughan, D. W., & Kass, D. A. (2004a). Role of Cardiac Myosin Binding Protein C in Sustaining Left Ventricular Systolic Stiffening. *Circ Res* **94**, 1249-1255.

Palmer, B. M., Noguchi, T., Wang, Y., Heim, J. R., Alpert, N. R., Burgon, P. G., Seidman, C. E., Seidman, J. G., Maughan, D. W., & LeWinter, M. M. (2004b). Effect of Cardiac Myosin Binding Protein-C on Mechanoenergetics in Mouse Myocardium. *Circ Res* **94**, 1615-1622.

Palmiter, K. A., Kitada, Y., Muthuchamy, M., Wieczorek, D. F., & Solaro, R. J. (1996). Exchange of beta- for alpha-tropomyosin in hearts of transgenic mice induces changes in thin filament response to Ca2+, strong cross-bridge binding, and protein phosphorylation. *J Biol.Chem.* **271**, 11611-11614.

Pan, B. S., Gordon, A. M., & Luo, Z. X. (1989). Removal of tropomyosin overlap modifies cooperative binding of myosin S-1 to reconstituted thin filaments of rabbit striated muscle. *J Biol.Chem.* **264**, 8495-8498.

Pan, B. S. & Solaro, R. J. (1987). Calcium-binding properties of troponin C in detergent-skinned heart muscle fibers. *J Biol.Chem.* **262**, 7839-7849.

Papp, Z., Csapo, K., Pollesello, P., Haikala, H., & Edes, I. (2005). Pharmacological mechanisms contributing to the clinical efficacy of levosimendan. *Cardiovasc.Drug Rev.* **23**, 71-98.

Papp, Z., van der Velden, J., & Stienen, G. J. (2000). Calpain-I induced alterations in the cytoskeletal structure and impaired mechanical properties of single myocytes of rat heart. *Cardiovasc Res* **45**, 981-93.

Pataricza, J., Hohn, J., Petri, A., Balogh, A., & Papp, J. G. (2000). Comparison of the vasorelaxing effect of cromakalim and the new inodilator, levosimendan, in human isolated portal vein. *J Pharm.Pharmacol.* **52**, 213-217.

Paulus, W. J. & Bronzwaer, J. G. (2004). Nitric oxide's role in the heart: control of beating or breathing? *Am.J Physiol Heart Circ.Physiol* **287**, H8-13.

Peckham, M., Young, P., & Gautel, M. (1997b). Constitutive and variable regions of Z-disk titin/connectin in myofibril formation: a dominant-negative screen. *Cell Struct.Funct.* **22**, 95-101.

Peckham, M., Young, P., & Gautel, M. (1997a). Constitutive and variable regions of Z-disk titin/connectin in myofibril formation: a dominant-negative screen. *Cell Struct.Funct.* **22**, 95-101.

Peng, J., Gurantz, D., Tran, V., Cowling, R. T., & Greenberg, B. H. (2002). Tumor necrosis factor-alpha-induced AT1 receptor upregulation enhances angiotensin II-mediated cardiac fibroblast responses that favor fibrosis. *Circ Res* **91**, 1119-26.

Perrie, W. T., Smillie, L. B., & Perry, S. V. (1972). A phosphorylated light-chain component of myosin. *Biochem.J* **128**, 105P-106P.

Pfeffer, M. A., Pfeffer, J. M., Fishbein, M. C., Fletcher, P. J., Spadaro, J., Kloner, R. A., & Braunwald, E. (1979). Myocardial infarct size and ventricular function in rats. *Circ.Res.* **44**, 503-512.

Pfeffer, M. A., Swedberg, K., Granger, C. B., Held, P., McMurray, J. J., Michelson, E. L., Olofsson, B., Ostergren, J., Yusuf, S., & Pocock, S. (2003). Effects of candesartan on mortality and morbidity in patients with chronic heart failure: the CHARM-Overall programme. *Lancet* **362**, 759-66.

Piacentino, V., III, Weber, C. R., Chen, X., Weisser-Thomas, J., Margulies, K. B., Bers, D. M., & Houser, S. R. (2003). Cellular basis of abnormal calcium transients of failing human ventricular myocytes. *Circ.Res.* **92**, 651-658.

Pieske, B., Beyermann, B., Breu, V., Loffler, B. M., Schlotthauer, K., Maier, L. S., Schmidt-Schweda, S., Just, H., & Hasenfuss, G. (1999). Functional effects of endothelin and regulation of endothelin receptors in isolated human nonfailing and failing myocardium. *Circulation* **99**, 1802-1809.

Pimentel-Muinos, F. X. & Seed, B. (1999). Regulated commitment of TNF receptor signaling: a molecular switch for death or activation. *Immunity* **11**, 783-93.

Pitt, B., Zannad, F., Remme, W. J., Cody, R., Castaigne, A., Perez, A., Palensky, J., & Wittes, J. (1999). The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators. *N Engl J Med* **341**, 709-17.

Podrasky, E., Xu, D., & Liang, B. T. (1997). A novel phospholipase C- and cAMP-independent positive inotropic mechanism via a P2 purinoceptor. *Am.J Physiol* **273**, H2380-H2387.

Pollesello, P., Ovaska, M., Kaivola, J., Tilgmann, C., Lundstrom, K., Kalkkinen, N., Ulmanen, I., Nissinen, E., & Taskinen, J. (1994). Binding of a new $Ca^{2+}$ sensitizer, levosimendan, to recombinant human cardiac troponin C. A molecular modelling, fluorescence probe, and proton nuclear magnetic resonance study [published erratum appears in J Biol Chem 1995 Feb 10;270(6):2880]. *J.Biol.Chem.* **269**, 28584-28590.

Prabhu, S. D., Chandrasekar, B., Murray, D. R., & Freeman, G. L. (2000). beta-adrenergic blockade in developing heart failure: effects on myocardial inflammatory cytokines, nitric oxide, and remodeling. *Circulation* **101**, 2103-9.

Pyle, W. G. & Solaro, R. J. (2004). At the Crossroads of Myocardial Signaling: The Role of Z-Discs in Intracellular Signaling and Cardiac Function. *Circ Res* **94**, 296-305.

Rauchhaus, M., Doehner, W., Francis, D. P., Davos, C., Kemp, M., Liebenthal, C., Niebauer, J., Hooper, J., Volk, H. D., Coats, A. J., & Anker, S. D. (2000). Plasma cytokine parameters and mortality in patients with chronic heart failure. *Circulation* **102**, 3060-7.

Razeghi, P., Young, M. E., Alcorn, J. L., Moravec, C. S., Frazier, O. H., & Taegtmeyer, H. (2001). Metabolic gene expression in fetal and failing human heart. *Circulation* **104**, 2923-2931.

Reeves, M. L., Leigh, B. K., & England, P. J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. Implications for the mechanism of action of selective phosphodiesterase inhibitors. *Biochem.J* **241**, 535-541.

Regnier, M., Rivera, A. J., Wang, C. K., Bates, M. A., Chase, P. B., & Gordon, A. M. (2002). Thin filament near-neighbour regulatory unit interactions affect rabbit skeletal muscle steady-state force-$Ca^{2+}$ relations. *J Physiol* **540**, 485-497.

Reiken, S., Gaburjakova, M., Guatimosim, S., Gomez, A. M., D'Armiento, J., Burkhoff, D., Wang, J., Vassort, G., Lederer, W. J., & Marks, A. R. (2003). Protein Kinase A Phosphorylation of the Cardiac Calcium Release Channel (Ryanodine Receptor) in Normal and Failing Hearts. Role of phosphatases and response to isoproterenol. *J.Biol.Chem.* **278**, 444-453.

Remondino, A., Kwon, S. H., Communal, C., Pimentel, D. R., Sawyer, D. B., Singh, K., & Colucci, W. S. (2003). Beta-adrenergic receptor-stimulated apoptosis in cardiac myocytes is mediated by reactive oxygen species/c-Jun NH2-terminal kinase-dependent activation of the mitochondrial pathway. *Circ Res* **92**, 136-8.

Rodriguez, E. K., Omens, J. H., Waldman, L. K., & McCulloch, A. D. (1993). Effect of residual stress on transmural sarcomere length distributions in rat left ventricle. *Am J Physiol* **264**, 1048-56.

Rozanski, G. J., Xu, Z., Whitney, R. T., Murakami, H., & Zucker, I. H. (1997). Electrophysiology of rabbit ventricular myocytes following sustained rapid ventricular pacing. *J Mol Cell Cardiol* **29**, 721-32.

Ryder, K. O., Bryant, S. M., & Hart, G. (1993). Membrane current changes in left ventricular myocytes isolated from guinea pigs after abdominal aortic coarctation. *Cardiovasc Res* **27**, 1278-87.

Sabbah, H. N., Sharov, V., Riddle, J. M., Kono, T., Lesch, M., & Goldstein, S. (1992). Mitochondrial abnormalities in myocardium of dogs with chronic heart failure. *J Mol Cell Cardiol* **24**, 1333-47.

Sabbah, H. N. (2000). Apoptotic cell death in heart failure. *Cardiovascular Research* **45**, 704-712.

Sanbe, A., Fewell, J. G., Gulick, J., Osinska, H., Lorenz, J., Hall, D. G., Murray, L. A., Kimball, T. R., Witt, S. A., & Robbins, J. (1999). Abnormal cardiac structure and function in mice expressing nonphosphorylatable cardiac regulatory myosin light chain 2. *J Biol.Chem.* **274**, 21085-21094.

Sato, S., Hassan Talukder, M. A., Sugawara, H., Sawada, H., & Endoh, M. (1998). Effects of Levosimendan on Myocardial Contractility and Ca2+Transients in Aequorin-loaded Right-ventricular Papillary Muscles and Indo-1-loaded Single Ventricular Cardiomyocytes of the Rabbit. *Journal of Molecular and Cellular Cardiology* **30**, 1115-1128.

Saupe, K. W., Spindler, M., Hopkins, J. C., Shen, W., & Ingwall, J. S. (2000). Kinetic, thermodynamic, and developmental consequences of deleting creatine kinase isoenzymes from the heart. Reaction kinetics of the creatine kinase isoenzymes in the intact heart. *J Biol Chem* **275**, 19742-6.

Scamps, F., Legssyer, A., Mayoux, E., & Vassort, G. (1990). The mechanism of positive inotropy induced by adenosine triphosphate in rat heart. *Circ.Res.* **67**, 1007-1016.

Scamps, F., Nilius, B., Alvarez, J., & Vassort, G. (1993). Modulation of L-type Ca channel activity by P2-purinergic agonist in cardiac cells. *Pflugers Arch.* **422**, 465-471.

Scamps, F., Rybin, V., Puceat, M., Tkachuk, V., & Vassort, G. (1992). A Gs protein couples P2-purinergic stimulation to cardiac Ca channels without cyclic AMP production. *J Gen Physiol* **100**, 675-701.

Scamps, F. & Vassort, G. (1990). Mechanism of extracellular ATP-induced depolarization in rat isolated ventricular cardiomyocytes. *Pflugers Arch.* **417**, 309-316.

Schaper, J., Froede, R., Hein, S., Buck, A., Hashizume, H., Speiser, B., Friedl, A., & Bleese, N. (1991). Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. *Circulation* **83**, 504-514.

Schaper, J., Hein, S., Scholz, D., & Mollnau, H. (1995). Multifaceted morphological alterations are present in the failing human heart. *J Mol Cell Cardiol.* **27**, 857-61.

Schaub, M. C., Hefti, M. A., Zuellig, R. A., & Morano, I. (1998). Modulation of contractility in human cardiac hypertrophy by myosin essential light chain isoforms. *Cardiovasc.Res.* **37**, 381-404.

Schmitt, J. P., Kamisago, M., Asahi, M., Li, G. H., Ahmad, F., Mende, U., Kranias, E. G., MacLennan, D. H., Seidman, J. G., & Seidman, C. E. (2003). Dilated cardiomyopathy and heart failure caused by a mutation in phospholamban. *Science* **299**, 1410-3.

Schroder, F., Handrock, R., Beuckelmann, D. J., Hirt, S., Hullin, R., Priebe, L., Schwinger, R. H., Weil, J., & Herzig, S. (1998). Increased availability and open probability of single L-type calcium channels from failing compared with nonfailing human ventricle. *Circulation* **98**, 969-76.

Schultheiss, H. P., Schulze, K., Schauer, R., Witzenbichler, B., & Strauer, B. E. (1995). Antibody-mediated imbalance of myocardial energy metabolism. A causal factor of cardiac failure? *Circ Res* **76**, 64-72.

Schwartz, K. & Mercadier, J. J. (1996). Molecular and cellular biology of heart failure. *Curr.Opin.Cardiol* **11**, 227-236.

Schwinger, R. H., Bohm, M., Koch, A., Schmidt, U., Morano, I., Eissner, H. J., Uberfuhr, P., Reichart, B., & Erdmann, E. (1994). The failing human heart is unable to use the Frank-Starling mechanism. *Circ Res.* **74**, 959-69.

Schwinger, R. H., Bohm, M., Schmidt, U., Karczewski, P., Bavendiek, U., Flesch, M., Krause, E. G., & Erdmann, E. (1995). Unchanged protein levels of SERCA II and phospholamban but reduced $Ca^{2+}$ uptake and $Ca^{2+}$-ATPase activity of cardiac sarcoplasmic reticulum from dilated cardiomyopathy patients compared with patients with nonfailing hearts. *Circulation* **92**, 3220-3228.

Scoote, M. & Williams, A. J. (2004). Myocardial calcium signalling and arrhythmia pathogenesis. *Biochem.Biophys.Res.Commun.* **322**, 1286-1309.

Shah, A., Kechejian, S. J., Kavaler, F., & Fisher, V. J. (1974). Effects of adenine nucleotides on contractility of normal and postischemic myocardium. *Am.Heart J* **87**, 740-749.

Shahid, M. & Nicholson, C. D. (1990). Comparison of cyclic nucleotide phosphodiesterase isoenzymes in rat and rabbit ventricular myocardium: positive inotropic and phosphodiesterase inhibitory effects of Org 30029, milrinone and rolipram. *Naunyn Schmiedebergs Arch.Pharmacol.* **342**, 698-705.

Shakur, Y., Fong, M., Hensley, J., Cone, J., Movsesian, M. A., Kambayashi, J., Yoshitake, M., & Liu, Y. (2002). Comparison of the effects of cilostazol and milrinone on cAMP-PDE activity, intracellular cAMP and calcium in the heart. *Cardiovasc.Drugs Ther.* **16**, 417-427.

Sharov, V. G., Sabbah, H. N., Shimoyama, H., Goussev, A. V., Lesch, M., & Goldstein, S. (1996). Evidence of cardiocyte apoptosis in myocardium of dogs with chronic heart failure. *Am J Pathol* **148**, 141-9.

Shen, W., Asai, K., Uechi, M., Mathier, M. A., Shannon, R. P., Vatner, S. F., & Ingwall, J. S. (1999). Progressive loss of myocardial ATP due to a loss of total purines during the development of heart failure in dogs: a compensatory role for the parallel loss of creatine. *Circulation* **100**, 2113-8.

Shimizu, H., Fujita, T., & Ishiwata, S. (1992). Regulation of tension development by MgADP and Pi without $Ca^{2+}$. Role in spontaneous tension oscillation of skeletal muscle. *Biophys.J* **61**, 1087-1098.

Shimoyama, M., Hayashi, D., Takimoto, E., Zou, Y., Oka, T., Uozumi, H., Kudoh, S., Shibasaki, F., Yazaki, Y., Nagai, R., & Komuro, I. (1999). Calcineurin plays a critical role in pressure overload-induced cardiac hypertrophy. *Circulation* **100**, 2449-2454.

Sicouri, S. & Antzelevitch, C. (1991). A subpopulation of cells with unique electrophysiological properties in the deep subepicardium of the canine ventricle. The M cell. *Circ.Res.* **68**, 1729-1741.

Siedner, S., Kruger, M., Schroeter, M., Metzler, D., Roell, W., Fleischmann, B. K., Hescheler, J., Pfitzer, G., & Stehle, R. (2003). Developmental changes in contractility and sarcomeric proteins from the early embryonic to the adult stage in the mouse heart. *J Physiol* **548**, 493-505.

Silver, M. A., Pick, R., Brilla, C. G., Jalil, J. E., Janicki, J. S., & Weber, K. T. (1990). Reactive and reparative fibrillar collagen remodelling in the hypertrophied rat left ventricle: two experimental models of myocardial fibrosis. *Cardiovasc Res* **24**, 741-7.

Slawsky, M. T., Colucci, W. S., Gottlieb, S. S., Greenberg, B. H., Haeusslein, E., Hare, J., Hutchins, S., Leier, C. V., LeJemtel, T. H., Loh, E., Nicklas, J., Ogilby, D., Singh, B., Smith, W., & on behalf of the study investigators (2000). Acute hemodynamic and clinical effects of levosimendan in patients with severe heart failure. Study Investigators. *Circulation* **102**, 2222-7.

Smith, D. A. & Geeves, M. A. (2003). Cooperative regulation of myosin-actin interactions by a continuous flexible chain II: actin-tropomyosin-troponin and regulation by calcium. *Biophys.J* **84**, 3168-3180.

Soeno, Y., Yajima, H., Kawamura, Y., Kimura, S., Maruyama, K., & Obinata, T. (1999). Organization of connectin/titin filaments in sarcomeres of differentiating chicken skeletal muscle cells. *Mol Cell Biochem.* **190**, 125-131.

Solaro, R. J., Moir, A. J., & Perry, S. V. (1976). Phosphorylation of troponin I and the inotropic effect of adrenaline in the perfused rabbit heart. *Nature* **262**, 615-617.

Solaro, R. J. & Van Eyk, J. (1996). Altered interactions among thin filament proteins modulate cardiac function. *J Mol Cell Cardiol* **28**, 217-230.

Solaro, R. J. & Rarick, H. M. (1998). Troponin and Tropomyosin : Proteins That Switch on and Tune in the Activity of Cardiac Myofilaments. *Circ Res* **83**, 471-480.

Sorsa, T., Pollesello, P., Permi, P., Drakenberg, T., & Kilpelainen, I. (2003). Interaction of levosimendan with cardiac troponin C in the presence of cardiac troponin I peptides. *Journal of Molecular and Cellular Cardiology* **35**, 1055-1061.

Sorsa, T., Heikkinen, S., Abbott, M. B., Abusamhadneh, E., Laakso, T., Tilgmann, C., Serimaa, R., Annila, A., Rosevear, P. R., Drakenberg, T., Pollesello, P., & Kilpelainen, I. (2001). Binding of Levosimendan, a Calcium Sensitizer, to Cardiac Troponin C. *J.Biol.Chem.* **276**, 9337-9343.

Spann, J. F., Jr., Buccino, R. A., Sonnenblick, E. H., & Braunwald, E. (1967). Contractile state of cardiac muscle obtained from cats with experimentally produced ventricular hypertrophy and heart failure. *Circ.Res.* **21**, 341-354.

Starling, R. C., Hammer, D. F., & Altschuld, R. A. (1998). Human myocardial ATP content and in vivo contractile function. *Mol Cell Biochem* **180**, 171-7.

Steinberg, S. F. & Brunton L.L. (2001). Compartmentation of G protein-coupled signaling pathways in cardiac myocytes. *Annu Rev Pharmacol Toxicol.* **41**, 751-73.

Stevens, C., Remme, E., LeGrice, I., & Hunter, P. (2003). Ventricular mechanics in diastole: material parameter sensitivity. *J Biomech.* **36**, 737-748.

Strauer, B. E. (1983). Left ventricular dynamics, energetics and coronary hemodynamics in hypertrophic heart disease. *Eur Heart J* **4 Suppl A**, 137-42.

Streeter, D. D., Jr., Spotnitz, H. M., Patel, D. P., Ross, J., Jr., & Sonnenblick, E. H. (1969). Fiber orientation in the canine left ventricle during diastole and systole. *Circ.Res.* **24**, 339-347.

Streeter, D. D., Jr., Vaishnav, R. N., Patel, D. J., Spotnitz, H. M., Ross, J., Jr., & Sonnenblick, E. H. (1970). Stress distribution in the canine left ventricle during diastole and systole. *Biophys.J* **10**, 345-363.

Stuyvers, B. D., Miura, M., & ter Keurs, H. E. (1997). Diastolic viscoelastic properties of rat cardiac muscle; involvement of Ca2+. *Adv.Exp.Med.Biol.* **430**, 13-28.

Sweeney, H. L., Bowman, B. F., & Stull, J. T. (1993). Myosin light chain phosphorylation in vertebrate striated muscle: regulation and function. *Am.J Physiol* **264**, C1085-C1095.

Sweeney, H. & Stull, J. (1990). Alteration of Cross-Bridge Kinetics by Myosin Light Chain Phosphorylation in Rabbit Skeletal Muscle: Implications for Regulation of Actin-Myosin Interaction. *PNAS* **87**, 414-418.

Swynghedauw, B. (1999). Molecular Mechanisms of Myocardial Remodeling. *Physiol.Rev.* **79**, 215-262.

Szilagyi, S., Pollesello, P., Levijoki, J., Kaheinen, P., Haikala, H., Edes, I., & Papp, Z. (2004). The effects of levosimendan and OR-1896 on isolated hearts, myocyte-sized preparations and phosphodiesterase enzymes of the guinea pig. *European Journal of Pharmacology* **486**, 67-74.

Takahashi, R., Talukder, M. A., & Endoh, M. (2000a). Effects of OR-1896, an active metabolite of levosimendan, on contractile force and aequorin light transients in intact rabbit ventricular myocardium. *J Cardiovasc.Pharmacol.* **36**, 118-125.

Takahashi, R., Talukder, M. A. H., & Endoh, M. (2000b). Inotropic effects of OR-1896, an active metabolite of levosimendan, on canine ventricular myocardium. *European Journal of Pharmacology* **400**, 103-112.

Takahashi, T., Allen, P. D., & Izumo, S. (1992). Expression of A-, B-, and C-type natriuretic peptide genes in failing and developing human ventricles. Correlation with expression of the $Ca^{2+}$-ATPase gene. *Circ Res* **71**, 9-17.

Takeda, N. (2003). Cardiomyopathy: molecular and immunological aspects (review). *Int J Mol Med* **11**, 13-6.

Takeda, S., Yamashita, A., Maeda, K., & Maeda, Y. (2003). Structure of the core domain of human cardiac troponin in the Ca(2+)-saturated form. *Nature* **424**, 35-41.

Tanaka, M., Ito, H., Adachi, S., Akimoto, H., Nishikawa, T., Kasajima, T., Marumo, F., & Hiroe, M. (1994). Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* **75**, 426-33.

Tavi, P., Han, C., & Weckstrom, M. (1998). Mechanisms of stretch-induced changes in $[Ca^{2+}]_i$ in rat atrial myocytes: role of increased troponin C affinity and stretch-activated ion channels. *Circ.Res.* **83**, 1165-1177.

The CONSENSUS Trial Study Group (1987). Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group. *N Engl J Med* **316**, 1429-35.

Thirlwell, H., Corrie, J. E., Reid, G. P., Trentham, D. R., & Ferenczi, M. A. (1994). Kinetics of relaxation from rigor of permeabilized fast-twitch skeletal fibers from the rabbit using a novel caged ATP and apyrase. *Biophys.J* **67**, 2436-2447.

Tian, R., Nascimben, L., Ingwall, J. S., & Lorell, B. H. (1997). Failure to maintain a low ADP concentration impairs diastolic function in hypertrophied rat hearts. *Circulation* **96**, 1313-9.

Tomanek, R. J., Searls, J. C., & Lachenbruch, P. A. (1982). Quantitative changes in the capillary bed during developing, peak, and stabilized cardiac hypertrophy in the spontaneously hypertensive rat. *Circ Res* **51**, 295-304.

Tomaselli, G. F. & Marban, E. (1999). Electrophysiological remodeling in hypertrophy and heart failure. *Cardiovascular Research* **42**, 270-283.

Tomaselli, G. F. & Zipes, D. P. (2004). What Causes Sudden Death in Heart Failure? *Circ Res* **95**, 754-763.

Torphy, T. J., Zhou, H. L., & Cieslinski, L. B. (1992). Stimulation of beta adrenoceptors in a human monocyte cell line (U937) up-regulates cyclic AMP-specific phosphodiesterase activity. *J Pharmacol Exp Ther* **263**, 1195-205.

Torre-Amione, G., Kapadia, S., Benedict, C., Oral, H., Young, J. B., & Mann, D. L. (1996a). Proinflammatory cytokine levels in patients with depressed left ventricular ejection fraction: a report from the Studies of Left Ventricular Dysfunction (SOLVD). *J Am Coll Cardiol* **27**, 1201-6.

Torre-Amione, G., Kapadia, S., Lee, J., Durand, J. B., Bies, R. D., Young, J. B., & Mann, D. L. (1996b). Tumor necrosis factor-alpha and tumor necrosis factor receptors in the failing human heart. *Circulation* **93**, 704-11.

Towbin, J. A. (1998). The role of cytoskeletal proteins in cardiomyopathies. *Curr.Opin.Cell Biol.* **10**, 131-139.

Traverse, J. H., Melchert, P., Pierpont, G. L., Jones, B., Crampton, M., & Bache, R. J. (1999). Regulation of myocardial blood flow by oxygen consumption is maintained in the failing heart during exercise. *Circ Res* **84**, 401-8.

Trinick, J., Knight, P., & Whiting, A. (1984). Purification and properties of native titin. *J Mol Biol.* **180**, 331-356.

Trombitas, K., Redkar, A., Centner, T., Wu, Y., Labeit, S., & Granzier, H. (2000). Extensibility of isoforms of cardiac titin: variation in contour length of molecular subsegments provides a basis for cellular passive stiffness diversity. *Biophys J* **79**, 3226-34.

Trombitas, K., Wu, Y., Labeit, D., Labeit, S., & Granzier, H. (2001). Cardiac titin isoforms are coexpressed in the half-sarcomere and extend independently. *Am J Physiol Heart Circ Physiol* **281**, 1793-9.

Trombitas, K., Wu, Y., McNabb, M., Greaser, M., Kellermayer, M. S., Labeit, S., & Granzier, H. (2003). Molecular basis of passive stress relaxation in human soleus fibers: assessment of the role of immunoglobulin-like domain unfolding. *Biophys.J* **85**, 3142-3153.

Tskhovrebova, L. & Trinick, J. (2003). Titin: properties and family relationships. *Nat Rev Mol Cell Biol* **4**, 679-89.

Tskhovrebova, L. & Trinick, J. (2004). Properties of titin immunoglobulin and fibronectin-3 domains. *J Biol.Chem.* **279**, 46351-46354.

Tsuchimochi, H., Kurimoto, F., Ieki, K., Koyama, H., Takaku, F., Kawana, M., Kimata, S., & Yazaki, Y. (1988). Atrial natriuretic peptide distribution in fetal and failed adult human hearts. *Circulation* **78**, 920-7.

Tsuji, Y., Opthof, T., Kamiya, K., Yasui, K., Liu, W., Lu, Z., & Kodama, I. (2000). Pacing-induced heart failure causes a reduction of delayed rectifier potassium currents along with decreases in calcium and transient outward currents in rabbit ventricle. *Cardiovasc Res* **48**, 300-9.

Tsutsui, H., Ishihara, K., & Cooper, G. (1993). Cytoskeletal role in the contractile dysfunction of hypertrophied myocardium. *Science* **260**, 682-687.

Tsutsui, H., Tagawa, H., Kent, R. L., McCollam, P. L., Ishihara, K., Nagatsu, M., & Cooper, G. (1994). Role of microtubules in contractile dysfunction of hypertrophied cardiocytes. *Circulation* **90**, 533-555.

Uozumi, H., Kudoh, S., Zou, Y., Harada, K., Yamazaki, T., & Komuro, I. (1998). Autocrine release of ATP mediates mechanical stress-induced cardiomyocytre hypertrophy. *Circulation* **98**, I-624.

Urboniene, D., Dias, F. A., Pena, J. R., Walker, L. A., Solaro, R. J., & Wolska, B. M. (2005). Expression of slow skeletal troponin I in adult mouse heart helps to maintain the left ventricular systolic function during respiratory hypercapnia. *Circ.Res.* **97**, 70-77.

van der Velden, J., de Jong, J. W., Owen, V. J., Burton, P. B., & Stienen, G. J. (2000). Effect of protein kinase A on calcium sensitivity of force and its sarcomere length dependence in human cardiomyocytes. *Cardiovasc.Res.* **46**, 487-495.

van der Velden, J., Moorman, A. F., & Stienen, G. J. (1998). Age-dependent changes in myosin composition correlate with enhanced economy of contraction in guinea-pig hearts. *J Physiol* **507**, 497-510.

van der Velden, J., Papp, Z., Boontje, N. M., Zaremba, R., de Jong, J. W., Janssen, P. M. L., Hasenfuss, G., & Stienen, G. J. M. (2003a). The effect of myosin light chain 2 dephosphorylation on Ca2+-sensitivity of force is enhanced in failing human hearts. *Cardiovascular Research* **57**, 505-514.

van der Velden, J., Papp, Z., Zaremba, R., Boontje, N. M., de Jong, J. W., Owen, V. J., Burton, P. B. J., Goldmann, P., Jaquet, K., & Stienen, G. J. M. (2003b). Increased Ca2+-sensitivity of the contractile apparatus in end-stage human heart failure results from altered phosphorylation of contractile proteins. *Cardiovasc Res* **57**, 37-47.

van der Ven, P. F., Bartsch, J. W., Gautel, M., Jockusch, H., & Furst, D. O. (2000). A functional knock-out of titin results in defective myofibril assembly. *J Cell Sci.* **113** , 1405-1414.

van der Ven, P. F., Ehler, E., Perriard, J. C., & Furst, D. O. (1999). Thick filament assembly occurs after the formation of a cytoskeletal scaffold. *J Muscle Res.Cell Motil.* **20**, 569-579.

Vanderheyden, M., Bartunek, J., & Goethals, M. (2004). Brain and other natriuretic peptides: molecular aspects. *Eur J Heart Fail* **6**, 261-8.

Vassort, G. (2001). Adenosine 5'-triphosphate: a P2-purinergic agonist in the myocardium. *Physiol Rev.* **81**, 767-806.

Verde, I., Vandecasteele, G., Lezoualc'h, F., & Fischmeister, R. (1999). Characterization of the cyclic nucleotide phosphodiesterase subtypes involved in the regulation of the L-type $Ca^{2+}$ current in rat ventricular myocytes. *Br J Pharmacol.* **127**, 65-74.

Vibert, P., Craig, R., & Lehman, W. (1997). Steric-model for activation of muscle thin filaments. *J Mol Biol.* **266**, 8-14.

Wakabayashi, ., Sugimoto, Y., Tanaka, H., Ueno, Y., Takezawa, Y., & Amemiya, Y. (1994). X-ray diffraction evidence for the extensibility of actin and myosin filaments during muscle contraction. *Biophys J.* **67**, 2422-35.

Walsh, R. A. (1997). Microtubules and pressure-overload hypertrophy. *Circ.Res.* **80**, 295-296.

Wang, K., Ramirez-Mitchell, R., & Palter, D. (1984). Titin is an extraordinarily long, flexible, and slender myofibrillar protein. *Proc.Natl.Acad.Sci.U.S.A* **81**, 3685-3689.

Wang, X., Li, F., Campbell, S. E., & Gerdes, A. M. (1999). Chronic Pressure Overload Cardiac Hypertrophy and Failure in Guinea Pigs: II. Cytoskeletal Remodeling. *Journal of Molecular and Cellular Cardiology* **31**, 319-331.

Watkins, H., Conner, D., Thierfelder, L., Jarcho, J. A., MacRae, C., McKenna, W. J., Maron, B. J., Seidman, J. G., & Seidman, C. E. (1995). Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. *Nat.Genet.* **11**, 434-437.

Wattanapermpool, J., Guo, X., & Solaro, R. J. (1995). The unique amino-terminal peptide of cardiac troponin I regulates myofibrillar activity only when it is phosphorylated. *J Mol Cell Cardiol* **27**, 1383-91.

Weber, K. T. (1997). Extracellular matrix remodeling in heart failure: a role for de novo angiotensin II generation. *Circulation* **96**, 4065-82.

Wehrens, X. H., Lehnart, S. E., Reiken, S. R., Deng, S. X., Vest, J. A., Cervantes, D., Coromilas, J., Landry, D. W., & Marks, A. R. (2004). Protection from cardiac arrhythmia through ryanodine receptor-stabilizing protein calstabin2. *Science* **304**, 292-296.

Weisberg, A. & Winegrad, S. (1996). Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle. *PNAS* **93**, 8999-9003.

Weisberg, A. & Winegrad, S. (1998). Relation Between Crossbridge Structure and Actomyosin ATPase Activity in Rat Heart. *Circ Res* **83**, 60-72.

Weishaar, R. E., Burrows, S. D., Kobylarz, D. C., Quade, M. M., & Evans, D. B. (1986a). Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. Isolation, characterization, and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. *Biochem Pharmacol* **35**, 787-800.

Weishaar, R. E., Burrows, S. D., Kobylarz, D. C., Quade, M. M., & Evans, D. B. (1986b). Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. Isolation, characterization, and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. *Biochem Pharmacol* **35**, 787-800.

Westfall, M. V., Turner, I. I., Albayya, F. P., & Metzger, J. M. (2001). Troponin I chimera analysis of the cardiac myofilament tension response to protein kinase A. *Am J Physiol Cell Physiol* **280**, 324-332.

Wettwer, E., Amos, G. J., Posival, H., & Ravens, U. (1994). Transient outward current in human ventricular myocytes of subepicardial and subendocardial origin. *Circ Res* **75**, 473-82.

Whiting, A., Wardale, J., & Trinick, J. (1989). Does titin regulate the length of muscle thick filaments? *J Mol Biol.* **205**, 263-268.

Williams, D. L., Jr., Greene, L. E., & Eisenberg, E. (1988). Cooperative turning on of myosin subfragment 1 adenosinetriphosphatase activity by the troponin-tropomyosin-actin complex. *Biochemistry* **27**, 6987-6993.

Winegrad, S. (1999). Cardiac Myosin Binding Protein C. *Circ Res* **84**, 1117-1126.

Witt, C. C., Gerull, B., Davies, M. J., Centner, T., Linke, W. A., & Thierfelder, L. (2001). Hypercontractile Properties of Cardiac Muscle Fibers in a Knock-in Mouse Model of Cardiac Myosin-binding Protein-C. *J.Biol.Chem.* **276**, 5353-5359.

Wolff, M. R., McDonald, K. S., & Moss, R. L. (1995). Rate of Tension Development in Cardiac Muscle Varies With Level of Activator Calcium. *Circ Res* **76**, 154-160.

Wolska, B. M., Keller, R. S., Evans, C. C., Palmiter, K. A., Phillips, R. M., Muthuchamy, M., Oehlenschlager, J., Wieczorek, D. F., de Tombe, P. P., & Solaro, R. J. (1999). Correlation between myofilament response to Ca2+ and altered dynamics of contraction and relaxation in transgenic cardiac cells that express beta-tropomyosin. *Circ.Res.* **84**, 745-751.

Wolska, B. M. & Wieczorek, D. M. (2003). The role of tropomyosin in the regulation of myocardial contraction and relaxation. *Pflugers Arch.* **446**, 1-8.

Wu, Y., Bell, S. P., Trombitas, K., Witt, C. C., Labeit, S., LeWinter, M. M., & Granzier, H. (2002). Changes in Titin Isoform Expression in Pacing-Induced Cardiac Failure Give Rise to Increased Passive Muscle Stiffness. *Circulation* **106**, 1384-1389.

Xu, X., Meiler, S. E., Zhong, T. P., Mohideen, M., Crossley, D. A., Burggren, W. W., & Fishman, M. C. (2002). Cardiomyopathy in zebrafish due to mutation in an alternatively spliced exon of titin. *Nat.Genet.* **30**, 205-209.

Yamaguchi, S., Yamaoka, M., Okuyama, M., Nitoube, J., Fukui, A., Shirakabe, M., Shirakawa, K., Nakamura, N., & Tomoike, H. (1999). Elevated circulating levels and cardiac secretion of soluble Fas ligand in patients with congestive heart failure. *Am J Cardiol* **83**, 1500-3.

Yamasaki, R., Berri, M., Wu, Y., Trombitas, K., McNabb, M., Kellermayer, M. S., Witt, C., Labeit, D., Labeit, S., Greaser, M., & Granzier, H. (2001). Titin-actin interaction in mouse myocardium: passive tension modulation and its regulation by calcium/S100A1. *Biophys.J* **81**, 2297-2313.

Yamasaki, R., Wu, Y., McNabb, M., Greaser, M., Labeit, S., & Granzier, H. (2002). Protein Kinase A Phosphorylates Titin's Cardiac-Specific N2B Domain and Reduces Passive Tension in Rat Cardiac Myocytes. *Circ Res* **90**, 1181-1188.

Yang, Q., Osinska, H., Klevitsky, R., & Robbins, J. (2001). Phenotypic deficits in mice expressing a myosin binding protein C lacking the titin and myosin binding domains. *J Mol Cell Cardiol* **33**, 1649-1658.

Yang, Q., Sanbe, A., Osinska, H., Hewett, T. E., Klevitsky, R., & Robbins, J. (1999). In vivo modeling of myosin binding protein C familial hypertrophic cardiomyopathy. *Circ.Res.* **85**, 841-847.

Yokoshiki, H., Katsube, Y., Sunagawa, M., & Sperelakis, N. (1997). The Novel Calcium Sensitizer Levosimendan Activates the ATP-Sensitive K+ Channel in Rat Ventricular Cells. *J Pharmacol Exp Ther* **283**, 375-383.

Young, P., Ehler, E., & Gautel, M. (2001). Obscurin, a giant sarcomeric Rho guanine nucleotide exchange factor protein involved in sarcomere assembly. *J Cell Biol.* **154**, 123-136.

Yu, H., Gao, J., Wang, H., Wymore, R., Steinberg, S., McKinnon, D., Rosen, M. R., & Cohen, I. S. (2000). Effects of the renin-angiotensin system on the current I(to) in epicardial and endocardial ventricular myocytes from the canine heart. *Circ Res* **86**, 1062-8.

Yue, P., Massie, B. M., Simpson, P. C., & Long, C. S. (1998). Cytokine expression increases in nonmyocytes from rats with postinfarction heart failure. *Am J Physiol* **275**, 250-8.

Zhang, J. & Bache, J. R. (1999). The Molecular Energetics of the Failing Heart from Animal Models—Large Animal Models. *Heart Failure Reviews* **4**, 255-267.

Zheng, J. S., Christie, A., De Young, M. B., Levy, M. N., & Scarpa, A. (1992). Synergism between cAMP and ATP in signal transduction in cardiac myocytes. *Am.J Physiol* **262**, C128-C135.

Zhu, W. Z., Wang, S. Q., Chakir, K., Yang, D., Zhang, T., Brown, J. H., Devic, E., Kobilka, B. K., Cheng, H., & Xiao, R. P. (2003). Linkage of beta1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca2+/calmodulin kinase II. *J Clin Invest* **111**, 617-25.

Zile, M. R., Tomita, M., Ishihara, K., Nakano, K., Lindroth, J., Spinale, F., Swindle, M., & Carabello, B. A. (1993). Changes in diastolic function during development and correction of chronic LV volume overload produced by mitral regurgitation. *Circulation* **87**, 1378-88.

Zimmermann, N., Boknik, P., Gams, E., Herzig, J. W., Neumann, J., & Scholz, H. (1998). Calcium sensitization as new principle of inotropic therapy in end-stage heart failure? *Eur J Cardiothorac Surg.* **14**, 70-5.

# LIST OF PUBLICATIONS

**In extenso publications related to this thesis**

1. Cazorla, O., **Szilagyi, S.**, Le Guennec, J.Y., Vassort, G., Lacampagne, A. (2005) Transmural stretch-dependent regulation of contractile properties in rat heart and its alteration after myocardial infarction. *FASEB J.* 19, 88-90. IF: 6.820
2. Cazorla, O.[†], **Szilagyi, S.**[†], Vignier, N., Salazar, G., Krämer, E., Vassort, G., Carrier, L., Lacampagne, A. (2005) Actiation of myofilaments in a hypertrophic mouse model lacking the cardiac myosin boinding protein C: effect of length and PKA phosphorylation. (submitted) [†] authors contributed equally to this work
3. **Szilagyi, S.**, Pollesello, P., Levijoki, J., Kaheinen, P., Haikala, H., Edes, I., Papp, Z. (2004) The effects of levosimendan and OR-1896 on isolated hearts, myocyte-sized preparations and phosphodiesterase enzymes of the guinea pig. *Eur J Pharmacol.* 486, 67-74. IF: 2.432
4. **Szilagyi, S.,** Pollesello, P., Levijoki, J., Haikala, H., Bak, I., Tosaki,A., Borbely, A., Edes, I., Papp, Z. (2005) Two inotropes with different mechanisms of action: contractile, PDE-inhibitory and direct myofibrillar effects of levosimendan and enoximone. *J Cardiovasc Pharmacol.* 46, 369-376. IF: 1.576

**Other in extenso publications**

1. Bodi, A., **Szilagyi, S.**, Edes, I., Papp Z. (2003) The cardiotonic effects of levosimendan in guinea pig hearts are modulated by beta-adrenergic stimulation. *Gen Physiol Biophys.* 22, 313-327. IF: 0.694
2. Kaheinen, P., Pollesello, P., Hertelendi, Z., Borbély, A., **Szilágyi, S.**, Édes, I., Nissinen, E., Haikala, H., Papp, Z. (2005) The positive inotropic effect of levosimendan is correlated to its sereoselective $Ca^{2+}$-sensitizing effect but not to stereoselective phosphodiesterase inhibition. *Basic Clin Pharmacol Toxicol.* (in press) IF: 1.342

**Published abstracts**

1. **Szilágyi**, **Sz.,** Vaszily, M., Édes, I., Papp, Z. (2002) Temperature-dependent changes of the contractile parameters in isolated human cardiomyocytes. *Cardiol. Hung.* Suppl.1., 88.
2. **Szilágyi**, **Sz.,** Vaszily, M., Édes, I., Papp, Z. (2002) Temperature-dependence of cross-bridge cycling rate and $Ca^{2+}$-sensitivity of force in human cardiomyocytes. *J. Mol. Cell. Cardiol.* 34, A62.
3. Bódi, A., **Szilágyi, S.**, Papp, Z., Édes, I. (2003) The cardiotonic effects of levosimendan in guinea pig hearts are modulated by beta-adrenergic stimulation. *Basic Res Cardiol* 98, A42.
4. Cazorla, O., **Szilagyi**, **S.**, Vassort, G., Lacampagne, A. (2003) Régionalisation des propriétés contractiles des cardiomyocytes et alteration au cours de l'insuffisance cardiaque chez le rat. *Arch. Mal. Cœur Vaiss.* 96, 396.
5. **Szilagyi, S**., Lacampagne, A., Kremer, E., Perriard, J.C., Vassort, G., Cazorla, O. (2003) Effects of the expression of a myomesin truncated form on cardiac contraction. *J. Muscle Res. Cell Motil.* 24, 340.

6. Cazorla, O., **Szilagyi, S.**, Vassort, G., Lacampagne, A. (2003) Regionalization of rat cardiomyocytes contractile properties : alterations in heart failure. *J. Muscle Res. Cell Motil.* 24, 343.
7. Cazorla, O., **Szilagyi**, **S.**, Vassort, G., Lacampagne, A. (2004) Myocyte contraction within the myocardium: tissular aspects. *Arch. Mal. Cœur Vaiss.* 97, 72.
8. **Szilágyi, S.**, Lacampagne, A., Kremer, E., Perriard, J.C., Vassort, G., Cazorla, O. (2004) Contractile properties of the myocardium following adenoviral expression of the myomesin My2 domain. *Cardiol. Hung.* 34, C32.
9. Molnár, A., **Szilágyi**, **S.**, Papp, Z., Vaszily, M., Édes, I., Tóth, A. (2004). The role of PKC izozymes on the regulation of human cardiomyocytes. *Cardiol. Hung.* 34, C87.
10. **Szilágyi, S.**, Pollesello, P., Levijoki, J., Kaheinen, P., Haikala, H., Édes, I., Papp, Z. (2004) Levosimendan and OR-1896 induced positive inotropy in guinea pig hearts. *J. Muscle Res. Cell Motil.* 25, 261.
11. Molnár, A., **Szilágyi, S.**, Papp, Z., Vaszily, M., Édes, I., Tóth, A. (2004) PKC mediated phosphorylation of human myofibrillar proteins. *J. Muscle Res. Cell Motil.* 25, 272.
12. Molnár, A., **Szilágyi, S.**, Borbély, A., Vaszily, M., Édes, I., Papp, Z., Tóth, A. (2005) Translocation and substrate specificity of protein kinase C alpha isozyme in human myocardium. *Cardiol. Hung.* 35, A24.
13. **Szilágyi, S.**, Cazorla, O., Le Guennec, J.-Y., Vassort, G., Lacampagne, A. (2005) Transmural alterations of stretch-dependent contractile properties in the failing rat heart. *Cardiol. Hung.* 35, A28.
14. Cazorla, O., **Szilágyi, S.**, Vignier, N., Vassort, G., Carrier, L., Lacampagne, A. (2005) Effects of PKA stimulation on the length-dependent of cardiac myosin binding protein C deficient cardiomayocytes. *J. Muscle Res. Cell Motil.* 26, 80.
15. Borbély, A., Hertelendi, Z., Tóth, A., **Szilágyi, S.**, Édes, I., Varró, I., Papp, J.Gy., Papp, Z. (2005) Contractile alterations in isolated human cardiomyocytes following oxidative protein modifications. *J. Muscle Res. Cell Motil.* 26, 81.
16. Molnár, A., **Szilágyi, S.**, Borbély, A., Vaszily, M., Varró, A., Papp, J.Gy., Édes, I., Papp, Z., Tóth, A. (2005) PKC alpha in the human myocardium: expression, translocation and possible functions. *J. Muscle Res. Cell Motil.* 26, 82.

**Oral presentations**

1. **Szilágyi**, **Sz.,** Vaszily, M., Édes, I., Papp, Z. (2002) Temperature-dependent changes of the contractile parameters in isolated human cardiomyocytes. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.
2. Bódi, A., **Szilágyi, S.**, Édes, I., van der Velden, J., Stienen, G.J.M., Papp, Z. (2002) Heart failure specific alterations in $Ca^{2+}$-dependencies of force generation and actin-myosin turnover rate in isolated human myocardial cells. 5$^{th}$ Czech-French-Slovak Symposium, Smolenice.
3. Bódi, A., **Szilágyi**, **S.**, Papp, Z., Édes, I. (2003) Cardiac β-adrenergic signalization and levosimendan. 11$^{th}$ Alpe Adria Cardiology Meeting, Balatonfüred.
4. Papp, Z., Édes, I., **Szilágyi**, **S.**, Pollesello, P., Levijoki, J., Kaheinen, P., Haikala, H. (2003) What is the mechanism of levosimendan induced positive inotropy? 11$^{th}$ Alpe Adria Cardiology Meeting, Balatonfüred.
5. **Szilágyi, S.**, Lacampagne, A., Kremer, E., Perriard, J.C., Vassort, G., Cazorla, O. (2004) Contractile properties of the myocardium following adenoviral expression of the myomesin My2 domain. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.

6. Molnár, A., **Szilágyi, S.**, Papp, Z., Vaszily, M., Édes, I., Tóth, A. (2004) The role of PKC izozymes on the regulation of human cardiomyocytes. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.
7. Molnár, A., **Szilágyi, S.**, Papp, Z., Vaszily, M., Édes, I., Tóth, A. (2004) PKC mediated phisphorylation of human myofibrillar proteins. 6th Meeting France-new CEE members "New frontiers in basic cardiovascular research", La Grande-Motte.
8. Cazorla, O., **Szilágyi, S.**, Vignier, N., Kulikovskaya, I. Winegrad, S., Vassort, G., Carrier, L., Lacampagne, A. (2004) Influence of vardiac myosin-binding protein C and PKA-dependent phosphorylation on length-dependent activation of myofilaments. 6th Meeting France-new CEE members "New frontiers in basic cardiovascular research", La Grande-Motte.
9. Molnár, A., **Szilágyi, S.**, Borbély, A., Vaszily, M., Édes, I., Papp, Z., Tóth, A. (2005) Translocation and substrate specificity of protein kinase C alpha isozyme in human myocardium. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.
10. **Szilágyi, S.**, Cazorla, O., Le Guennec, J.-Y., Vassort, G., Lacampagne, A. (2005) Transmural alterations of stretch-dependent contractile properties in the failing rat heart. Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.
11. Cazorla, O., **Szilágyi, S.**, Vignier, N., Vassort, G., Carrier, L., Lacampagne, A. (2005) Effects of PKA stimulation on the length-dependent of cardiac myosin binding protein C deficient cardiomayocytes. XXXIV European Muscle Conference, Máta.

**Poster presentations**

1. Magyar, J., Bányász, T., Kern, A., **Szilágyi, Sz.**, Bakk, J., Szigligeti, P., Körtvély, Á., Nánási, P.P. The effects of timol on contractility and electrophysiological properties of mammalian myocardium. (1999) Annual Meeting of the Hungarian Physiological Society, Budapest.
2. Magyar, J., Bányász, T., Kern, A., **Szilágyi, Sz.,** Bakk, J., Szigligeti, P., Körtvély, Á., Nánási, P.P. (1999) The effects of timol on the mechanical and electrophysiological parameters of canine heart. 23rd Meeting of the European Working Group on Cardiac Cellular Electrophysiology, Oxford.
3. Magyar, J., Bányász, T., Kern, A., **Szilágyi, Sz.,** Bakk, J., Szigligeti, P., Körtvély, Á., Nánási, P.P. (2000) The effects of timol on the mechanical and electrophysiological parameters of canine heart. (2000). Annual Meeting of the Hungarian Society of Cardiologists, Balatonfüred.
4. **Szilágyi, Sz.,** Vaszily, M., Édes, I., Papp, Z. (2002) Temperature-dependence of cross-bridge cycling rate and $Ca^{2+}$-sensitivity of force in human cardiomyocytes. 22nd European Section Meeting of the International Society for Heart Research, Szeged.
5. Cazorla, O., **Szilagyi, S.**, Lacampagne, A., Kremer, E., Perriard, J.C., Vassort, G. (2003) Effects of expression of a myomesin truncated form in the heart on cardiac contraction. 4th International Ascona Workshop on Cardiomyocyte Cell Motility, Ascona.
6. Cazorla, O., **Szilagyi**, S., Vassort, G., Lacampagne, A. (2003) Régionalisation des propriétés contractiles des cardiomyocytes et alteration au cours de l'insuffisance cardiaque chez le rat. 20e Congrès du Groupe de Réflexion sur la Recherche Cardiovasculaire, Grenoble.
7. Bódi, A., **Szilágyi**, **S**., Papp, Z., Édes, I. (2003) The cardiotonic effects of levosimendan in guinea pig hearts are modulated by beta-adrenergic stimulation. Heart Failure 2003/ISHR-ES, Strasbourg.

8. **Szilagyi, S.**, Lacampagne, A., Kremer, E., Perriard, J.C., Vassort G., Cazorla, O. (2003) Effects of the expression of a myomesin truncated form on cardiac contraction. 32nd European Muscle Conference, Montpellier.
9. Cazorla, O., **Szilagyi, S.,** Vassort, G., Lacampagne, A. (2003) Regionalization of rat cardiomyocytes contractile properties : alterations in heart failure. 32nd European Muscle Conference, Montpellier.
10. **Szilágyi, S.**, Pollesello, P., Levijoki, J., Kaheinen, P., Haikala, H., Édes, I., Papp, Z. (2004) Levosimendan and OR-1896 induced positive inotropy in guinea pig hearts. XXIII European Muscle Conference, Elba.
11. Molnár, A., **Szilágyi, S.**, Papp, Z., Vaszily, M., Édes, I., Tóth, A. (2004) PKC mediated phosphorylation of human myofibrillar proteins. XXIII European Muscle Conference, Elba.
12. Borbély, A., Hertelendi, Z., Tóth, A., **Szilágyi, S.**, Édes, I., Varró, I., Papp, J.Gy., Papp, Z. (2005) Contractile alterations in isolated human cardiomyocytes following oxidative protein modifications. XXXIV European Muscle Conference, Máta.
13. Molnár, A., **Szilágyi, S.**, Borbély, A., Vaszily, M., Varró, A., Papp, J.Gy., Édes, I., Papp, Z., Tóth, A. (2005) PKC alpha in the human myocardium: expression, translocation and possible functions. XXXIV European Muscle Conference, Máta.

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