Dissertation

Nucleo-to-cytoplasmic Ca\textsuperscript{2+} gradients in cardiomyocytes from non-failing and failing mouse and human hearts

submitted by

Senka Ljubojević

for the Academic Degree of

Doctor of Philosophy
(Ph.D.)

at the
Medical University of Graz
Division of Cardiology

under the supervision of
Univ. Prof. Dr. med. Burkert Pieske
I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of “Good Scientific Practice”.

Graz, 21.02.2012
Acknowledgement

First I would like to express very special thanks to my supervisor Prof. Burkert Pieske. With his infectiously enthusiastic spirit he managed to inspire me under any circumstances. "Always look at the bright side, work hard and aim big" is his recipe for success I always keep in mind.

Another special thanks goes to my second supervisor Prof. Jens Kockskamper. It was a real pleasure to be his student in any possible way. For me, he is a role model for being a great professional and a great person.

Completion of this thesis would be impossible without my colleagues from the Division of Cardiology. I am extremely grateful for all the knowledge Simon, Steffi, Paulina, Gudrun, Frank, Heiner, Ecko, Dirk, Peter and Albrecht provided me with. I also want to mention the unforgettable moments spent all around the world with Simon, Steffi, Yvonne, Michael#1, Michael#2, Markus, Felix, Robbie and Eva and hopefully more memories are about to come.

Very special thank goes to my table tennis team. Gabriel, Wolfi, Fritz, Sea, Sami, Karolf and all the others made me feel like home and kept my winning spirit awake all the time.

I also want to acknowledge the great friends I have, nothing would be the same without Cici and Slatka – my simply the best for the past ~20 years. Kaca, Jelena, The Buba, The Zec, Tomica, Samra, Admir, Zimon, Niki, Ljilja, Lada and Ivana, thank you for being around!

My biggest thank goes to my family. Thank to my cool mum, my greatest emotional teacher, for all her love and support. Thanks dad, my greatest professional success teacher, I know that you are around when I recognize your essence in my own behavior. My dear Ana, my big sister and someone to always look up to, thank you for being there for me since as long as my memory goes back 😊. Our sisterhood is essential for me.

Mkabghj nhfdgf mdithd hg iksjhu are the words for my Michi, simply because there are no real words to describe what he means to me. It is more than the world…
Summary

Nucleoplasmic calcium concentration ([Ca$^{2+}$]$_{\text{nuc}}$) in cardiac myocytes (CMs) regulates transcription and its alterations are involved in remodelling processes leading to hypertrophy and heart failure (HF). Quantification of subcellularly resolved Ca$^{2+}$ signals in CMs is therefore essential for understanding alterations of Ca$^{2+}$ fluxes and indentifying potential therapeutic targets in cardiac failure. Thus, the aim of this project was to quantify changes in Ca$^{2+}$ homeostasis in two distinct cellular compartments, the nucleus vs. the cytoplasm, in early – compensated and terminal – decompensated stages of HF, as well as to determine whether [Ca$^{2+}$] can be regulated differentially in these compartments under different physiological and pathophysiological conditions.

Properties of fluorescent Ca$^{2+}$-indicators in intracellular compartments may differ, thus affecting the translation of qualitative Ca$^{2+}$-dependent fluorescence changes into quantitative [Ca$^{2+}$] changes. Therefore, we determined the in situ characteristics of a frequently used Ca$^{2+}$ indicator, Fluo-4, and a ratiometric Ca$^{2+}$ indicator, Asante Calcium Red, and evaluated their use in reporting and quantifying cytoplasmic and nucleoplasmic Ca$^{2+}$ signals in isolated CMs. Ca$^{2+}$ calibration curves revealed significant differences in the apparent Ca$^{2+}$ dissociation constants of Fluo-4 and Asante Calcium Red between cytoplasm and nucleoplasm. Obtained parameters were applied for transformation of fluorescence into nucleoplasmic and cytoplasmic [Ca$^{2+}$]. Using this approach, I was able to demonstrate, for the first time, substantial differences in quantitative Ca$^{2+}$ handling between cytoplasm and nucleoplasm: resting and diastolic [Ca$^{2+}$] were always higher in the nucleoplasm, while systolic [Ca$^{2+}$] was usually higher in the cytoplasm (some cells (15%) exhibited higher systolic [Ca$^{2+}$] in the nucleoplasm). Ca$^{2+}$ store depletion or blockade of Ca$^{2+}$ leak pathways eliminated the resting [Ca$^{2+}$] gradient between nucleoplasm and cytoplasm, whereas inhibition of inositol 1,4,5-trisphosphate receptors by 2-APB reversed it. The results suggest that there are significant nucleoplasmic to cytoplasmic [Ca$^{2+}$] gradients in resting myocytes and during the cardiac cycle. Nucleoplasmic [Ca$^{2+}$]
in cardiomyocytes may be regulated via two mechanisms: diffusion from the cytoplasm and active Ca\textsuperscript{2+} release via inositol 1,4,5-trisphosphate receptors from perinuclear Ca\textsuperscript{2+} stores.

With the established method, we then characterized alterations in cytoplasmic and nucleoplasmic Ca\textsuperscript{2+} handling after pressure overload-induced hypertrophy in murine CMs and in CMs isolated from failing and non-failing human hearts. In the early stage of hypertrophy, i.e. (1) one week after transverse aortic constriction (TAC) intervention in mouse and (2) in moderately failing human hearts, slowing of the kinetics and a decline in the amplitude of CaTs was found selectively in the nucleus. Seven weeks after TAC, as well as in severely failing human hearts, similar changes of CaTs also occurred in the cytoplasm. At higher stimulation frequencies, CMs from TAC mice showed significant alterations in Ca\textsuperscript{2+} cycling compared to healthy controls. Due to its slower kinetics, dysfunction of Ca\textsuperscript{2+} handling in the nucleoplasmic compartment was even more pronounced than in the cytoplasm when higher stimulation frequencies were applied.

To understand potential mechanisms for altered nucleoplasmic Ca\textsuperscript{2+} handling, we investigated possible changes in nuclei dimensions, the nuclear envelope morphology and expression pattern of Ca\textsuperscript{2+} regulatory proteins during the hypertrophy progress. In CMs from Sham animals, staining of perinuclear Ca stores revealed a nuclear envelope and tubular structures transversing the nucleus. A significant increase in number of tubules per nucleus was observed during physiological growth, while nuclear dimensions remained unaltered. In TAC CMs, the number of tubules per nucleus progressively decreased.

Immunostaining of Ca\textsuperscript{2+} regulatory proteins showed distinct expression patterns in perinuclear regions in failing hearts as compared to the controls.

In conclusion, perinuclear Ca stores and nucleoplasmic CaTs undergo significant changes during pressure overload-induced hypertrophy, which appear to precede changes in cytoplasmic Ca regulation. Similar changes can also be observed in failing human myocardium. These results raise the possibility that altered nucleoplasmic [Ca] may contribute to the development and/or progression of hypertrophy.
Zusammenfassung

Die Konzentration von nukleoplasmatischem Kalzium ([Ca$^{2+}$]$_{\text{nuc}}$) reguliert im Zellkern von Kardiomyocyten dessen Genexpression und Transkription. Veränderte [Ca$^{2+}$]$_{\text{nuc}}$ Konzentrationen und Signale können an Prozessen beteiligt sein, die die Herzstruktur verändern und dadurch zu Hypertrophie und Herzinsuffizienz führen. Die Quantifizierung subzellulärer Ca$^{2+}$ Signale könnte daher ein wichtiger Schritt zu einem besseren Verständnis kardialer Remodelingprozesse führen.

Das Ziel dieser Dissertation bestand darin Änderungen im Ca Haushalt in unterschiedlichen Zellkompartimenten, dem Zellkern und dem Zytoplasma, zu quantifizieren. Um Einblicke in die Entstehung der Herzinsuffizienz zu erlangen wurde ein Vergleich zwischen der frühen Phase kompensierter Herzinsuffizienz und der späten Phase dekompensierter Herzinsuffizienz vorgenommen. Zusätzlich wurde experimentell erfasst, ob die Regulierung von Ca$^{2+}$ Signalen unter physiologischen oder pathophysiologischen Bedingungen verändert ist.

Die Eigenschaften von Ca$^{2+}$-sensitiven Fluorophoren sind abhängig von ihrer intrazellulären Umgebung, wodurch eine subzelluläre quantitative Erfassung von Ca$^{2+}$ Signalen erschwert wird. Um dennoch eine quantitative Messung von subzellulären Ca in human und murinen Kardiomyocyten zu erlauben, wurden die in situ Eigenschaften der Ca$^{2+}$ Sensoren Fluo-4 und des ratiometrischen Ca$^{2+}$ Indikators Asante Calcium Red erfasst. Aus den Kalibrierungsgeraden wurde ersichtlich, dass die Ca$^{2+}$ Dissoziationskonstanten für Fluo-4 und Asante Calcium Red im Zytoplasma und im Nukleoplasma unterschiedlich sind. Die ermittelten Ca$^{2+}$ Dissoziationskonstanten wurden verwendet um die gemessene Fluoreszenz in tatsächliche Ca$^{2+}$-Konzentrationen umzurechnen, wodurch eine quantitative Bestimmung unabhängig von der zellulären Umgebung ermöglicht wurde.

Mit Hilfe dieser in situ Kalibrierung, war es mir erstmals möglich quantitative Unterschiede zwischen zytoplasmatischen und nukleoplasmatischen Ca$^{2+}$-handling zu zeigen. Die diastolische [Ca$^{2+}$]$_{\text{nuc}}$ war signifikant höher als im
Zytoplasma. Im Gegensatz dazu war das systolische Calcium meistens höher im Zytoplasma, mit der Ausnahme von rund 15 % der Zellen, die erhöhtes systolisches \([\text{Ca}^{2+}]_{\text{nuc}}\) aufwiesen. Die Depletion von \(\text{Ca}^{2+}\) oder die Blockade des \(\text{Ca}^{2+}\) „leaks“ führte zum Verschwinden des \(\text{Ca}^{2+}\) Gradienten zwischen Nukleoplasma und dem Zytoplasma. Im Gegensatz dazu wurde dieser Effekt durch die Inhibierung des 1,4,5-trisphosphate Rezeptors mittels 2-APB wieder aufgehoben. Diese Resultate weisen auf einen signifikanten Unterschied der \(\text{Ca}^{2+}\) Konzentrationen zischen dem Nukleus und dem Zytoplasma in ruhenden Kardiomyocyten hin. Die Regulation dieses \(\text{Ca}^{2+}\) Gradienten könnte über 2 Signalwegen geregelt werden. Zum einen durch die Diffusion von \(\text{Ca}^{2+}\) aus dem Zytoplasma und zum anderen durch die aktive Freisetzung von \(\text{Ca}^{2+}\) aus perinukleären \(\text{Ca}^{2+}\) Speichern über den 1,4,5-trisphosphate Rezeptor.

Des Weiteren haben wir Veränderungen in \(\text{Ca}^{2+}\) Signalen von murinen Kardiomyocyten nach „pressure overload“ induzierter Hypertrophie quantifiziert. Zusätzlich wurden humane Kardiomyozyten analysiert die aus gesunden und insuffizienten Herzen isoliert wurden.

In der Frühphase kardialen Remodelings, wie z.B. (1) eine Woche nach „transverse aortic constriction“ (TAC) in einem Mausmodell und (2) in moderat insuffizienten humanen Herzen, zeigte sich eine Verlangsamung der Kinetik, sowie ein Abfall der CaT-Amplitude welche sich auf das Nukleoplasma beschränkt. Bei weiter fortgeschrittener Herzinsuffizienz (z.B. sieben Wochen nach TAC oder in insuffizienten humanen Herzen) zeigten sich auf zellulärer Ebene ähnliche Veränderungen auch im Zytoplasma. Die Stimulation mit höheren Frequenzen führte in Kardiomyocyten von TAC Mäusen zu einer unterschiedlichen \(\text{Ca}^{2+}\) handling im Vergleich mit gesunden Kontrollen. Dabei waren die diastolischen \(\text{Ca}^{2+}\) Spiegel erhöht, die systolischen \(\text{Ca}^{2+}\) Spiegel unverändert und die Amplitude des \(\text{Ca}^{2+}\) Transienten reduziert. Diese langsamere Kinetik war eindeutig stärker ausgeprägt im Nukleus.

Um die kinetischen Änderungen auf einer strukturellen Ebene zu verstehen, wurden die Größe, die Morphologie und das Expressionsmuster von \(\text{Ca}^{2+}\) regulierenden Proteinen im Zellkern von Kardiomyocyten untersucht. In

Diese Ergebnisse zeigen die Möglichkeit auf das Veränderungen im nukleoplasmatischen Ca\(^{2+}\) kausal an der Entstehung und dem Fortschreiten der Herzinsuffizienz beteiligt sind.
Index

List of Tables ........................................................................................................XII
List of Figures ..........................................................................................................XIII
List of abbreviations: ............................................................................................. XV
1. Introduction ..............................................................................................................1
  1.1. Cardiac calcium cycling and its regulation .................................................2
  1.2. Heart failure ......................................................................................................4
    1.2.1. Definition and etiology ................................................................................4
    1.2.2. Prevalence, incidence and prognosis of heart failure .........................5
    1.2.3. Pathophysiology of heart failure .................................................................6
    1.2.4. Molecular basis of mechanisms involved in hypertrophy, remodelling and failure ........................................................................................................8
    1.2.5. Ca^{2+} in the regulation of transcription and hypertrophy ................11
    1.2.6. Nucleoplasmic vs. cytoplasmic Ca^{2+} signaling ..................................12
    1.2.7. Quantification of nucleoplasmic vs. cytoplasmic [Ca^{2+}] ...............15
  1.3. Aims and description of thesis projects ....................................................17
2. Materials and Methods ......................................................................................19
  2.1. Cell Preparation ............................................................................................20
    2.1.1. Murine and rat myocyte isolation ..............................................................20
    2.1.2. Human myocyte isolation ........................................................................20
  2.2. Confocal Ca^{2+} imaging ..............................................................................22
    2.2.1. Confocal Ca^{2+} imaging of subcellular CaTs ........................................22
    2.2.2. Measurement of the point spread function (PSF) ................................23
    2.2.3. Spectral properties of Fluo-4 in the presence of 2-ABP, CPA, and tetracaine .................................................................24
  2.3. In situ calibration of Ca^{2+} indicators in adult CMs ..................................24
    2.3.1. In situ calibration of Fluo-4 and ACaR fluorescence .........................24
2.3.2. Compartmentalization and leakage of Fluo-4 ................................................................. 25
2.4. Minimally invasive transverse aortic constriction .............................................................. 26
2.5. Echocardiography ..................................................................................................................... 26
2.6. Perinuclear Ca\(^{2+}\) stores visualization ....................................................................................... 27
2.7. Immunocytochemistry ................................................................................................................ 27
2.8. Electron microscopy .................................................................................................................... 28
2.9. Drugs and solutions ..................................................................................................................... 29
2.10. Statistics ..................................................................................................................................... 29
3. Results ........................................................................................................................................... 30

3.1. *In situ* calibration of Ca\(^{2+}\) inhibitors in adult CMs ............................................................... 31
   3.1.1. Point spread function (PSF) and axial resolution ................................................................. 31
   3.1.2. *In situ* calibration of Fluo-4 fluorescence in adult CM ...................................................... 32
   3.1.3. Compartmentalization and leakage of Fluo-4 ..................................................................... 35
   3.1.4. *In situ* calibration of ACaR fluorescence in adult CMs .................................................... 36
3.2. Quantification and characterisation of subcellular Ca\(^{2+}\) handling in resting and electrically stimulated CMs ................................................................................................................................. 38
   3.2.1. Quantification of cytoplasmic and nucleoplasmic CaTs in electrically stimulated CMs ................................................................................................................................................. 38
   3.2.2. Correlation between nucleoplasmic and cytoplasmic [Ca\(^{2+}\)] ........................................ 40
   3.2.3. Resting [Ca\(^{2+}\)]\(_{cyto}\) and [Ca\(^{2+}\)]\(_{nuc}\) before and following depletion of intracellular Ca\(^{2+}\) stores .............................................................................................................................................. 42
   3.2.4. Effects of 2-APB and tetracaine on the nucleo-to-cytoplasmic [Ca\(^{2+}\)] gradient ......................................................................................................................................................... 43
   3.2.5. Excitation and emission spectra of Fluo-4 in the absence and presence of drugs ..................................................................................................................................................... 45
   3.2.6. Stimulation frequency-dependent changes in [Ca\(^{2+}\)]\(_{nuc}\) vs. [Ca\(^{2+}\)]\(_{cyto}\) .. 46
3.3. Alterations in cytoplasmic vs. nucleoplasmic Ca\(^{2+}\) handling in hypertrophy and failure in an animal model of pressure overload and in failing human hearts .......................................................................................................................... 48
   3.3.1. Characterisation of pressure overload-induced hypertrophy in mice . 48
3.3.2. Quantification of electrically stimulated CaTs in the nucleus vs. cytoplasm after pressure overload-induced hypertrophy in mouse CMs ........50

3.3.3. Quantification of electrically stimulated CaTs in the nucleus vs. cytoplasm in CMs isolated from non-failing, moderately failing and severely failing human hearts ........................................................................................................................................54

3.3.4. Stimulation frequency-dependent changes of nucleoplasmic vs. cytoplasmic CaTs after pressure overload-induced hypertrophy in mouse CMs ........................................................................................................................................56

3.4. Structural and biochemical alterations of nuclear envelope in hypertrophy and failure in mouse model of pressure overload and in failing human hearts ........................................................................................................................................60

3.4.1. Perinuclear Ca stores and nuclear size in hypertrophy and failure in mouse model of pressure overload and in failing human hearts ..................60
3.4.2. Subcellular localization and expression of Ca$^{2+}$ regulating proteins in control and failing mouse and human CMs ........................................................................................................62

4. Discussion ........................................................................................................................................65

4.1. In situ calibration of Ca$^{2+}$ inhibitors in adult CMs .................................................66

4.2. Quantification and characterisation of subcellular Ca$^{2+}$ handling in resting and electrically stimulated CMs ........................................................................................................................................68

4.2.1. The nuclear CaT consists of two components ........................................68
4.2.2. Perinuclear Ca$^{2+}$ store content and Ca$^{2+}$ leak determine the resting nucleo-to-cytoplasmic [Ca$^{2+}$] gradient ........................................................................................................................................69
4.2.3. Stimulation frequency affects [Ca$^{2+}$]$_{nuc}$ and [Ca$^{2+}$]$_{cyto}$ differentially ..........70

4.3. Alterations in cytoplasmic vs. nucleoplasmic Ca$^{2+}$ handling in hypertrophy and failure in an animal model of pressure overload and in failing human hearts ........................................................................................................................................71

4.3.1. Changes of electrically stimulated CaTs during the development and progression of hypertrophy in mouse and human hearts occur first in the nucleus ........................................................................................................................................71
4.3.2. $[\text{Ca}^{2+}]_{\text{nuc}}$ is affected more than $[\text{Ca}^{2+}]_{\text{cyto}}$ by increased stimulation frequency after pressure overload-induced hypertrophy in electrically stimulated mouse CMs .......................................................... 72

4.4. Structural and biochemical alterations of nuclear envelope in hypertrophy and failure in mouse model of pressure overload and in failing human hearts ............................................................................................................. 74

   4.4.1. Calcium perinuclear stores suffer progressive loss of nucleoplasmic tubular network during hypertrophy and heart failure ................................................. 74

   4.4.2. Remodelling of $\text{Ca}^{2+}$ release channels expression pattern in perinuclear region during hypertrophy and heart failure .............................................. 75

5. Conclusion ................................................................................................................. 77

6. References .................................................................................................................. 79
List of Tables

Table 1-1: Basic characteristic of patients undergoing transplantation or heart donors.................................................................21

Table 3-1: Left ventricular echocardiographic parameters in control and 1 and 6 weeks after TAC intervention.................................................................48

Table 3-2: Stimulation frequency-dependent changes of CaTs after pressure-overload induced hypertrophy in mouse CMs......................................................57
List of Figures

1. Introduction ............................................................................................................. 1

Figure 1-1: Excitation-contraction coupling..............................................................3
Figure 1-2: Overview of heart failure development .....................................................7
Figure 1-3: Neurohormonal activation during cardiac stress ........................................8
Figure 1-4: Ca$^{2+}$-dependent transcriptional regulation in CMs. ...............................11
Figure 1-5: Nucleoplasmic [Ca$^{2+}$] regulation .........................................................14

3. Results ..................................................................................................................... 30

Figure 3-1 Point spread function (PSF).....................................................................31
Figure 3-2: In situ calibration of fluorescent Ca$^{2+}$ indicator Fluo-4 in the nucleus vs. the cytoplasm of mouse and rat cardiac myocytes ................................................33
Figure 3-3: Lack of significant compartmentalization of Fluo-4. ...............................35
Figure 3-4: In situ calibration of the ratiometric Ca$^{2+}$ indicator ACaR in the nucleus vs. cytoplasm of rat ventricular myocytes .........................................................37
Figure 3-5: Quantification and characterization of cytoplasmic and nucleoplasmic CaTs in electrically stimulated cardiac myocytes .....................................................39
Figure 3-6: Recording of nucleoplasmic vs. cytoplasmic [Ca$^{2+}$] using the ratiometric Ca$^{2+}$ indicator ACaR .................................................................40
Figure 3-7: Relationship between nucleoplasmic and cytoplasmic [Ca$^{2+}$] in diastole and systole. ..................................................................................................41
Figure 3-8: Resting cytoplasmic and nucleoplasmic [Ca$^{2+}$] before and following depletion of intracellular Ca$^{2+}$ stores ..................................................................42
Figure 3-9: Effects of 2-APB and tetracaine on the nucleo-to-cytoplasmic [Ca$^{2+}$] gradient ............................................................................................................44
Figure 3-10: Lack of effect of 2-APB, CPA, and tetracaine on the excitation and emission spectra of Fluo-4 .....................................................................................46
Figure 3-11: Frequency-dependent increases in diastolic and systolic [Ca$^{2+}$] in the nucleus vs. cytoplasm .................................................................47
Figure 3-12: Characterisation of pressure overload-induced hypertrophy in adult WT mice………………………………………………………………………………………………..49

Figure 3-13: Quantification and characterisation of cytoplasmic and nucleoplasmic CaTs after pressure overload-induced hypertrophy in electrically stimulated adult mouse CMs……………………………………………………………………………………………………..50

Figure 3-14: Sub-cytoplasmic analyses of electrically stimulated CaTs after pressure overload-induced hypertrophy in mouse CMs…………………………………………………………………………………………………………..52

Figure 3-15: Sub-nucleoplasmic analyses of electrically stimulated CaTs after pressure overload-induced hypertrophy in mouse CMs…………………………………………………………………………………………………………53

Figure 3-16: Quantification and characterisation of cytoplasmic and nucleoplasmic CaTs of CMs isolated from non-failing, moderately failing and severely failing human hearts. …………………………………………………………………………………………………..54

Figure 3-17: Hypertrophy-induced increase in diastolic [Ca$^{2+}$] in the nucleus versus cytoplasm of mouse and human ventricular myocytes. ………………………………………….55

Figure 3-18: Stimulation frequency-dependent changes of nucleoplasmic and cytoplasmic CaTs after pressure overload-induced hypertrophy in adult mouse CMs. …………………………………………………………………………………………………………………..58

Figure 3-19: Perinuclear Ca stores and nuclei size after pressure overload-induced hypertrophy in mouse CMs and in CMs from non-failing and failing human hearts………………………………………………………………………………………………..61

Figure 3-20: Correlation between number of tubules per nucleus and nuclei length. …………………………………………………………………………………………………………………………………………….62

Figure 3-21: Subcellular localization and expression of Ca$^{2+}$ regulating proteins in control and failing mouse and human CMs. ……………………………………………………………….63
List of abbreviations:

$\alpha_1$-AR and $\beta$-AR  $\alpha_1$ and $\beta$ adrenergic receptor
$[\text{Ca}^{2+}]$  calcium concentration
$[\text{Ca}^{2+}]_{\text{cyto}}$  cytoplasmic calcium concentration
$[\text{Ca}^{2+}]_{\text{nuc}}$  nucleoplasmic calcium concentration
$[\text{Ca}^{2+}]_{\text{rest}}$  resting calcium concentration
2-APB  2-aminoethoxydiphenyl borate
AC  adenyl-cyclase
ACaR  Asante Calcium Red
ACE  angiotensin-converting enzyme
AP  action potential
AT  angiotensin
AT$_1$  AT II receptor type 1
ATP  adenosine triphosphate
BDM  butanedione monoxime
BSA  bovine serum albumin
Ca$^{2+}$  calcium ion
CAD  coronary artery disease
CaM  calmodulin
CaMKII  calmodulin-dependent protein kinase II
cAMP  cyclic adenosine monophosphate
CaN  calcineurin
CaT  calcium concentration transients
CICR  calcium induced calcium release
CM  cardiac myocyte
CPA  cyclopiazonic acid
CVD  cardiovascular disease
DAG  diacylglycerol
DCM  dilated cardiomyopathy
DMSO  dimethyl sulfoxide
ECC  excitation-contraction coupling
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF</td>
<td>ejection fraction</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>ETAR and ETBR</td>
<td>endothelin-A and endothelin-B receptors</td>
</tr>
<tr>
<td>$F_{\text{max}}$</td>
<td>fluorescence at saturating free calcium concentration</td>
</tr>
<tr>
<td>$F_{\text{min}}$</td>
<td>fluorescence at zero free calcium concentration</td>
</tr>
<tr>
<td>$F_{\text{rest}}$</td>
<td>fluorescence at resting free calcium concentration</td>
</tr>
<tr>
<td>FS</td>
<td>fractional shortening</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width at half maximum</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HCM</td>
<td>hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone-deacetylase</td>
</tr>
<tr>
<td>HF</td>
<td>heart failure</td>
</tr>
<tr>
<td>ICM</td>
<td>ischemic cardiomyopathy</td>
</tr>
<tr>
<td>$IP_3$</td>
<td>inositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>$IP_3R$</td>
<td>inositol-1,4,5-trisphosphate receptors</td>
</tr>
<tr>
<td>IVS</td>
<td>intraventricular septum</td>
</tr>
<tr>
<td>$K_d$</td>
<td>calcium dissociation constant of the indicator</td>
</tr>
<tr>
<td>$K_{d,\text{app}}$</td>
<td>apparent calcium dissociation constant of the indicator</td>
</tr>
<tr>
<td>LCC</td>
<td>L-type calcium channel</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>LVEDD</td>
<td>left ventricular end-diastolic diameter</td>
</tr>
<tr>
<td>LVESD</td>
<td>left ventricular end-systolic diameter</td>
</tr>
<tr>
<td>LV$_{\text{mass}}$</td>
<td>left ventricular mass</td>
</tr>
<tr>
<td>LVPW</td>
<td>left ventricular posterior wall</td>
</tr>
<tr>
<td>MEF</td>
<td>myocyte enhancer factor</td>
</tr>
<tr>
<td>NCX</td>
<td>sodium-calcium exchanger</td>
</tr>
<tr>
<td>NucE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>NFAT</td>
<td>nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NICM</td>
<td>non-ischemic cardiomyopathy</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization sequence</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complexes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NT</td>
<td>normal Tyrode’s solution</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA and PKC,</td>
<td>protein kinase A and C</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholamban</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>plasma membrane ATPase</td>
</tr>
<tr>
<td>PSF</td>
<td>point spread function</td>
</tr>
<tr>
<td>R</td>
<td>fluorescence signal normalized to $F_{\text{rest}}$</td>
</tr>
<tr>
<td>RCM</td>
<td>restrictive cardiomyopathy</td>
</tr>
<tr>
<td>Rel. WT</td>
<td>relative wall thickness</td>
</tr>
<tr>
<td>$R_f$</td>
<td>dynamic range</td>
</tr>
<tr>
<td>$R_{\text{max}}$</td>
<td>fluorescence ratio at saturating free calcium concentration</td>
</tr>
<tr>
<td>$R_{\text{min}}$</td>
<td>fluorescence ratio at zero free calcium concentration</td>
</tr>
<tr>
<td>RV</td>
<td>right ventricle</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco-endoplasmic reticulum calcium-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TAC</td>
<td>transverse aortic constriction</td>
</tr>
</tbody>
</table>
1. Introduction
1.1. Cardiac calcium cycling and its regulation

The function of the heart essentially depends on the ability of individual heart muscle cells, cardiac myocytes (CM), to translate an electrical signal, i.e. the action potential (AP), into mechanical contraction. The complex chain of events that drives the translation of an AP into mechanical activity of the myocyte is known as excitation-contraction coupling (ECC) (for review see (1,2)) and it enables the chambers of the heart to rhythmically contract and relax. The ubiquitous second messenger, calcium ion (Ca$^{2+}$), has a central role in cardiac electrical activity and is the direct activator of myofilament interaction (2). A schematic model of ECC is illustrated in Fig. 1-1.

During the cardiac AP, myocyte membrane depolarization leads to opening of the voltage-dependent L-type calcium channels (LCC), inducing an inward Ca$^{2+}$ current. The increase in intracellular Ca$^{2+}$ triggers the release of Ca$^{2+}$ stored in the sarcoplasmic reticulum (SR) through Ca$^{2+}$ release channels – ryanodine receptors (RyR) – in a positive feedback fashion. About 75% of Ca$^{2+}$ present in the cytoplasm during contraction is released from the SR and it increases the free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) from about 100 nM to 1 µM. This mechanism, where conduction of Ca$^{2+}$ through the sarcolemma triggers release of Ca$^{2+}$ ions from the SR into the cytoplasm, is termed calcium-induced calcium release (CICR). The transient increase in cytoplasmic free [Ca$^{2+}$] allows Ca$^{2+}$ binding to the myofilament protein troponin C. The conformational change of troponin regulatory complex leads to hydrolysis of high-energy phosphate bonds of adenosine triphosphate (ATP) and initiation of cross-bridge formation between actin and myosin. The actin and myosin filaments slide past each other thereby causing shortening of the sarcomere and myocyte contraction.

For relaxation to occur, free cytoplasmic [Ca$^{2+}$] has to decline and allow Ca$^{2+}$ to dissociate from troponin. Intracellular Ca$^{2+}$ is mostly taken up by an ATP-dependent calcium pump, the sarco-endoplasmic reticulum calcium-ATPase (SERCA), into the SR. To a quantitatively smaller extent, cytoplasmic Ca$^{2+}$ is removed from the cell by the electrogenic sodium-calcium exchanger (NCX).
About 1% of Ca\(^{2+}\) is stored by mitochondria or extruded via the sarcolemmal Ca\(^{2+}\)-ATPase (3).

During the steady state, the amount of Ca\(^{2+}\) that enters the myocyte with each AP matches exactly the amount of Ca\(^{2+}\) extruded from the myocyte during the relaxation. The minute control of Ca\(^{2+}\) fluxes underlies rhythmic contraction and relaxation, thereby allowing proper systolic ejection and diastolic filling of the heart.

**Figure 1-1: Excitation-contraction coupling.** AP, Action potential; LCC, L-type calcium channel; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA, SR-Ca\(^{2+}\)-ATPase; NCX, Na\(^{+}\)/Ca\(^{2+}\) exchanger; PLB, phospholamban; PMA, plasma membrane ATPase; ECM, extracellular matrix. Ca\(^{2+}\) upstroke during systole (red arrows) and Ca\(^{2+}\) removal during diastole (green arrows).

The dynamic regulation of calcium transport mechanisms is essential at varying heart rates (4). To achieve this, several cellular kinases and phosphatases
regulate proteins involved in ECC, providing their suitable activation and inhibition under different physiological conditions. In healthy hearts, Ca\(^{2+}\)-handling proteins are primarily regulated by adrenergic stimulation upon the activation of sympathetic nerves. Adrenergic stimulation triggers \(G_q\) and \(G_s\) protein-coupled signaling cascades to increase Ca\(^{2+}\) entry through LCCs and phosphorylate phospholamban (PLB) that normally inhibits calcium uptake via SERCA. Disinhibition of PLB leads to an increased rate of calcium uptake by the SR. Adrenergic stimulation can also promote the release of calcium from the SR through inositol-1,4,5-trisphosphate receptors (IP\(_3\)R) located on the SR membrane. The increase in free cytoplasmic [Ca\(^{2+}\)] through LCCs and IP\(_3\)Rs enhances the force generated by the actin and myosin interactions, as well as the velocity of shortening. Therefore, adrenergic stimulation increases the force and shortening velocity of contraction (i.e., positive inotropy), and the rate of relaxation (i.e., positive lusitropy) (5).

In contrast to physiological regulation, mishandling of Ca\(^{2+}\) homeostasis in CMs is a source of major pathophysiological events – such as contractile dysfunction and arrhythmias – in heart failure (1).

1.2. Heart failure

1.2.1. Definition and etiology

HF is a syndrome in which the patients should have the following features: symptoms of HF, typically shortness of breath at rest or during exertion, and/or fatigue; signs of fluid retention such as pulmonary congestion or ankle swelling; and objective evidence of an abnormality of the structure or function of the heart at rest (6). It may be divided into diastolic heart failure (also termed heart failure with preserved left ventricular ejection fraction) and systolic heart failure. In systolic HF, there is reduced cardiac contractility which results in inadequate systemic perfusion, whereas in diastolic HF impaired cardiac relaxation and
abnormal ventricular filling result in elevated diastolic pressure in the left ventricle despite essentially normal end diastolic volume.
The most frequent form of heart failure is left ventricular (LV) systolic dysfunction (~60% of patients). It is commonly the outcome of coronary artery disease (CAD), with either myocardial infarction or chronic hypoperfusion. Other causes of LV systolic dysfunction include valvular heart disease, hypertension, congenital heart disease, inflammation and chemotherapeutic drugs or other toxic agents.
Right ventricular (RV) systolic dysfunction is usually a consequence of LV systolic dysfunction. It can also occur as a result of right ventricular infarction, pulmonary hypertension, chronic tricuspid regurgitation, or arrhythmogenic right ventricular dysplasia (7).
Predominant diastolic dysfunction is relatively uncommon in younger patients but increases in importance in the elderly (8). It is mostly related to chronic hypertension and diabetes mellitus (7).
Cardiomyopathies, or “severe myocardial diseases leading to heart failure” (9), can be divided into non-ischemic cardiomyopathies (NICM) and ischemic cardiomyopathies (ICM). The class of NICM is further subdivided according to functional disturbances into dilated (DCM), hypertrophic (HCM) and restrictive (RCM) cardiomyopathies. Although the etiology of diseases in each group may vary, the strength of this classification is that virtually all cardiomyopathies can be readily placed in one of three categories and the therapeutic approaches to each category are distinctly different (10).

1.2.2. Prevalence, incidence and prognosis of heart failure

Heart failure is more common than most cancers, including breast, testicular, cervical and bowel cancers (11) with a prevalence of ~1-2% in the overall population and >10% in people older than 75 years (12). It is the only major cardiovascular disease (CVD) whose prevalence and incidence are increasing, partly as a result of the ageing population and a lack of curative therapies (13).
It has been estimated that approximately 14 million people in Europe currently suffer from heart failure and over 3.6 million new cases are being reported each year. Admission to hospital with HF has more than doubled in the last 20 years and, by 2020, the number of deaths attributed to heart failure could reach nine million per year (14).

The long-term prognosis associated with HF is uniformly poor despite recent improvements in diagnosis and therapeutic treatment. It is estimated that only 25% of men and 38% of women carrying a diagnosis of HF will survive more than five years following the onset of disease, whereas of those diagnosed with severe HF, more than 50% will die within one year after first hospitalization (15-18). Novel treatments for HF can slow, but not arrest the progression of the disease. Despite numerous advantages of modern approaches, quality of life for patients surviving HF-related hospitalizations is low and discomfort and distress is often worse than in many other chronic diseases (11,18).

1.2.3. Pathophysiology of heart failure

Various triggers such as CAD, ischemia, hypertension, inflammation or toxic agents can cause myocardial damage. To maintain cardiac output and proper organ perfusion in such conditions, several compensatory mechanisms are activated in order to increase muscle contractility and circulating blood volume. Compensatory mechanisms include activation of neurohormonal pathways such as the renin-angiotensin and the endothelin system. This compensatory regulation may maintain cardiovascular homeostasis during short-term cardiac stress, and the patient may remain asymptomatic over a variably long period of time. However, in this early, “compensated”, stage of HF development a process called “cardiac remodeling” already takes place. It is scaled to the cellular and molecular level and includes myocyte hypertrophy and elongation, as well as changes in gene expression in the direction of a “fetal gene program” reactivation. Prolonged, continuous neurohormonal stimulation leads to further cardiac enlargement and a progressive decline in contractile function, eventually
resulting in “decompensated” HF with its signs and symptoms (Fig. 1-2). In addition to neurohormonal stimuli, a systemic pro-inflammatory state occurs with activation of cytokines and other inflammatory mediators. This systemic response to a local cardiac defect results in progressive systemic phenotypic alterations, including the vasculature, kidneys, skeletal muscle and other organs. In combination with cardiac dysfunction, these global changes eventually result in the syndrome of heart failure.

Figure 1-2: Overview of heart failure development. The figure summarizes events leading to HF, which have been recently reviewed in detail (19-21).
1.2.4. Molecular basis of mechanisms involved in hypertrophy, remodelling and failure

As mentioned above, cardiac remodeling in response to injury initially aims at restoring and maintaining cardiac output. Short-term neurohumoral adaptation may contribute to maintenance of perfusion of vital organs in two ways: (1) by maintaining arterial pressure during inadequate blood flow by vasoconstriction, resulting in redistribution of the flow to vital organs and (2) by restoration of cardiac output by increasing myocardial contractility and heart rate and by expansion of the extracellular fluid volume (22,23). The neurohumoral mediators capable of maintaining systemic arterial pressure are potent vascular constrictors like angiotensin (AT) and endothelin (ET), the action of sympathetic nervous system and the secretion of aldosterone (Fig. 1-3).

Figure 1-3: Neurohormonal activation during cardiac stress leads to positive inotropic and lusitropic effect. ECM, extracellular matrix; ET-1, endothelin-1; ETAR and ETBR, endothelin-A and endothelin-B receptors; ATII, angiotensin II; AT₁, AT II receptor type 1; E, epinephrine; NE, norepinephrine; α₁-AR and β-AR, α₁ and β adrenergic receptor; PLC, phospholipase C; IP₃, inositol-1,4,5-trisphosphate; IP₃R, IP₃ receptors; DAG, diacylglycerol; PKC, protein kinase C; LCC, L-type calcium channel; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA, SR-Ca²⁺-ATPase; PLB, phospholamban; AC, adenyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; P, phosphate. Note the increase in free cytoplasmic and nucleoplasmic [Ca²⁺].
**Endothelin** is a strong vasoconstrictor primarily expressed in the endothelium and present in three different isoforms ET-1, ET-2, and ET-3. Of the three isoforms, ET-1 is expressed in cardiac myocytes where it has potent inotropic activity (24). ET-1 exerts its function by binding to endothelin-A and endothelin-B receptors (ETARs and ETBRs) (25,26). ETARs and ETBRs are G_q protein-coupled receptors that activate phospholipase C (PLC) and cause hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) (27). IP_3 translocates from the plasma membrane to the cytoplasm and binds to IP_3 receptors (IP_3R) located at the SR membrane and the nuclear envelope (NucE). IP_3Rs are Ca^{2+} release channels and, unlike the short bursts and removals of Ca^{2+} induced by CICR, IP_3-mediated Ca^{2+} signaling results in a lower amplitude, but sustained Ca^{2+} release (27). On the other hand, DAG activates several isoforms of protein kinase C (PKC), which also regulate myocyte contractility.

**Angiotensin** is a peptide hormone that causes vasoconstriction, increased blood pressure, and release of aldosterone from the adrenal cortex. The active form of the hormone, angiotensin II (ATII), is derived by removal of two C-terminal residues from angiotensin I by the angiotensin-converting enzyme (ACE). Similarly to ET, it signals primarily via the G_q-coupled ATII receptor type 1 (AT_1) and activates PLC signaling pathway and IP_3-mediated Ca^{2+} release.

**Epinephrine** and **norepinephrine**, catecholaminergic mediators of the sympathetic nervous system, modulate target cells via action on two families of adrenergic receptors (AR). Epinephrine can activate both, α and β-ARs, whereas norepinephrine targets only α-ARs. Signaling via G_q-coupled α1-AR, leads to the activation of PLC/IP_3/IP_3R pathway and mobilization of Ca^{2+} from the stores. On the other hand, stimulation of the β-AR subtype most abundant (~80% in the healthy heart) AR, β1-AR, activates G_s protein, which acts on adenylyl-cyclase (AC), causing the production of cAMP. This stimulates cAMP-dependent PKA, which phosphorylates and controls the function of numerous substrates important for SR Ca^{2+} regulation, including the LCC, RyR and PLB (27,28). These PKA-mediated effects regulate the inotropic and chronotropic functions of the healthy
heart, but chronic activation of β1-AR signaling pathways may lead to substantial alterations of Ca\(^{2+}\) homeostasis in cardiac cells (27).

Even though these neurohormonal pathways may initially be compensatory and beneficial, there is a number of maladaptive consequences eventually leading to the development of hypertrophy and its progression into HF. For example, overexpression of human β1ARs in transgenic mice has various negative effects on the heart (29) and the activation of the sympathetic nervous system in patients with HF contributes to the poor prognosis associated with severe heart failure (30). Several studies have demonstrated that plasma ET levels are elevated in patients with HF (31,32) and in animal models of HF (33). As reported by Kockskämper and colleagues (34) and Wu and colleagues (35), ET-1 causes hypertrophy – at least in part – by activating IP\(_3\)-mediated Ca\(^{2+}\) release from IP\(_3\)Rs located at the NucE. ATII and ACE are both present at high levels during cardiac hypertrophy (36,37). Overexpression of AT\(_1\)Rs in the heart results in hypertrophy and fibrosis in otherwise healthy mice. These mice also show enhanced hypertrophic responses to pressure overload and die prematurely in response to heart failure (38).

The improvement in patient survival associated with the use of ACE inhibitors, AT\(_1\)R blockers, β-blockers, and aldosterone antagonists provides indirect evidence for the long-term deleterious effect of neurohumoral overstimulation and anti-neuroendocrine therapy is the basis for modern treatment of HF. However, while the pathophysiology of HF also implicates ET-1 in the progression of disease, the promise of ET inhibition as a novel therapeutic strategy has not been confirmed in clinical trials (39).

Although the exact mechanism of how the process of cardiac hypertrophy and remodeling is initiated and maintained is still not totally resolved, it is known that a key subcellular mediator for adaptation factors is Ca\(^{2+}\) and its regulation. Increased free cytoplasmic and nucleoplasmic [Ca\(^{2+}\)] was previously shown to be causally involved in the initiation and progression of heart failure (40) and altered Ca\(^{2+}\) homeostasis has been identified in hypertrophic and failing human hearts, as well as in many different animal models of HF (41,42).
1.2.5. **Ca\textsuperscript{2+} in the regulation of transcription and hypertrophy**

Increased intracellular Ca\textsuperscript{2+} concentration, [Ca\textsuperscript{2+}], can activate two major Ca\textsuperscript{2+}-dependent hypertrophic signaling pathways: Ca\textsuperscript{2+}-calmodulin (CaM)-CaM-dependent protein kinase II (CaMKII)-histone-deacetylase (HDAC) and Ca\textsuperscript{2+}-CaM-calcineurin (CaN)-nuclear factor of activated T cells (NFAT) pathways (Fig. 1-4)(1).

**Figure 1-4: Ca\textsuperscript{2+}-dependent transcriptional regulation in CMs.** ECM, extracellular matrix; ET-1, endothelin-1; ATII, angiotensin II; NE, norepinephrine; GPCR, G protein-coupled receptor; PLC, phospholipase C; IP\textsubscript{3}, inositol-1,4,5-trisphosphate; IP\textsubscript{3}R, IP\textsubscript{3} receptors; RyR, ryanodine receptor; DAG, diacylglycerol; TRPC, transient receptor potential cation channel; CaM, calmodulin; CaN, calcineurin; NFAT, nuclear factor of activated T cells; CaMKII, calmodulin-dependent protein kinase II; HDAC, histone-deacetylase; MEF2, myocyte enhancer factor 2.

CaM is a multifunctional second messenger that transduces Ca\textsuperscript{2+} signals in all eukaryotic cells. Upon binding of Ca\textsuperscript{2+} ions, CaM undergoes conformational
I. Introduction

changes which allow the interaction of Ca\(^{2+}\)-CaM complex with different target proteins, initiating various signaling cascades. Transgenic overexpression of CaM causes hypertrophy *per se* (43), and CaM inhibition can block myocyte hypertrophy (44).

CaM is known to stimulate CaMKII, a serine/threonine protein kinase that can phosphorylate several target proteins in the cell. The major CaMKII isoform expressed in cardiac myocytes is CaMKII\(\delta\) (45,46). Two splice variants of CaMKII\(\delta\) have been identified: (1) CaMKII\(\delta\)B which contains a nuclear localization sequence (NLS) and therefore compartmentalizes to the nucleus and (2) CaMKII\(\delta\)C with no NLS which concentrates in the cytoplasm, but neither localization is exclusive (47). CaMKII\(\delta\)C mostly affects ECC by modulation of Ca\(^{2+}\) handling proteins such as PLB, RyR, and L-type Ca\(^{2+}\) channel, whereas CaMKII\(\delta\)B targets proteins involved in the regulation of various transcription factors, e.g. HDACs class II. Phosphorylation of HDACs and subsequent nuclear export relieves HDAC-dependent suppression of myocyte enhancer factor 2 (MEF2)-driven transcription. Liberated MEF2 is strongly implicated in the development and progression of hypertrophy (48).

Another common target for CaM is calcineurin, (CaN), a Ca\(^{2+}\)/CaM complex-dependent serine/threonine protein phosphatase. CaN has a much higher affinity for Ca\(^{2+}\)/CaM complex than CaMKII. Thus, whereas CaMKII may respond to high-amplitude Ca\(^{2+}\) changes, CaN may be better in sensing smaller, sustained Ca\(^{2+}\) elevations (1). Activated CaN binds to phosphorylated NFAT, which is predominantly located in the cytoplasm. After dephosphorylation, NFAT exposes a NLS, which causes its import into the nucleus, where NFAT can – together with GATA4 – activate hypertrophic gene transcription (49).

1.2.6. **Nucleoplasmic vs. cytoplasmic Ca\(^{2+}\) signaling**

Nuclear Ca\(^{2+}\) regulates key cellular processes including gene expression, apoptosis, assembly of the nuclear envelope and nucleo-cytoplasmic transport (1,50-53).
As mentioned before, in CMs there is a transient rise in the cytoplasmic free $\text{Ca}^{2+}$ concentration, $[\text{Ca}^{2+}]_{\text{cyto}}$, during each heart beat. Each cytoplasmic $[\text{Ca}^{2+}]$ transient (CaT) also elicits a nucleoplasmic CaT (34,54). However, previous studies have shown that nucleoplasmic $[\text{Ca}^{2+}]$ transients (CaT) follow significantly slower kinetics comparing to the kinetics of cytoplasmic CaTs (34,55). The reason is the insulation of the nucleus from the surrounding cytoplasm by the nuclear envelope (NucE), which not only contributes to nuclear structural integrity, but also controls bidirectional transport of ions (including $\text{Ca}^{2+}$) and macromolecular cargo, acts as a functional $\text{Ca}^{2+}$ store to regulate nucleoplasmic $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_{\text{nuc}}$) and gene expression, and operates as a mechanotransducer and a platform for signaling (56). The NucE consists of the inner and outer nuclear membrane, and is interrupted by macromolecular nuclear pore complexes (NPC) connecting the nucleoplasm and the cytoplasm and allowing the passive diffusion of small molecules and ions between these two compartments. NucE is not simply a smooth-surfaced outer boundary, but is interrupted by invaginations that reach deep into the nucleoplasm and may even traverse the nucleus completely. The presence of such NucE invaginations has been demonstrated in a number of cell types, including cardiomyocytes (56-60). The NucE invaginations are lined by both, the inner and the outer nuclear membranes and filled with cytosol, SR and even mitochondria (58). Such a network of invaginations provides sites capable of carrying out the NucE functions deep within the nucleus in regions that would otherwise be remote from the nuclear periphery (56).

Whether cytoplasmic CaTs transmit passively to the nucleus by $\text{Ca}^{2+}$ diffusion through nuclear pore complexes or whether $[\text{Ca}^{2+}]_{\text{nuc}}$ can also be regulated actively and – if so – by which mechanisms, is not fully understood. The notion that $[\text{Ca}^{2+}]_{\text{nuc}}$ does not just passively follow the $[\text{Ca}^{2+}]_{\text{cyto}}$ changes, but may instead be regulated actively and independently, is supported by evidence showing that the nuclear envelope expresses $\text{Ca}^{2+}$-regulating proteins including the sarcoplasmic/endoplasmic reticulum $\text{Ca}^{2+}$-ATPase (SERCA), $\text{Ca}^{2+}$ release channels, and $\text{Ca}^{2+}$-buffering proteins (59,61-63). Furthermore, the NucE
contains a set of G protein-coupled receptors (GPCRs), including receptors for ET-1, and angiotensin II, and the entire machinery for IP₃ signaling directed to the nucleoplasm. It has been previously shown that ET-1 – via IP₃R-mediated Ca²⁺ release from the nuclear envelope – may increase [Ca²⁺]nuc independently from [Ca²⁺]cyto (34).

Thus, nucleoplasmic [Ca²⁺] is the sum of Ca²⁺ that enters the nucleoplasm by diffusion from the cytoplasm through NPCs and Ca²⁺ that is released from the NucE in a specific and regulated manner upon different stimuli. That means that

**Figure 1-5: Nucleoplasmic [Ca²⁺] regulation.** ECM, extracellular matrix; ET-1, endothelin-1; ATII, angiotensin II; NE, norepinephrine; GPCR, G protein-coupled receptor; PLC, phospholipase C; IP₃, inositol-1,4,5-trisphosphate; IP₃R, IP₃ receptors; RyR, ryanodine receptor; SERCA, SR-Ca²⁺-ATPase; DAG, diacylglycerol; TRPC, transient receptor potential cation channel; LCC, L-type calcium channel.
any event which either leads to increase in cytoplasmic $[Ca^{2+}]$ (i.e. mechanical stretch, increased heart rate or non-excitatory stimulation) or stimulates active $Ca^{2+}$ release from NucE (i.e. neurohormonal activation) will also lead to an increase in nucleoplasmic $[Ca^{2+}]$ (Fig. 1-5).

Increase in nucleoplasmic $[Ca^{2+}]$ may activate transcription factors, thereby modulating the gene expression (40,64), and it has also been implicated in the development of cardiac hypertrophy and the progression of heart failure (65-67). However, little is known about the autonomous regulation and alterations of $[Ca^{2+}]_{nuc}$ homeostasis under different physiological and pathophysiological conditions, thus underlining the importance of better understanding the relationship between $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{cyto}$ and how $Ca^{2+}$-dependent signaling in the nucleus is regulated. This, nonetheless, requires accurate measurements of $[Ca^{2+}]$ in both the nucleoplasmic and cytoplasmic compartments.

**1.2.7. Quantification of nucleoplasmic vs. cytoplasmic $[Ca^{2+}]$**

Quantification of subcellular $Ca^{2+}$ concentration and fluxes is of crucial importance for understanding physiological and pathological processes in the heart (68,69).

The fluorescein-derived fluorescent indicators Fluo-3 and Fluo-4 are widely used to monitor $[Ca^{2+}]$ in the cytoplasm and nucleoplasm of various cell types. Determination of $[Ca^{2+}]_{nuc}$ vs. $[Ca^{2+}]_{cyto}$ using these indicators, however, encounters several technical difficulties and has provided quite variable results. Some reports have indicated that resting $[Ca^{2+}]_{nuc}$ is higher than $[Ca^{2+}]_{cyto}$ (70), but also the opposite has been observed (71). In other cases no difference between $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{cyto}$ has been found (72) or it has been attributed to artifacts (73). This discrepancy is in part caused by the fact that estimates of $[Ca^{2+}]_{nuc}$ changes are usually based on the assumption of identical behavior of the $Ca^{2+}$ indicators in different cellular compartments. This assumption, however, is not valid if fluorescence properties of the dyes are altered differentially by the cytoplasmic and nucleoplasmic environment, as observed for Fluo-3.
fluorescence in *Xenopus* oocyte nucleoplasmic and cytoplasmic homogenates (74). *In situ* studies with three different mouse cell lines loaded with either Fluo-3 or Fluo-4 yielded similar results (75). A comparison of fluorescent Ca$^{2+}$ indicator properties in HeLa cells further confirmed distinct characteristics of Fluo-3 and Fluo-4 in the cytoplasmic vs. nucleoplasmic compartment (72). Further problems can arise from the sequestration of Ca$^{2+}$ indicators into intracellular organelles, such as the endoplasmic reticulum or mitochondria (73,76). Indicators have also been reported to leak from the cytoplasm to the extracellular medium facilitated by sarcolemmal anion transporters. The consequence of all these observations is that without proper independent determination of the indicator properties in the nucleoplasm vs. the cytoplasm, any quantitative analysis of [Ca$^{2+}$]$_{\text{nuc}}$ vs. [Ca$^{2+}$]$_{\text{cyto}}$ is not reliable. Thus, addressing quantitative changes of [Ca$^{2+}$]$_{\text{nuc}}$ and [Ca$^{2+}$]$_{\text{cyto}}$ requires transformation of raw fluorescence signals into calibrated [Ca$^{2+}$], taking into account the effects of the different subcellular environments on the characteristics of the indicator. In CMs, however, the Ca$^{2+}$ binding affinities and fluorescent properties of Fluo indicators in different intracellular compartments have not been determined yet.
1. Introduction

1.3. Aims and description of thesis projects

Adaptation of the heart to stress involves cardiac hypertrophy and remodeling. Adaptation may be beneficial in the short term, but eventually activate maladaptive pathways that ultimately result in heart failure. There is evidence that cytoplasmic Ca\(^{2+}\) handling is altered during adaptive and eventually maladaptive processes. In addition, specific hypertrophic signaling cascades intimately depend on and are activated by cytoplasmic Ca\(^{2+}\) ions. Recent work showed that also nuclear Ca\(^{2+}\) handling involves active regulation processes, and that also nuclear Ca\(^{2+}\) handling may directly impact gene expression. However, there is almost no information on cytoplasmic vs. nucleoplasmic Ca\(^{2+}\) handling in physiological and pathophysiological conditions. Also, the mechanisms that govern nuclear Ca\(^{2+}\) handling, as well as potential alterations in these mechanisms during the progression to heart failure are unknown. Part of this agnostic state is related to difficulties in measuring and quantifying [Ca\(^{2+}\)] in distinct subcellular spaces.

Therefore, the aim of my work was 4-fold:

1. To establish a novel technique that allows the reliable subcellular quantification of [Ca\(^{2+}\)] in the cytoplasm vs. the nucleus
2. To characterize and quantify cytosolic vs. nucleoplasmic Ca\(^{2+}\) handling in isolated myocytes from non-failing mammalian and human hearts during varying physiological conditions
3. To describe specific alterations in cytoplasmic vs. nucleoplasmic Ca\(^{2+}\) handling in hypertrophy and failure in an animal model of pressure overload and in failing human hearts
4. To assess underlying structural and biochemical alterations that contribute to changes in nuclear Ca\(^{2+}\) handling.
The **first part** of the thesis, thus, describes in detail the newly developed method for transformation of the fluorescence signal of a frequently used Ca\(^{2+}\) indicator, Fluo-4, and the newly available ratiometric indicator Asante Calcium Red into absolute \([\text{Ca}^{2+}]\) and nucleoplasmic-to-cytoplasmic Ca\(^{2+}\) gradients. The **second part** describes the application of the developed method for quantifying subcellular Ca\(^{2+}\) signals in resting myocytes and during the cardiac cycle within a range of increasing stimulation frequencies. Part 1 and 2 of my thesis were previously published as an original article (55) and served as the basis for quantification of subcellular Ca\(^{2+}\) signals in early – compensated and terminal – decompensated stage of heart failure.

The **third part** of the thesis details my findings on distinct alterations in nucleoplasmic vs. cytoplasmic Ca\(^{2+}\) handling during the development and progression of heart failure in myocyte isolated from mouse and human hearts.

In the **fourth part**, I describe specific structural and biochemical remodelling of the nuclear envelope during the development and progression of heart failure in myocyte isolated from mouse and human hearts. Part 3 and 4 are currently under preparation for the submission in the peer reviewed journal.

Collectively, the results revealed that Fluo-4 and Asante Calcium Red exhibit distinctively different fluorescent and Ca\(^{2+}\) binding properties between cytoplasm and nucleoplasm of CMs and that there are significant nucleo-to-cytoplasmic \([\text{Ca}^{2+}]\) gradients in resting CMs, that are dependent on intact 2-APB-sensitive Ca\(^{2+}\) stores. Perinuclear Ca stores and nucleoplasmic CaTs undergo significant changes during pressure overload-induced hypertrophy in mice, as well as in moderately and severely failing human hearts. These changes appear to precede changes in cytoplasmic Ca\(^{2+}\) regulation, and raise the possibility that altered nucleoplasmic \([\text{Ca}^{2+}]\) may contribute to the development and/or progression of cardiac hypertrophy.
2. Materials and Methods
2. Materials and Methods

2.1. Cell Preparation

2.1.1. Murine and rat myocyte isolation

The experimental procedures used for isolation of atrial and ventricular myocytes from adult rat and mouse hearts were approved by the local Animal Care and Use Committees according to criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences (National Institutes of Health publication No. 85-23, revised 1996). Male adult rats were terminally anaesthetized by injection of urethane (1 g kg\(^{-1}\) intraperitoneally). Mice of either sex were anaesthetized with isoflurane prior to cervical dislocation. The thoracic cavity was open to excise the hearts and the aorta was rapidly cannulated. Atrial and ventricular myocytes from adult rat and mouse hearts were isolated by a standard collagenase or liberase-based Langendorff perfusion protocol as described in detail previously (77,78). Freshly isolated myocytes were plated on laminin-coated glass-bottomed culture dishes and allowed to attach to the bottom for at least 30 min. Only quiescent, Ca\(^{2+}\)-tolerant, rod-shaped myocytes with clear cross striations were used for experiments within 4 hours. All experiments were conducted at room temperature (22-24°C).

2.1.2. Human myocyte isolation

The procedure was approved by the Ethical Committee of the University Hospital (ref. nr: 20-277 ex 08/09) and was carried out in accordance with the *Declaration of Helsinki*. Myocardial tissue samples were obtained from the explanted failing hearts at the time of transplantation (n = 4) and from unused donor hearts (n = 8). Heart function was evaluated by transthoracic echocardiography at the Division of Cardiology, Medical University of Graz, and divided in three groups: non-failing (EF≥55%), moderately failing (55%>EF>35%) and severely failing (EF≤35%). Patient characteristics are summarized in Table III-1.
2. Materials and Methods

Table 2-1: Basic characteristic of patients undergoing transplantation or heart donors. EF, ejection fraction; Meds, medications; CAT, catecholamine; AB, antibiotics; PP, phenprocoumon; ACEI, ACE inhibitors; BB, β-blockers; D, diuretics.

<table>
<thead>
<tr>
<th></th>
<th>Non-failing</th>
<th>Moderately failing</th>
<th>Severely failing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td>Age, y</td>
<td>EF, %</td>
<td>Sex</td>
</tr>
<tr>
<td>m</td>
<td>62</td>
<td>65</td>
<td>m</td>
</tr>
<tr>
<td>f</td>
<td>57</td>
<td>65</td>
<td>f</td>
</tr>
<tr>
<td>m</td>
<td>46</td>
<td>65</td>
<td>f</td>
</tr>
<tr>
<td>f</td>
<td>64</td>
<td>55</td>
<td>f</td>
</tr>
<tr>
<td>m</td>
<td>54</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>57±3</td>
<td>61±2</td>
<td>50%</td>
</tr>
<tr>
<td><strong>50%</strong></td>
<td>43±12</td>
<td>14±1</td>
<td></td>
</tr>
</tbody>
</table>

Mean data are presented as mean±S.E.M., *P<0.05 vs. non-failing.

The procedure for cell isolation was modified after Sipido et al (79). Immediately following the resection, hearts were perfused with Custodiol® cardioplegic solution in the operating room at the time of surgery. Hearts were kept at 4°C in Custodiol® cardioplegic solution supplemented with 2,3-butanedione-monoxime (BDM) and transported to the laboratory. A wedge of the left ventricular wall with its perfusing coronary artery was carefully excised, the artery was cannulated and perfused with Tyrode’s solution containing: 130 mM NaCl, 5.4 mM KCl, 1.2 mM MgSO₄, 1.8 mM CaCl₂, 20 mM HEPES, 10 mM taurine, 12.5 mM glucose, 2 mM pyruvate and 1 mM Na-L-lactate; pH 7.2 with NaOH. If possible the left anterior descending coronary was used; otherwise the left circumflex or a posterior branch was used. After cannulation the tissue wedge was perfused at 37°C with a Ca²⁺-free Tyrode’s solution for 10 min followed by enzyme perfusion for 25 min (liberase 0.15 mg/ml, trypsin 0.056 mg/ml, in 0.05 mM CaCl₂ Tyrode’s solution). The enzyme was washed out by 0.05 mM CaCl₂ Tyrode’s solution perfusion for 5 min. The tissue was then sectioned in slices of 5–7 mm and slices with visible signs of digestion were selected and minced in low Ca²⁺ solution. The preparation was filtered and resuspended in 0.1 mM CaCl₂ Tyrode’s solution. Small part of cell yield was fixed for immunocytochemistry and the rest of the solution was stepwise replaced by a Tyrode’s solution containing 1.8 mM CaCl₂. Ca²⁺-tolerant cell yield was 5-20% and these cells were used for CaT recordings. Freshly isolated cells were stored at room temperature and studied within 5 h after isolation.
2.2. Confocal Ca\textsuperscript{2+} imaging

2.2.1. Confocal Ca\textsuperscript{2+} imaging of subcellular CaTs

Cells were loaded with the Ca\textsuperscript{2+}-sensitive fluorescent dye Fluo-4 (Molecular Probes, Leiden, The Netherlands) by 30 min incubation in normal Tyrode’s (NT) solution (rat cells: 130 mM NaCl, 5.4 mM KCl, 1.5 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 22 mM glucose, 10 mM HEPES, 5 mM glutamine and 0.01 U/ml insulin; mouse cells: 140 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM glucose, and 10 mM HEPES; pH 7.4 with NaOH) containing 8 μM of the acetoxymethylester of the dye (dissolved in 20% (w/v) Pluronic-127/dimethyl sulfoxide (DMSO); Molecular Probes, Leiden, The Netherlands). Further 30 min were allowed for de-esterification. When Asante Calcium Red (ACaR) was used, myocytes were incubated with 10 μM ACaR AM (TEFLabs, Austin, TX, USA) for 90 min, and 45 min were allowed for de-esterification.

Fast, two dimensional (2D) confocal [Ca\textsuperscript{2+}] imaging was performed as described previously (80) using a confocal imaging setup (VisiTech International, Sunderland, UK) that consisted of an inverted microscope (Nikon, Tokyo, Japan) equipped with a ×40 oil-immersion objective lens (N.A. 1.3), a Nipkow dual disc-based confocal unit, and an ICCD camera with a temporal resolution of 120 Hz. Fluo-4 was excited by the 488 nm light from an argon-ion laser and fluorescence was collected at wavelengths >515 nm.

Alternatively, a Zeiss LSM 510 Meta confocal laser point scanning system (Carl Zeiss, Jena, Germany) equipped with a ×40 oil-immersion objective lens (N.A. 1.3) and an argon-ion laser was used. For Fluo-4, excitation and emission wavelengths were 488 nm and >515 nm, respectively. For ACaR, excitation occurred at 488 nm and emission was collected at >650 nm (F\textsubscript{1}) and at 475-525 nm (F\textsubscript{2}). A 512 pixel scan line was drawn along the longitudinal axis of the cell and scanned every 1.91 milliseconds. Consecutive scan lines were stacked over time and visualized as 2D image.
The z-axis resolution of images obtained with either confocal system was 0.9-1.4 μm. The height of the nuclei amounted to 5-7 μm. For measuring $[\text{Ca}^{2+}]_{\text{nuc}}$, the confocal plane was set to the middle (z axis) of the nuclei thus ensuring that only fluorescence originating from the nucleoplasm was collected.

Cells loaded with the indicator were placed on the stage of the microscope and superfused with NT solution delivered by a gravity-driven superfusion pipette. For quantification of nucleoplasmic vs. cytoplasmic CaTs cells were field-stimulated via two platinum electrodes at 1 Hz. Resting $[\text{Ca}^{2+}]$ was defined as $[\text{Ca}^{2+}]$ 1 min after cessation of stimulation. To compare nucleoplasmic vs. cytoplasmic resting $[\text{Ca}^{2+}]$ after Ca$^{2+}$ store depletion, cyclopiazonic acid (CPA, 5 μM), an inhibitor of the SERCA, was applied in Ca$^{2+}$-free Tyrode's solution (containing 1mM EGTA and no Ca$^{2+}$ added). To measure the frequency dependent changes in CaTs, stimulation frequency was gradually increased from 0.5 Hz to 5 Hz. Isoprenaline (30 nm) was used to investigate the effects of β-adrenergic stimulation. Part of the frequency response experiments in control hearts were performed by Mojib Asgarzoei at the Division of Cardiology, Medical University of Graz.

### 2.2.2. Measurement of the point spread function (PSF)

The PSF measurements were performed by imaging green subresolution fluorescent beads (PS-Speck Microscope Point Source Kit, Molecular Probes, Leiden, The Netherlands) with excitation/emission maxima at 505/515 nm and a diameter of 0.175±0.005 μm. For PSF measurements 2 μl of ready-to-use PS-Speck microsphere suspension was mounted to poly-L-lysine-coated glass-bottomed culture dishes and allowed to attach and air dry. When the sample was completely dry, a small drop of mounting medium was added to cover the spot; a coverslip was placed on the dish and sealed. The PSF of the Zeiss LSM 510 Meta confocal microscope was determined by imaging beads in three dimensions. The beads were imaged with a resolution of 0.05 μm x 0.05 μm x 0.1 μm per voxel. Images of the beads were captured using the same parameters as for the cardiomyocyte images (i.e. laser excitation at 488 nm,
objective lens, pinhole size, zoom factor). Twenty singular beads, i.e. beads with no neighbors in the field of view, were selected to provide sufficient signal-to-noise ratio for the reconstruction of the PSF. An ImageJ plug-in to measure 3D point spread functions (MOSAIC Group, www.mosaic.inf.ethz.ch, Switzerland) was used for analysis and reconstruction of the z-stacks. ImageJ software (National Institutes of Health, USA) was used for the intensity analysis of the generated PSF map. Optical section thickness, i.e. axial resolution (z resolution), was determined as full width at half maximum (FWHM).

2.2.3. Spectral properties of Fluo-4 in the presence of 2-ABP, CPA, and tetracaine

To evaluate the effects of 2-APB (3 μM), CPA (5 μM) and tetracaine (1 mM) on Fluo-4 physico-chemical properties that can affect confocal Ca^{2+} measurements, excitation and emission spectra were recorded as follows. Internal solutions containing 8 μM of Fluo-4 pentapotassium salt and either no or a saturating free [Ca^{2+}] (2 mM) were prepared and Fluo-4 emission and excitation spectra were recorded in the absence and presence, respectively, of the tested substances. Emission spectra were recorded between 430-700 nm (5 nm increments) at an excitation wavelength of 488 nm. The excitation spectra were recorded between 300-600 nm (5 nm increments) at an emission wavelength of 520 nm. All spectra were recorded in duplicate on a Flexstation II fluorimeter (Molecular devices, Ismaning, Germany) at the Institute of Experimental and Clinical Pharmacology of the Medical University of Graz.

2.3. In situ calibration of Ca^{2+} indicators in adult CMs

2.3.1. In situ calibration of Fluo-4 and ACaR fluorescence

Calibration solutions were made using the procedure described by Bers and McGuigan (81,82). Briefly, total [Ca^{2+}] required to obtain the desired free [Ca^{2+}] in
the presence of 1 mM EGTA was calculated using the MaxChelator program (52) (http://www.stanford.edu/~cpatton/maxc.html). Two solutions were prepared: (1) EGTA solution contained (mM): 130 NaCl, 5.4 KCl, 0.5 MgCl₂, 1 EGTA, 15 BDM (2,3 butanedione monoxime), 25 Hepes, 0.01 A23187, 1.8 2-deoxy-Dglucose, 0.01 rotenone, 0.005 CPA, pH 7.4; (2) CaEGTA solution contained 2 mM CaCl₂ in addition. Known quantities of EGTA and CaEGTA solutions were mixed to obtain calibration solutions with free [Ca²⁺] of 0 nM, 50 nM, 250 nM, 750 nM, 1500 nM, 3000 nM and 1 mM. The free [Ca²⁺] was confirmed with a Ca²⁺-sensitive electrode (Orion 97-20 ionplus; Thermo Electron Co., Beverly, MA, USA). Calibration solutions also contained the Ca²⁺ ionophore A23187, metabolic inhibitors (2-deoxy-D-glucose, 1.8 mM; rotenone, 10 μM) and the SERCA inhibitor (CPA, 5 μM) in order to block active Ca²⁺ transport systems and allow equilibration of [Ca²⁺] between the extracellular medium and the cell interior (72). Finally, the calibration solutions contained butanedione monoxime (BDM) to prevent contracture of the myocytes at high [Ca²⁺]. Cells were equilibrated in each calibration solution for 8 min. During this period, fluorescence was averaged over 1 s intervals every 2 min. Minimal Fluo-4 or ACaR fluorescence (F_min) was measured during exposure to Ca²⁺-free calibration solution and maximal Fluo-4 or ACaR fluorescence (F_max) during exposure to a calibration solution containing a saturating free [Ca²⁺] of 1 mM.

2.3.2. Compartmentalization and leakage of Fluo-4

Compartmentalization of Fluo-4 was assessed using the Zeiss LSM 510 Meta confocal microscope equipped with a ×40 oil-immersion objective lens (N.A. 1.3) running a time-series protocol (512 pixels x 512 pixels at 0.5 Hz). Myocytes were permeabilized with digitonin (5 μM, 1 s) at different time points (20, 30 and 40 min) after dye de-esterification and washed with internal solution (140 mM KCl, 0.5 mM KH₂PO₄, 5 mM EGTA, 10 mM HEPES, 2 mM malic acid, 2 mM glutamic acid, 5 mM Na-pyruvate, 3 mM MgATP, 15 mM BDM, and 1.6 mM total CaCl₂ (free [Ca²⁺] computed to be 95.5 nM); pH 7.4 with KOH). For each cell, the Fluo-4
fluorescence remaining after digitonin treatment was calculated as the fraction of Fluo-4 fluorescence before the treatment. In some experiments, 250 μM sulfinpyrazone, an inhibitor of organic anion transporters, was included in the calibration solutions to assess the effect of dye export from the cytoplasm to the extracellular medium and active dye sequestration into cellular organelles.

2.4. Minimally invasive transverse aortic constriction

The surgical procedure was approved by the responsible Ethical Committee and government agencies (ref. nr: BMWF-66.010/0062-II/10b/2010). Cardiac hypertrophy was induced by minimally invasive transverse aortic constriction (TAC) as described previously (83,84). Briefly, ketamine/xylazine-anaesthetized (80 mg/kg and 5 mg/kg body weight, respectively), C57BL/6 wild-type mice of 21±1 g (females) and 24±1 g (males) body weight underwent TAC surgery. The jugulum was horizontally incised to visualize the aorta. A 27-gauge needle was placed parallel to the aorta and tied against the transversal portion of the aorta using a non-absorbable suture. After removal of the needle, the wound was closed. Mice were kept on a warming pad at 37°C in a cage supplied with oxygen-enriched atmosphere until recovering from anaesthesia. An age-matched group of mice underwent sham procedure consisting of aortic exposure without ligation and it served as control. The surgery was performed by Dr. Simon Sedej from the Division of Cardiology of the Medical University of Graz. Ventricular cardiomyocytes were isolated 1 and 7 weeks after TAC/Sham.

2.5. Echocardiography

1 and 6 weeks after Sham/TAC intervention, transthoracic echocardiography was performed by Dr. Albrecht Schmidt, Division of Cardiology, Medical University of Graz, as previously described (85). Mice were initially anaesthetized with 1.5-2% isoflurane and maintained lightly sedated with 0.5% isoflurane during a high
resolution imaging using a 30 MHz linear array transducer (Visual Sonics, Canada). Heating pad was set to maintain body temperature constant at 37°C. M-mode tracings of the left ventricle (short axis) were recorded and end-diastolic (LVEDD) and end-systolic diameters (LVESD) were averaged from three consecutive cardiac cycles. Left ventricular fractional shortening was calculated by the difference between LVEDD and LVESD, divided by LVEDD. Left ventricular mass (LV_{mass}) was calculated according to:

\[
LV_{mass} = 1.005 \times ((IVS + LVPW + LVEDD)^3 - (LVEDD)^3)
\] (86),

where IVS is the intraventricular septum; LVPW is the left ventricular posterior wall and LVEDD is the left ventricular end-diastolic diameter.

**2.6. Perinuclear Ca^{2+} stores visualization**

Perinuclear Ca stores were visualized using confocal imaging system (Zeiss LSM 510 Meta) with a Plan Neofluar 63x/1.3 NA oil-immersion objective and staining with the low affinity Ca^{2+} indicator Mag-Fluo-4/AM (10μM, 90-120min, excitation at 488 nm, emission >515 nm). The pinhole was set to 1 Airy unit, resulting in an optical slice thickness of 0.6 μm. 2D images (2048x2048 pixel, average of 4 frames) through the middle of nuclei were collected. The length and width of the nuclei were measured with LSM 510 software. The longitudinal axis was drawn through the middle of the nuclei and tubular structures were counted along the half of the nuclear envelope which had more invaginations.

**2.7. Immunocytochemistry**

Isolated cardiomyocytes were fixed with 2% paraformaldehyde for 35 min. After fixation, the cells were rinsed with phosphate-buffered saline (PBS), and permeabilized with 0.5% Triton-X in PBS for 15 min. After washing, cells were incubated with a blocking solution (5% BSA in PBS) for 1 h at room temperature.
Cells were then incubated with the primary antibodies for 2 h at room temperature and washed with PBS. Subsequently, the samples were incubated with the secondary antibody for 1 h at room temperature and washed in PBS. Primary and secondary antibodies were diluted 1:300 and 1:500 in PBS/5% BSA (v/v), respectively. Images were recorded by confocal imaging system (Zeiss LSM 510 Meta) with a Plan Neofluar 63x/1.3 oil-immersion objective.

The following antibodies were used: mouse monoclonal anti-nuclear pore complex proteins antibody (ab60080, Abcam, Cambridge, UK), mouse monoclonal anti-SERCA2 antibody (MA-3-919, Thermo Scientific, Rockford, IL, USA), mouse monoclonal anti-RyR antibody (MA3-925, Thermo Scientific, Rockford, IL, USA) and goat polyclonal anti-IP$_3$R antibody (NB100-2466, Novus Biologicals, Littleton, CO, USA).

### 2.8. Electron microscopy

Human ventricular trabeculae were used for electron microscopic analysis of perinuclear structures. Human endocardial trabeculae (cross-sectional area <0.6 mm$^2$) were dissected under a stereo-microscope and prepared as previously described (87). The samples were fixed at 4°C over night in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde and 2.5% formaldehyde. The samples were post fixed for 2 h at room temperature in the same buffer additionally containing 2% osmium tetroxide, rinsed and dehydrated in a series of graded ethanol solutions. Using propylene oxide as an intermedium, the samples were embedded in TAAB epoxy resin (TAAB, Aldermaston, UK). 60 nm thick sections were cut on a Leica UCT ultramicrotome (Leica Mikrosysteme Handelsges.m.b.H., Vienna, Austria). The sections were stained in lead citrate and uranyl acetate using a Leica EM AC20 contrasting instrument, and then visualised using a FEI Tecnai G²20 transmission electron microscope (FEI, Eindhoven, Netherlands), as in Leitinger et al., 2012 (88). Recordings were performed at the Institute of Cell Biology, Histology and Embryology of the Medical University of Graz by Dr. Gerd Leitinger.
2.9. Drugs and solutions

2-APB, A23187, CPA, rotenone, and tetracaine were obtained from Calbiochem (Darmstadt, Germany). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Drugs were dissolved in DMSO as concentrated stock solutions and diluted at least 1000-fold into the medium. This yielded a final DMSO concentration of 0.1% or less which on its own showed no effect on myocyte morphology and contractile function.

2.10. Statistics

The data are presented as mean ± SE. Differences between data sets were evaluated by Student’s t-test or ANOVA and considered significant when P<0.05.
3. Results
3. Results

3.1. *In situ* calibration of Ca\textsuperscript{2+} inhibitors in adult CMs

3.1.1. Point spread function (PSF) and axial resolution

To ensure that our experimental setup is suitable for subcellularly resolved Ca\textsuperscript{2+} signals, we first determined the PSF of the LSM 510 Meta confocal microscope (for experimental details refer to section 2.3.). Average images obtained from 20 individual subresolution fluorescent beads are illustrated in Figure 3-1, *left*. Resolution was measured as full width at half maximum (FWHM). As evident from the profile plots (*right*) along the dashed lines in the images, lateral (xy) resolution was 0.48 μm and axial (xz) resolution was 0.88 μm. At the tails of the z profile plot (*bottom right*), the maximal distention was ≤4 μm. As the height of the nuclei in cardiomyocytes amounted to ~5-7 μm (89), the axial resolution was sufficient for recording nucleoplasmic fluorescence (i.e. Ca\textsuperscript{2+}) signals without contamination from cytoplasmic signals.

![Point spread function (PSF)](image)

*Figure 3-1 Point spread function (PSF).* Point spread function (PSF) of the LSM 510 Meta confocal microscope measured using subresolution fluorescent beads. *xy (top left)* and *xz (bottom left)* images (obtained by averaging images of 20 individual beads) and the according profile plots (*right*) along the dashed lines shown in the images. Resolution was determined as full width at half maximum (FWHM). Lateral (xy) resolution amounted to 0.48 μm and axial (xz) to 0.88 μm.
3.1.2. *In situ* calibration of Fluo-4 fluorescence in adult CM

Mouse ventricular CMs as well as rat atrial and ventricular CMs were loaded with the fluorescent Ca\(^{2+}\) indicator Fluo-4 by incubation with the acetoxymethyl ester form of the dye. Since Fluo-4 fluorescence changes do not directly report changes in the free [Ca\(^{2+}\)], the fluorescence signals had to be transformed into calibrated [Ca\(^{2+}\)]. The most common approach to transforming the Fluo-4 fluorescence into the free [Ca\(^{2+}\)] is the equation first formulated by Grynkiewicz and colleagues (90). For non-ratiometric dyes the formula is as follows:

\[
[\text{Ca}^{2+}] = K_d (F - F_{\text{min}}) / (F_{\text{max}} - F)
\]  

where \(K_d\) is the apparent Ca\(^{2+}\) dissociation constant of the indicator, \(F_{\text{min}}\) and \(F_{\text{max}}\) are the fluorescence intensities at zero and saturating [Ca\(^{2+}\)], respectively, and \(F\) is the fluorescence intensity at any given time. Obviously, the derivation of [Ca\(^{2+}\)] depends on two parameters, \(F_{\text{min}}\) and \(F_{\text{max}}\), which have to be measured for each cell independently. As we sought to define the equation in a way that includes only the indicator’s properties that can be predetermined, we transformed the original formula using the equation for the resting [Ca\(^{2+}\)], \([\text{Ca}^{2+}]_{\text{rest}}\):

\[
[\text{Ca}^{2+}]_{\text{rest}} = K_d (F_{\text{rest}} - F_{\text{min}}) / (F_{\text{max}} - F_{\text{rest}})
\]  

and the dynamic range of the indicator, \(R_f\):

\[
R_f = F_{\text{max}} / F_{\text{min}}
\]  

Elimination of the parameters \(F_{\text{min}}\) and \(F_{\text{max}}\) from equation [1], by their substitution with equations [2] and [3], led to the final formula used for transformation of the fluorescence signals into absolute [Ca\(^{2+}\)]:

\[
[\text{Ca}^{2+}] = K_d \frac{(R (K_d + R_f [\text{Ca}^{2+}]_{\text{rest}}) - (K_d + [\text{Ca}^{2+}]_{\text{rest}}))}{(R_f (K_d + [\text{Ca}^{2+}]_{\text{rest}}) - R (K_d + R_f [\text{Ca}^{2+}]_{\text{rest}}))}
\]  

where \(R\) is the "normalized fluorescence" signal, \(F/F_{\text{rest}}\).
The key parameters in equation [4] are $K_d$, $[\text{Ca}^{2+}]_{\text{rest}}$, and $R_f$. Therefore, fluorescent signals from nucleoplasmic and cytoplasmic compartments with 7 different $[\text{Ca}^{2+}]$ ranging from 0 nM to 1 mM were obtained from mouse ventricular (n=15) and rat atrial (n=8) and ventricular (n=15) cardiac myocytes (Fig. 3-2).

Figure 3-2: In situ calibration of fluorescent $\text{Ca}^{2+}$ indicator Fluo-4 in the nucleus vs. the cytoplasm of mouse and rat cardiac myocytes. (A) Original 2D images of Fluo-4 fluorescence of a mouse ventricular myocyte at various $[\text{Ca}^{2+}]$ during the calibration protocol. (B) Concentration response curves with 7 different $[\text{Ca}^{2+}]$ illustrating the in situ $\text{Ca}^{2+}$-dependent fluorescence of Fluo-4 in the nucleus (red) vs. the cytoplasm (black) of mouse ventricular, rat atrial, and rat ventricular myocytes. (C) Apparent dissociation constants for $\text{Ca}^{2+}$ binding ($K_{d,\text{app}}$) and (D) dynamic range of Fluo-4 fluorescence in the nucleus (red) vs. the cytoplasm (black). Data in (B) to (D) from a total of 15 mouse ventricular, 8 rat atrial, and 15 rat ventricular myocytes. Asterisks indicate $P<0.05$ vs. cytoplasm.

The calibration solutions contained a $\text{Ca}^{2+}$ ionophore, metabolic inhibitors, an inhibitor of SERCA, and BDM, thus allowing rapid and complete equilibration of $[\text{Ca}^{2+}]$ between the extracellular solution and the cell interior and preventing any
active Ca\textsuperscript{2+} transport as well as movement and contracture of the cells at higher [Ca\textsuperscript{2+}]. Figure 3-2A shows original images of a mouse cardiac myocyte obtained with selected [Ca\textsuperscript{2+}] solutions during the calibration protocol. Fluo-4 fluorescence from the nucleus was higher than from the surrounding cytoplasm at each [Ca\textsuperscript{2+}] used and this was true for all cell types studied. Nucleoplasmic and cytoplasmic Fluo-4 fluorescence was plotted vs. [Ca\textsuperscript{2+}] (Fig. 3-2B). These concentration-response curves were fitted using the Hill equation:

\[
F = \{(F_{\text{max}} - F_{\text{min}})/(1+(K_d/[\text{Ca}^{2+}])^n)\} + F_{\text{min}}
\]  

[5]

allowing determination of the in situ Ca\textsuperscript{2+} dissociation constant (K_d) and the Hill coefficient (n). Photobleaching of the Ca\textsuperscript{2+} indicator Fluo-4 was also assessed over the course of the protocol and found to be negligible (not shown). The obtained calibration curves were used to calculate the apparent K_d and the dynamic range, R_f. Fluo-4 showed significantly different Ca\textsuperscript{2+} binding affinities between the nucleoplasmic and cytoplasmic compartments in all cell types studied (Fig. 3-2C). Higher apparent K_d values were found in the nucleoplasm of mouse and rat ventricular myocytes, but not in rat atrial myocytes, where the apparent K_d in the nucleoplasm was lower than in the cytoplasm. Furthermore, significantly higher values for R_f were found in nucleoplasmic as compared to cytoplasmic compartments of all three cell types studied (Fig. 3-2D). Within a given cell type results were uniform, independent of dye loading and gain settings. Once determined average values for K_{d,app}, [Ca\textsuperscript{2+}]_{rest}, and R_f were used in equation [4] for further transformation of the fluorescence signals. For cells in which all calibration parameters had been measured, we compared the [Ca\textsuperscript{2+}] calculated by equation [1] and equation [4]. We observed no significant difference for electrically stimulated CaTs calculated by these two methods. This confirms that the use of K_{d,app}, R_f, and [Ca\textsuperscript{2+}]_{rest} – as determined with the calibration protocol – in combination with equation [4] is well suited for calculation of [Ca\textsuperscript{2+}]\textsubscript{cyto} and [Ca\textsuperscript{2+}]\textsubscript{nuc} in the same type of myocytes, isolated under the same experimental conditions, in which no calibration was conducted.
3. Results

3.1.3. Compartmentalization and leakage of Fluo-4

CMs loaded with Fluo-4 generally displayed uniform cellular fluorescence patterns without obvious accumulation in subcellular organelles (except for the nuclei). The nuclei of cells loaded with Fluo-4 possessed a higher fluorescence than that of the surrounding cytoplasm. Cells left at room temperature for 30 min showed no difference in fluorescence compared to freshly loaded cells.

In order to address whether the difference in nucleoplasmic and cytoplasmic fluorescence levels depended upon compartmentalization of the dye in cytoplasmic organelles, such as mitochondria (73,76), we performed measurements before and after permeabilization of the cells with digitonin. As can be seen in Figure 3-3, this treatment caused loss of >90% of fluorescence from both, nucleoplasmic and cytoplasmic compartments.

Figure 3-3: Lack of significant compartmentalization of Fluo-4. (A) Original recording of nucleoplasmic (red) and cytoplasmic (black) [Ca^{2+}] changes in a digitonin (dig, 5 μM)-treated mouse ventricular myocyte. (B) Corresponding 2D fluorescence images at selected time points showing the loss of >90% of fluorescence from both the nucleoplasmic and the cytoplasmic compartment. Note that the higher fluorescence in the nucleus observed in the beginning of the recording vanishes following digitonin treatment. (C) Digitonin-insensitive fluorescence at any chosen time point after the dye de-esterification period was <10% of the initial fluorescence and essentially identical in the nucleus and the cytoplasm. Data from 6 myocytes for each cell type.
The remaining fluorescence did not increase over time, meaning that there was no significant dye sequestration in cytoplasmic organelles over the time course of the calibration protocol. To further test the possibility that the low fluorescence from cytoplasmic organelles is due to the low resting \([\text{Ca}^{2+}]\) in these cellular compartments, we performed similar experiments with intracellular solutions containing up to 800 nM \(\text{Ca}^{2+}\). These concentrations have been previously shown to induce calcium uptake by mitochondria (91). No selective increase in cytoplasmic digitonin-insensitive fluorescence was observed (not shown), confirming that the amount of Fluo-4 trapped in cytoplasmic organelles under our conditions cannot explain the difference in fluorescence between the cytoplasm and the nucleoplasm.

Addition of sulfinpyrazone to the calibration solutions had no effect on the parameters measured, suggesting that the higher nucleoplasmic fluorescence is not a consequence of the indicator’s clearance by anion transporters present in the plasma membrane.

### 3.1.4. *In situ* calibration of ACaR fluorescence in adult CMs

To validate the results obtained with the non-ratiometric \(\text{Ca}^{2+}\) indicator Fluo-4, we also conducted *in situ* calibration experiments using the ratiometric \(\text{Ca}^{2+}\) indicator ACaR. Figure 3-4A illustrates original images of a ventricular myocyte obtained before (diastole) and during the calibration procedure at various \([\text{Ca}^{2+}]\). Fluorescence emission at >650 nm (\(F_1\), left) and at 475-525 nm (\(F_2\), middle) is shown, as well as the ratio \(F_1/F_2\) (right). With increasing \([\text{Ca}^{2+}]\), \(F_1\) increased, whereas \(F_2\) decreased, as expected from the emission spectra of ACaR. Consequently, the ratio \(F_1/F_2\) also increased with increasing \([\text{Ca}^{2+}]\). Similar to Fluo-4, ACaR exhibited higher fluorescence in the nucleoplasm than in the cytoplasm. The ratio \(F_1/F_2\) was plotted as a function of \([\text{Ca}^{2+}]\) (Fig. 3-4B) and the Hill equation was used for fitting and obtaining the \(K_{d,\text{app}}\) for \(\text{Ca}^{2+}\) binding. In a total of 15 ventricular myocytes, \(K_{d,\text{app}}\) amounted to 2183±55 nM in the nucleoplasm and to 1336±38 nM in the cytoplasm (Fig. 3-4C). Thus, similar to
Fluo-4, ACaR displayed different $K_{d,\text{app}}$ for $Ca^{2+}$ binding in the nucleoplasm vs. cytoplasm. Having obtained the *in situ* $K_{d,\text{app}}$ for $Ca^{2+}$ binding, we used the Grynkiewicz formula for ratiometric dyes for calculating $[Ca^{2+}]_{\text{cyto}}$ and $[Ca^{2+}]_{\text{nuc}}$:

$$[Ca^{2+}] = K_d \beta \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}$$

where $R = F_1/F_2$, $R_{\text{min}} = F_1/F_2$ in the absence of $Ca^{2+}$, $R_{\text{max}} = F_1/F_2$ in the presence of saturating $[Ca^{2+}]$, $\beta$ = proportionality factor determined by the ratio of the $F_2$ intensities of free and $Ca^{2+}$-bound dye, respectively.

![Figure 3-4: In situ calibration of the ratiometric Ca²⁺ indicator ACaR in the nucleus vs. cytoplasm of rat ventricular myocytes. (A) Original 2D images of ACaR fluorescence at >650 nm ($F_1$, left) and at 475-525 nm ($F_2$, middle), as well as the ratio $F_1/F_2$ (right), of a rat ventricular myocyte at various $[Ca^{2+}]$ during the calibration protocol. (B) Concentration response curves of the ratio $F_1/F_2$ as a function of $[Ca^{2+}]$ in the nucleus (blue) vs. the cytoplasm (black) of rat ventricular myocytes. (C) $K_{d(\text{app})}$ for $Ca^{2+}$ binding in the nucleus (blue) vs. the cytoplasm (black). Data from 15 rat ventricular myocytes. Asterisk indicates P<0.05 vs. cytoplasm.]
3.2. Quantification and characterisation of subcellular Ca\textsuperscript{2+} handling in resting and electrically stimulated CMs

3.2.1. Quantification of cytoplasmic and nucleoplasmic CaTs in electrically stimulated CMs

The established \textit{in situ} calibration curves were used to transform the raw fluorescence signal during an electrically stimulated CaT into \([\text{Ca}^{2+}]_{\text{nuc}}\) and \([\text{Ca}^{2+}]_{\text{cyto}}\).

Figure 3-5A shows original traces from nucleus and cytoplasm of three mouse cardiac myocytes. Traces on the left show a typical example of CaTs as observed in most of the cells studied (85%): diastolic \([\text{Ca}^{2+}]_{\text{nuc}}\) was higher (in all cells studied), whereas systolic \([\text{Ca}^{2+}]_{\text{nuc}}\) was lower than in the cytoplasm. Interestingly, however, some of the cells (15%) showed higher systolic \([\text{Ca}^{2+}]\) in the nucleus as compared to the cytoplasm (\textit{traces in the middle}). On average, there was a significant difference in diastolic and systolic \([\text{Ca}^{2+}]\) between cytoplasmic and nucleoplasmic compartments in all three types of cardiac myocytes: diastolic \([\text{Ca}^{2+}]_{\text{nuc}}\) was higher, whereas systolic \([\text{Ca}^{2+}]_{\text{nuc}}\) was lower in the nucleus (Fig. 3-5B). The kinetics of the CaTs were significantly slower in the nucleoplasm than in the cytoplasm, and this was true for all myocytes studied (n=70, Fig. 3-5C).

To validate the results obtained with the non-ratiometric Ca\textsuperscript{2+} indicator Fluo-4, we also recorded and quantified electrically stimulated CaTs and resting values from cytoplasmic and nucleoplasmic compartments using the ratiometric Ca\textsuperscript{2+} indicator ACaR.

Figure 3-6A represents an original 2D image of a rat ventricular myocyte during rest with overlaid ACaR fluorescence from two channels (\(F_1\) at >650 nm and \(F_2\) at 475-525 nm). The red arrow was placed through the nucleus and the surrounding cytoplasm. The corresponding fluorescence traces below show the differences of the indicator’s properties in the two compartments. The fluorescence signal from channel 1 (\(F_1\)) was higher in the nucleus, while the
3. Results

opposite was true for the signal from channel 2 ($F_2$). During electrical stimulation $F_1$ increased whereas $F_2$ decreased with increasing $[\text{Ca}^{2+}]$ (Fig. 3-6B).

**Figure 3-5: Quantification and characterization of cytoplasmic and nucleoplasmic CaTs in electrically stimulated cardiac myocytes. (A)** Original recordings of electrically stimulated CaTs in the nucleus (red, blue) vs. cytoplasm (black) of three mouse ventricular myocytes. (B) Diastolic and systolic $[\text{Ca}^{2+}]$ as well as (C) kinetic parameters (time to peak (left) and $DT_{50}$ (right)) of the CaTs. Data in (B) and (C) from a total of 15 mouse ventricular, 8 rat atrial, and 15 rat ventricular myocytes for Fluo-4, and 15 rat ventricular myocytes for ACaR. Asterisks indicate $P<0.05$ vs. cytoplasm.
3. Results

Measurements with the ratiometric indicator ACaR (n=15) yielded almost identical results for diastolic and systolic calibrated \([\text{Ca}^{2+}]\) as compared to non-ratiometric indicator Fluo-4: diastolic \([\text{Ca}^{2+}]\) was higher, whereas systolic \([\text{Ca}^{2+}]\) was lower in the nucleus (Fig. 3-5A,B,C, right). Two out of fifteen cells (13%) showed higher systolic \([\text{Ca}^{2+}]\) in the nucleoplasm. This is in perfect agreement with \([\text{Ca}^{2+}]\) measurements using Fluo-4, where 15% of the cells exhibited higher systolic \([\text{Ca}^{2+}]\) in the nucleoplasm.

Figure 3-6: Recording of nucleoplasmic vs. cytoplasmic \([\text{Ca}^{2+}]\) using the ratiometric \(\text{Ca}^{2+}\) indicator ACaR. (A) Original 2D image of a rat ventricular myocyte during rest with overlaid fluorescence from two channels, i.e. \(F_i (>650 \text{ nm})\) and \(F_2 (475-525 \text{ nm})\). The red arrow is positioned through the nucleus and the surrounding cytoplasm. The traces below show the corresponding fluorescence profiles at \(F_i\) and \(F_2\). (B) Original recording of two electrically stimulated \(\text{CaTs}\) in the cytoplasm (left) and nucleus (right) obtained at \(F_i\) (red) and \(F_2\) (green). (C) \(\text{CaTs}\) in nucleus (blue) and cytoplasm (black) calculated as fluorescence ratio \((F_i/F_2; \text{left})\) and calibrated \([\text{Ca}^{2+}]\) (right).

3.2.2. Correlation between nucleoplasmic and cytoplasmic \([\text{Ca}^{2+}]\)

Figure 3-7 summarizes nucleoplasmic and cytoplasmic \(\text{CaT}\) data obtained from 70 CMs loaded with Fluo-4. A comparison between single cells revealed that the ratio of diastolic \([\text{Ca}^{2+}]\) in the nucleus to diastolic \([\text{Ca}^{2+}]\) in the cytoplasm, as well as the systolic peak of the nucleoplasmic \(\text{CaT}\) relative to the systolic peak of the cytoplasmic \(\text{CaT}\) was quite variable (Fig. 3-7A). Diastolic \([\text{Ca}^{2+}]\) in the nucleus was consistently higher than cytoplasmic \([\text{Ca}^{2+}]\) in all cells studied, while the systolic ratio of nucleoplasmic to cytoplasmic \([\text{Ca}^{2+}]\) was <1 in most CMs (85%).
3. Results

Figure 3-7B presents nucleoplasmic diastolic \([\text{Ca}^{2+}]\) as a function of cytoplasmic diastolic \([\text{Ca}^{2+}]\) (n=70). The cytoplasmic compartment showed a fairly narrow range of diastolic \([\text{Ca}^{2+}]\), while the values in the nuclei were much more variable, with about 15% of cells showing values higher than 300 nM. There was a weak but significant correlation between nucleoplasmic and cytoplasmic diastolic \([\text{Ca}^{2+}]\) (Figure 3-7B), and a much higher correlation between nucleoplasmic and cytoplasmic systolic \([\text{Ca}^{2+}]\) (Figure 3-7C). The amplitude (systolic minus diastolic) ratio of nucleoplasmic to cytoplasmic \([\text{Ca}^{2+}]\) transients was rather constant (not shown), meaning that an increase in cytoplasmic peak \([\text{Ca}^{2+}]\) during electrically stimulated \([\text{Ca}^{2+}]\) transients was followed by a proportional increase in nucleoplasmic peak \([\text{Ca}^{2+}]\). The major difference between single cells arose from the prominently higher diastolic values in the nuclei of some cells (15%), followed by an increase in systolic nucleoplasmic \([\text{Ca}^{2+}]\) above the cytoplasmic values.

Figure 3-7: Relationship between nucleoplasmic and cytoplasmic \([\text{Ca}^{2+}]\) in diastole and systole. (A) Histogram showing the distribution of the ratio of nucleoplasmic to cytoplasmic \([\text{Ca}^{2+}]\) for diastole (black) and systole (grey). Nucleoplasmic to cytoplasmic \([\text{Ca}^{2+}]\) ratio was always >1 in diastole, but usually <1 in systole. Note, however, that in 15% of the cells the systolic ratio was >1. (B) Diastolic nucleoplasmic \([\text{Ca}^{2+}]\) as a function of diastolic cytoplasmic \([\text{Ca}^{2+}]\) and (C) systolic nucleoplasmic \([\text{Ca}^{2+}]\) as a function of systolic cytoplasmic \([\text{Ca}^{2+}]\). In both cases, there was a positive correlation (with the correlation coefficient \(r\) as given in the figure), suggesting that the nucleoplasmic \([\text{Ca}^{2+}]\) was dependent, in part, on the cytoplasmic \([\text{Ca}^{2+}]\). Data in (A) to (C) from a total of 70 cardiac myocytes.

Taken together, these data imply the following: [1] diastolic and systolic \([\text{Ca}^{2+}]\) in the nucleus depend to some extent on \([\text{Ca}^{2+}]\) in the cytoplasm; [2] diastolic \([\text{Ca}^{2+}]\) in the nucleus is always higher than diastolic \([\text{Ca}^{2+}]\) in the cytoplasm suggesting
active release of Ca$^{2+}$ into and/or slowed removal of Ca$^{2+}$ from the nucleus; and [3] there may be both a passive and an active component of Ca$^{2+}$ increase during the spread of the cytoplasmic CaT into the nucleus.

3. Results

3.2.3. Resting [Ca$^{2+}$]$_{cyto}$ and [Ca$^{2+}$]$_{nuc}$ before and following depletion of intracellular Ca$^{2+}$ stores

To elucidate the reason for the higher diastolic [Ca$^{2+}$] in the nucleus, we investigated in more detail the resting [Ca$^{2+}$] values in the two compartments. Figure 3-8A shows that even in resting myocytes the gradient between nucleus and cytoplasm persisted, i.e. [Ca$^{2+}$]$_{nuc}$ was still significantly higher than [Ca$^{2+}$]$_{cyto}$ in all cell types studied, irrespective of the Ca$^{2+}$ indicator used, Fluo-4 or ACaR.

Figure 3-8: Resting cytoplasmic and nucleoplasmic [Ca$^{2+}$] before and following depletion of intracellular Ca$^{2+}$ stores. (A) Calibrated resting [Ca$^{2+}$] in mouse ventricular (n=15), rat atrial (n=8), and rat ventricular (n=15 for both Fluo-4 and ACaR) myocytes show significantly higher values in the nucleus than in the cytoplasm. Asterisks indicate P<0.05 vs. cytoplasm. (B) Original recording of nucleoplasmic (red) and cytoplasmic (black) [Ca$^{2+}$] of a mouse ventricular myocyte before (NT) and during the depletion of intracellular Ca$^{2+}$ stores by means of CPA and zero Ca$^{2+}$ solution. Note that the difference between nucleoplasmic and cytoplasmic [Ca$^{2+}$] evident in NT vanishes during depletion of the Ca$^{2+}$ stores. (C) Resting [Ca$^{2+}$] in a total of 12 mouse ventricular myocytes following depletion of intracellular Ca$^{2+}$ stores. Resting [Ca$^{2+}$]$_{nuc}$ and [Ca$^{2+}$]$_{cyto}$ were essentially identical under these conditions, indicating that the nucleo-cytoplasmic [Ca$^{2+}$] gradient observed in NT depended on intracellular Ca$^{2+}$ store content.
This indicates that the difference in diastolic [Ca$^{2+}$] between the two compartments during electrical stimulation (Fig. 3-5) cannot be explained entirely by differences in the kinetics of the CaTs (see also below). We hypothesized that the difference in resting [Ca$^{2+}$] was caused by Ca$^{2+}$ release from the perinuclear calcium stores into the nucleoplasm. If such a nucleoplasmic Ca$^{2+}$ leak was higher than the cytoplasmic Ca$^{2+}$ leak, i.e. Ca$^{2+}$ released from the SR into the cytoplasm, then this could explain the diastolic [Ca$^{2+}$] gradient between the nucleus and the cytoplasm. In order to test this hypothesis, we depleted the intracellular Ca$^{2+}$ stores using CPA and Ca$^{2+}$-free solution. Figure 3-8B illustrates an original recording from a mouse ventricular myocyte. In the resting cell in the presence of NT, [Ca$^{2+}$]$_{nuc}$ was higher than [Ca$^{2+}$]$_{cyto}$. Shortly after the addition of CPA in Ca$^{2+}$-free solution, [Ca$^{2+}$] rose both in the nucleus and the cytoplasm, presumably because of an imbalance between uptake and release from the internal stores with uptake being blocked by CPA. [Ca$^{2+}$]$_{nuc}$ still exceeded [Ca$^{2+}$]$_{cyto}$ during this phase. The Ca$^{2+}$ elevation was transient though, because plasma membrane Ca$^{2+}$ transport systems were still functioning and capable of extruding the ions out of the cell.

Finally, [Ca$^{2+}$]$_{nuc}$ and [Ca$^{2+}$]$_{cyto}$ returned close to the resting values. Importantly, however, the gradient between nucleus and cytoplasm had vanished. Figure 3-8C shows that – following Ca$^{2+}$ store depletion – [Ca$^{2+}$]$_{nuc}$ and [Ca$^{2+}$]$_{cyto}$ were essentially identical (n=12). This indicates that the nucleoplasmic to cytoplasmic [Ca$^{2+}$] gradient in resting myocytes depends on intact intracellular Ca$^{2+}$ stores.

3.2.4. Effects of 2-APB and tetracaine on the nucleo-to-cytoplasmic [Ca$^{2+}$] gradient

We hypothesized that tonic Ca$^{2+}$ leak from perinuclear Ca$^{2+}$ stores caused the nucleo-to-cytoplasmic [Ca$^{2+}$] gradient observed in resting myocytes. To identify the Ca$^{2+}$ release channel(s) involved we conducted experiments with 2-APB (3 μM), a blocker of IP$_3$Rs, and tetracaine (1 mM), a blocker of ryanodine receptors (RyRs). Figure 3-9A shows an original recording from a ventricular myocyte.
3. Results

Average results are presented in Figure 3-9C. In NT, $[\text{Ca}^{2+}]_{\text{nuc}}$ was higher than $[\text{Ca}^{2+}]_{\text{cyto}}$. Exposure of the cell to 2-APB decreased $[\text{Ca}^{2+}]_{\text{nuc}}$, whereas $[\text{Ca}^{2+}]_{\text{cyto}}$ remained almost unchanged. Thus, the nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient was completely reversed. Additional application of tetracaine reduced predominantly $[\text{Ca}^{2+}]_{\text{cyto}}$. In the presence of both 2-APB and tetracaine, there was no nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient.

Figure 3-9: Effects of 2-APB and tetracaine on the nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient. (A) and (B) Original recordings and of resting $[\text{Ca}^{2+}]_{\text{nuc}}$ (red) vs. $[\text{Ca}^{2+}]_{\text{cyto}}$ (black) of two rat ventricular myocytes before (NT) and following application of 2-APB and tetracaine. (C) and (D) Corresponding average values. Data from a total of 10 rat ventricular myocytes for each series. Asterisks indicate P<0.05 vs. cytoplasm. (E) Original recordings of $[\text{Ca}^{2+}]_{\text{nuc}}$ (red) vs. $[\text{Ca}^{2+}]_{\text{cyto}}$ (black) transients in an electrically stimulated rat ventricular myocyte before and following application of 2-APB. (F) Average values of diastolic and systolic $[\text{Ca}^{2+}]_{\text{nuc}}$ (red) vs. $[\text{Ca}^{2+}]_{\text{cyto}}$ (black) from a total of 15 electrically stimulated rat ventricular myocytes before and following application of 2-APB. Asterisk indicates P<0.05 versus $[\text{Ca}^{2+}]_{\text{nuc}}$ under control conditions.
When tetracaine was applied first (Fig. 3-9B and D), there was a decrease in both nucleo- and cytoplasmic [Ca\(^{2+}\)]. Importantly, however, [Ca\(^{2+}\)]\(_{\text{nuc}}\) remained higher than [Ca\(^{2+}\)]\(_{\text{cyto}}\). Additional application of 2-APB caused a selective decrease in [Ca\(^{2+}\)]\(_{\text{nuc}}\). Again, in the presence of both inhibitors, there was no nucleo-to-cytoplasmic [Ca\(^{2+}\)] gradient. These results show that the nucleo-to-cytoplasmic [Ca\(^{2+}\)] gradient depends on Ca\(^{2+}\) leak from intracellular Ca\(^{2+}\) stores. Since 2-APB caused selective decreases of [Ca\(^{2+}\)]\(_{\text{nuc}}\) and could even reverse the gradient, this suggests that there is a tonic IP\(_3\)R-mediated Ca\(^{2+}\) leak from perinuclear Ca\(^{2+}\) stores which is responsible for the higher [Ca\(^{2+}\)]\(_{\text{nuc}}\) in resting myocytes. When electrically stimulated ventricular myocytes were exposed to 2-APB (3 μM), there was a selective decrease of diastolic nucleoplasmic [Ca\(^{2+}\)] (Fig. 3-9E and F). Systolic [Ca\(^{2+}\)]\(_{\text{nuc}}\) as well as [Ca\(^{2+}\)]\(_{\text{cyto}}\) both in diastole and systole remained unaffected. Thus, consistent with the results in resting myocytes, an IP\(_3\)R-mediated Ca\(^{2+}\) leak/release from perinuclear Ca\(^{2+}\) stores appeared to contribute to nucleoplasmic Ca\(^{2+}\) regulation particularly in diastole, while systolic [Ca\(^{2+}\)] in the nucleoplasm seemed to be governed predominantly by the cytoplasmic [Ca\(^{2+}\)] increase and diffusion of Ca\(^{2+}\) through nuclear pores into the nucleoplasm (see also Figure 3-7).

### 3.2.5. Excitation and emission spectra of Fluo-4 in the absence and presence of drugs

In order to exclude the possibility that the drugs used for the experiments had altered the fluorescent properties of Fluo-4, we recorded the excitation and emission spectra of Fluo-4 in the absence (Control) and presence, respectively, of 2-APB (3 μM), CPA (5 μM), and tetracaine (1 mM). Figure 3-10A shows the excitation spectra, Figure 3-10B the emission spectra at either 0 or 2 mM [Ca\(^{2+}\)]. In the absence of Ca\(^{2+}\), excitation and emission intensities were negligible. At 2 mM Ca\(^{2+}\), excitation and emission spectra could be recorded closely resembling the spectra published by the manufacturer (92). Importantly, when compared to
control conditions, neither of the substances tested significantly altered the excitation or emission spectra of Fluo-4.

Figure 3-10: Lack of effect of 2-APB, CPA, and tetracaine on the excitation and emission spectra of Fluo-4. (A) Excitation spectra of Fluo-4 (emission at 520 nm) at 0 or 2 mM Ca^{2+} under control conditions or in the presence of 3 μM 2-APB, 5 μM CPA, and 1 mM tetracaine. (B) Emission spectra of Fluo-4 (excitation at 488 nm) at 0 or 2 mM Ca^{2+} under control conditions or in the presence of 3 μM 2-APB, 5 μM CPA, and 1 mM tetracaine.

3.2.6. Stimulation frequency-dependent changes in \([\text{Ca}^{2+}]_{\text{nuc}}\) vs. \([\text{Ca}^{2+}]_{\text{cyto}}\)

Heart rate is an important determinant of cardiac function. In cardiac myocytes, increases in stimulation frequency cause increases in diastolic and systolic \([\text{Ca}^{2+}]\). Because of the markedly slower kinetics of nucleoplasmic CaTs as compared to cytoplasmic CaTs (Fig. 3-5), we hypothesized that an increase in stimulation frequency would affect \([\text{Ca}^{2+}]_{\text{nuc}}\) and \([\text{Ca}^{2+}]_{\text{cyto}}\) differentially, in particular the diastolic \([\text{Ca}^{2+}]\). Figure 3-11A shows an original recording from a mouse ventricular myocyte. Stimulation frequency was gradually increased from
0.5 Hz to 4 Hz. Both, diastolic and systolic [Ca$^{2+}$] in the nucleus and cytoplasm rose with the increase in frequency. However, the increase was most pronounced for diastolic [Ca$^{2+}$] in the nucleus. Fig. 3-11B shows average data from 20 myocytes confirming this observation. Systolic [Ca$^{2+}$] in the cytoplasm and nucleoplasm increased in parallel, whereas the diastolic [Ca$^{2+}$] increase in the nucleus by far exceeded that in the cytoplasm. These results show that, because of the slower CaT kinetics in the nucleus, an increase in stimulation frequency is sufficient for differential regulation of diastolic [Ca$^{2+}$] in the nucleus vs. cytoplasm.

![Graph A](image1.png)  
![Graph B](image2.png)  
![Graph C](image3.png)

Figure 3-11: Frequency-dependent increases in diastolic and systolic [Ca$^{2+}$] in the nucleus vs. cytoplasm. (A) Original recording of electrically stimulated CaTs in the nucleus (red) vs. cytoplasm (black) of a mouse ventricular myocyte during gradual increases of stimulation frequency from 0.5 Hz to 4 Hz. Average values of diastolic and systolic [Ca$^{2+}$] from a total of 20 mouse ventricular myocytes are shown in (B) and (C), respectively. The frequency-dependent increase in diastolic [Ca$^{2+}$] in the nucleus was significantly larger (P<0.05) than in the cytoplasm.
3. Results

3.3. Alterations in cytoplasmic vs. nucleoplasmic Ca\(^{2+}\) handling in hypertrophy and failure in an animal model of pressure overload and in failing human hearts

3.3.1. Characterisation of pressure overload-induced hypertrophy in mice

After 1 and 6 weeks, TAC-operated mice were subjected to transthoracic echocardiography to determine cardiac function and structure. Sham-operated hearts served as controls and showed virtually identical left ventricular chamber dimensions and systolic ventricular function at any time point after the surgery (not shown). Comparable heart rates were observed in control and either group of TAC-operated mice (Sham: 514±22 bpm; TAC 1w: 483±21 bpm and TAC 6w: 491±2 bpm). Assessment of LVEDD and LVESD revealed that 1 week after TAC mice effectively compensated the pressure overload (n=13), while deterioration of the left ventricle chamber diameters was observed 6 weeks after TAC (n=2; Table 2). Ventricular systolic function progressively declined, as indicated by reduced relative ejection fraction by ~8% one week and ~60% six weeks after TAC (Table 2).

<table>
<thead>
<tr>
<th>Table 3-1: Left ventricular echocardiographic parameters in control and 1 and 6 weeks after TAC intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameter</strong></td>
</tr>
<tr>
<td>LVEDD (mm)</td>
</tr>
<tr>
<td>LVESD (mm)</td>
</tr>
<tr>
<td>IVS (mm)</td>
</tr>
<tr>
<td>PW (mm)</td>
</tr>
<tr>
<td>FS (%)</td>
</tr>
<tr>
<td>EF (%)</td>
</tr>
<tr>
<td>Rel. WT</td>
</tr>
<tr>
<td>LV mass (mg)</td>
</tr>
</tbody>
</table>

Data are mean±S.E.M., *P<0.05 vs. Sham 1 week.
Increased dilation in conjunction with increased LV thickness, measured as the sum of intraventricular septum thickness and posterior wall thickness, also suggested that TAC hearts underwent transition from stress-induced hypertrophy to overt heart failure (Fig. 3-12A).

Pressure overload-induced hypertrophy was confirmed by an increased heart weight normalized to left tibia length (n=5-6; Fig. 3-12B) or body weight (n=6-10; Fig. 3-12C). Six weeks after the intervention, TAC mice developed pulmonary edema, as manifested by an elevated lung weight/left tibia length ratio (not shown).

Collectively, these data indicate that pressure overload-induced myocardial remodelling is associated with an early onset of hypertrophy and systolic dysfunction, ultimately resulting in overt heart failure.

Figure 3-12: Characterisation of pressure overload-induced hypertrophy in adult WT mice. (A) LVEDD (dilation) in conjunction with increased LV thickness (hypertrophy), measured as the sum of intraventricular septum thickness and posterior wall thickness for sham (n=16) and TAC-operated mice (after 1 week n=14 and after 6 weeks n=2). Heart weight normalized to left tibia length (n=5-6 per group) or body weight (n=6-10 per group) are shown in (B) and (C), respectively. Asterisks indicate P<0.05 versus sham 1 week control group.
3.3.2. Quantification of electrically stimulated CaTs in the nucleus vs. cytoplasm after pressure overload-induced hypertrophy in mouse CMs

Using the previously described method, raw fluorescence signals recorded during an electrically stimulated CaT were transformed into \([\text{Ca}^{2+}]_{\text{nuc}}\) and \([\text{Ca}^{2+}]_{\text{cyto}}\). Figure 3-13A shows original traces averaged from nucleus and cytoplasm of ventricular myocytes from sham-operated animals (n=15, left), isolated 1 (top) and 7 (bottom) weeks after the intervention and of ventricular myocytes from post-intervention time matched TAC-operated mice (n=15, right).

Figure 3-13: Quantification and characterisation of cytoplasmic and nucleoplasmic CaTs after pressure overload-induced hypertrophy in electrically stimulated adult mouse CMs. (A) Averaged original recordings of electrically stimulated \([\text{Ca}^{2+}]\) transients in the nucleus (red) versus cytoplasm (black) of mouse ventricular CMs from sham-operated animals (left), isolated 1 (up) and 7 (down) weeks after the intervention and CMs from post-intervention time matched TAC-operated mice (right). (B) Diastolic and systolic \([\text{Ca}^{2+}]\), (C) amplitude and nucleoplasmic to cytoplasmic amplitude ratio and (D) kinetic parameters (time to peak (left) and DT\(_{50}\) (right)) of the \([\text{Ca}^{2+}]\) transients. Data in (A) to (D) from a total of 15 mouse ventricular myocytes. Asterisks indicate P<0.05 versus sham 1 week control group.
3. Results

No differences were observed between the two groups of sham-operated mice (Fig. 3-13). On the other hand, one week after TAC, diastolic $[\text{Ca}^{2+}]_{\text{nuc}}$ and $[\text{Ca}^{2+}]_{\text{cyto}}$ were significantly increased and increased further 7 weeks after TAC (Fig. 3-13B, left). Systolic $[\text{Ca}^{2+}]$ remained unchanged in both compartments (Fig. 3-13B, right). Interestingly, kinetic parameters and CaTs amplitude were changed only in the nucleoplasm at the early stage of hypertrophy. Seven weeks after TAC intervention similar changes of CaTs also occurred in the cytoplasm (Fig. 3-13C and D).

To test the possibility that the slower rise of the CaTs that occurred in the nucleoplasm 1 week post-TAC was due to the slower spread of the CaT through the cytoplasm (i.e. CaTs from the cytoplasm reach the nuclear envelope with a delay), further sub-cytoplasmic and sub-nucleoplasmic analyses were performed. Figure 3-14 shows that time to peak and DT$_{50}$ of the CaT through the cytoplasm remained essentially unchanged in the 1 week post-TAC group (n=10). This suggested that the origin of the alterations in the nucleoplasmic CaTs lays in a slower spread of $\text{Ca}^{2+}$ through the nucleoplasm. In agreement with this hypothesis, there was a selective slowing of the kinetics of the central nucleoplasmic CaT 1 week after TAC (Fig. 3-15). The velocity of the spread of the CaT in the nucleoplasm (calculated as the difference of the time to peak between the sub-nucleolemmal and central nucleoplasmic region divided by their distance) was significantly reduced 1 week post-TAC (n=10; Fig. 3-15). Seven weeks after TAC intervention the velocity was further reduced. At this time, a similar slowing of the central CaT was also observed in the cytoplasm (Fig. 3-14) presumably caused by a reduction in T-tubular density that was shown to occur in heart failure (93).

Interestingly, the TAC-induced increase in diastolic $[\text{Ca}^{2+}]_{\text{nuc}}$ also differed between the sub-nuclear regions (Fig. 3-15B). It was more pronounced in the central area starting already 1 week after TAC with a further increase observed 7 weeks after TAC.
Figure 3-14: Sub-cytoplasmic analyses of electrically stimulated CaTs after pressure overload-induced hypertrophy in mouse CMs. (A) Line scan confocal imaging of subcytoplasmic CaTs in an electrically stimulated ventricular CM. (B) Averaged CaTs of distinct subcellular regions, as indicated in the schematic representation of the cell in (A): central (blue) versus subsarcolemmal region (black) from sham-operated animals (left) and TAC-operated animals isolated 1 (middle) and 7 (right) weeks after the intervention. (C) Diastolic and systolic $[\text{Ca}^{2+}]$, (D) central to subsarcolemmal region amplitude ratio and (E) kinetic parameters (time to peak (left) and DT_{50} (right)) of the $[\text{Ca}^{2+}]$ transients. Data in (B) to (E) from a total of 10 mouse ventricular myocytes. Asterisks indicate P<0.05 versus sham 1 week control group and pound signs indicate P<0.05 versus cytoplasm.
Figure 3-15: Sub-nucleoplasmic analyses of electrically stimulated CaTs after pressure overload-induced hypertrophy in mouse CMs. (A) Averaged original recordings of electrically stimulated [Ca$^{2+}$] transients in the central (green) versus subnucleolemmal (black) region of mouse ventricular nuclei from sham-operated animals (left) and TAC-operated animals isolated 1 (middle) and 7 (right) weeks after the intervention. (B) Diastolic and systolic [Ca$^{2+}$], as well as central to subnucleolemmal region amplitude ratio and (C) kinetic parameters (time to peak (left), DT$_{50}$ (middle) and velocity of the spread (right)) of the [Ca$^{2+}$] transients. Data in (A) to (C) from a total of 10 mouse ventricular myocytes. Asterisks indicate P<0.05 versus sham 1 week control group and pound signs indicate P<0.05 versus cytoplasm.

Taken together, these results indicate that the changes in nucleoplasmic [Ca$^{2+}$] occurring during hypertrophy precede the ones in the cytoplasm, suggesting that they may contribute to the development and/or progression of hypertrophy.
3. Results

3.3.3. Quantification of electrically stimulated CaTs in the nucleus vs. cytoplasm in CMs isolated from non-failing, moderately failing and severely failing human hearts

Figure 3-16: Quantification and characterisation of cytoplasmic and nucleoplasmic CaTs of CMs isolated from non-failing, moderately failing and severely failing human hearts. (A) Averaged original recordings of electrically stimulated [Ca^{2+}] transients in the nucleus (red) versus cytoplasm (black) of ventricular CMs from healthy controls (n=6, left), moderately failing (n=2, middle) and severe failing human hearts (n=4, right). (B) Diastolic and systolic [Ca^{2+}]. (C) amplitude and nucleoplasmic to cytoplasmic amplitude ratio and (D) kinetic parameters (time to peak (left) and RT_{50} (right)) of the [Ca^{2+}] transients. Data in (A) to (D) from a total of 6-10 ventricular myocytes. Asterisks indicate P<0.05 versus non-failing control group.
Figure 3-16 shows the averaged original CaT traces from 6-8 CMs isolated from non-failing (n=6; left), moderately failing (n=2; middle) and severely failing (n=4; right) human hearts. Raw fluorescence was transformed into \([\text{Ca}^{2+}]\) using the method described before. Diastolic \([\text{Ca}^{2+}]\) was found to be significantly increased in both compartments of CMs from patients with moderately failing hearts, and it further increased in CMs from group of patients with severely failing hearts (Fig. 3-16B, left). Systolic \([\text{Ca}^{2+}]\) was unaltered (Fig. 3-16B, right). CaT amplitude and TTP were selectively affected in the nucleus of CMs from the intermediate group, while the CaT decay was altered in both compartments (Fig. 3-16C and D). In line with observations from mouse CMs, amplitude and kinetic parameters of both, cytoplasmic and nucleoplasmic CaTs, were strongly altered in CMs from the end-stage failing group.

Alterations of diastolic \([\text{Ca}^{2+}]_{\text{nuc}}\) (Fig. 3-17) and kinetic parameters (not shown) in both models we studied were more pronounced in the nucleoplasm as compared to the cytoplasm. This observation might be of a particular relevance at higher stimulation frequencies, when nucleoplasmic \([\text{Ca}^{2+}]\) is more affected due to its slower kinetics (55).

Figure 3-17: Hypertrophy-induced increase in diastolic \([\text{Ca}^{2+}]\) in the nucleus versus cytoplasm of mouse and human ventricular myocytes. Increase in diastolic \([\text{Ca}^{2+}]\) calculated as the difference to sham-operated mice or non-failing human hearts. Data from a total of 15 mouse and 6-10 human ventricular myocytes. Asterisks indicate \(P<0.05\) versus 1 week post-TAC or moderately failing group and pound signs indicate \(P<0.05\) versus cytoplasm.
3.3.4. **Stimulation frequency-dependent changes of nucleoplasmic vs. cytoplasmic CaTs after pressure overload-induced hypertrophy in mouse CMs**

The original recordings of a typical experiment are illustrated in Figure 3-18A. The stimulation frequency was gradually increased from 0.5 Hz to 5 Hz. In CMs from sham-operated mice, both diastolic and systolic [Ca$^{2+}$] in the nucleus and cytoplasm rose with the increase in frequency (Fig. 3-18B, left and middle). The increase was most pronounced, however, for diastolic [Ca$^{2+}$] in the nucleus. The amplitude of the CaT in both compartments was greatest at 2 Hz and was unchanged with further increase to 5 Hz (Fig. 3-18B, right). In CMs from 1 week TAC group diastolic [Ca$^{2+}$] in the nucleus and cytoplasm also increased with the stimulation frequency (Fig. 3-18B, left). At low frequency of 0.5 Hz, diastolic [Ca$^{2+}$] in the cytoplasm was the same as in the control group, but it was significantly higher than the control between 1 and 5 Hz. Diastolic [Ca$^{2+}$] in the nucleus was higher as compared to the control at any frequency measured. Systolic [Ca$^{2+}$] in both cellular compartments increased between 0.5 and 2 Hz, but no further increase occurred at 5 Hz (Fig. 3-18B, middle). Systolic peak [Ca$^{2+}$] was comparable to the control group. The amplitude of the cytoplasmic CaTs was unchanged between 0.5 and 2 Hz, and significantly decreased at 5 Hz (Fig. 3-18B, right). Reduction of the nucleoplasmic amplitude occurred already at 2 Hz and was further decreased at 5 Hz. In the late-stage failing group, 7 weeks post-TAC, diastolic [Ca$^{2+}$] rose with increasing frequency and was significantly higher than in the control group in both cellular compartments, although the increase in the nucleus exceeded that in the cytoplasm (Fig. 3-18B, left). Changes of the systolic [Ca$^{2+}$] were comparable to those observed in the compensated hypertrophy group (Fig. 3-18B, middle). At low stimulation frequencies, 0.5 and 1 Hz, the amplitude of cytoplasmic and nucleoplasmic CaTs was unchanged, but significantly decreased at higher frequencies. However, the amplitude was lower then the control group at any frequency studied (Fig. 3-18B, right).
Table 3-2
Stimulation frequency-dependent changes of CaTs after pressure overload-induced hypertrophy in adult mouse CMs

<table>
<thead>
<tr>
<th></th>
<th>Normal Tyrode</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cytoplasm</td>
<td>nucleus</td>
<td>cytoplasm</td>
<td>nucleus</td>
</tr>
<tr>
<td>Diastolic [Ca(^{2+})] (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>88.29 ± 5.2</td>
<td>125.73 ± 3.8</td>
<td>112.31 ± 10.5</td>
<td>152.08 ± 10.0(\dagger)</td>
</tr>
<tr>
<td>1 Hz</td>
<td>95.95 ± 6.1*</td>
<td>151.12 ± 7.0*</td>
<td>134.06 ± 8.1*(\dagger)</td>
<td>200.56 ± 13.5*(\dagger)</td>
</tr>
<tr>
<td>2 Hz</td>
<td>118.48 ± 5.3*</td>
<td>207.23 ± 9.4*</td>
<td>175.17 ± 8.7*(\dagger)</td>
<td>276.74 ± 11.9*(\dagger)</td>
</tr>
<tr>
<td>5 Hz</td>
<td>199.08 ± 11.0*</td>
<td>378.59 ± 8.9*</td>
<td>309.13 ± 35.1*(\dagger)</td>
<td>507.56 ± 24.6*(\dagger)</td>
</tr>
<tr>
<td>Systolic [Ca(^{2+})] (nM)</td>
<td>687.47 ± 50.5</td>
<td>499.02 ± 45.3</td>
<td>711.09 ± 45.0</td>
<td>516.44 ± 35.4</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>723.75 ± 46.4*</td>
<td>539.28 ± 44.5*</td>
<td>730.55 ± 47.5*</td>
<td>553.83 ± 36.2*</td>
</tr>
<tr>
<td>1 Hz</td>
<td>795.28 ± 56.0*</td>
<td>639.29 ± 32.3*</td>
<td>779.26 ± 35.1*</td>
<td>616.57 ± 37.8*</td>
</tr>
<tr>
<td>2 Hz</td>
<td>867.86 ± 62.7*</td>
<td>733.63 ± 40.6*</td>
<td>797.12 ± 28.7</td>
<td>715.11 ± 36.7</td>
</tr>
<tr>
<td>5 Hz</td>
<td>911.14 ± 66.7*</td>
<td>801.99 ± 43.4*</td>
<td>928.95 ± 34.8</td>
<td>830.74 ± 40.3*</td>
</tr>
<tr>
<td>Amplitude (nM)</td>
<td>599.18 ± 45.8</td>
<td>373.29 ± 43.9</td>
<td>598.79 ± 48.7</td>
<td>364.36 ± 36.3</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>627.80 ± 40.6*</td>
<td>388.17 ± 44.2*</td>
<td>596.49 ± 51.9</td>
<td>353.26 ± 35.5</td>
</tr>
<tr>
<td>1 Hz</td>
<td>676.80 ± 53.6*</td>
<td>432.05 ± 35.4*</td>
<td>604.08 ± 39.4</td>
<td>329.82 ± 41.6*(\dagger)</td>
</tr>
<tr>
<td>2 Hz</td>
<td>668.78 ± 53.3</td>
<td>355.03 ± 41.9</td>
<td>487.99 ± 58.2*(\dagger)</td>
<td>207.55 ± 43.2*(\dagger)</td>
</tr>
<tr>
<td>Isoprenaline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic [Ca(^{2+})] (nM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>221.34 ± 10.3</td>
<td>231.60 ± 13.0</td>
<td>253.22 ± 19.6</td>
<td>275.27 ± 23.4</td>
</tr>
<tr>
<td>1 Hz</td>
<td>221.66 ± 10.0</td>
<td>250.97 ± 17.9</td>
<td>269.51 ± 20.5*</td>
<td>363.95 ± 24.0*(\dagger)</td>
</tr>
<tr>
<td>2 Hz</td>
<td>223.20 ± 10.5</td>
<td>291.91 ± 19.3*</td>
<td>299.67 ± 21.7*(\dagger)</td>
<td>508.01 ± 26.2*(\dagger)</td>
</tr>
<tr>
<td>5 Hz</td>
<td>225.17 ± 10.4</td>
<td>374.66 ± 24.1*</td>
<td>499.47 ± 19.3*</td>
<td>725.76 ± 28.5*</td>
</tr>
<tr>
<td>Systolic [Ca(^{2+})] (nM)</td>
<td>1742.49 ± 25.9</td>
<td>1556.30 ± 36.4</td>
<td>1526.51 ± 74.9*</td>
<td>1187.39 ± 90.9*</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>1743.29 ± 29.3</td>
<td>1551.46 ± 40.8</td>
<td>1532.85 ± 81.8*</td>
<td>1206.91 ± 98.3*</td>
</tr>
<tr>
<td>1 Hz</td>
<td>1750.33 ± 27.8</td>
<td>1568.43 ± 42.7</td>
<td>1541.47 ± 86.9*</td>
<td>1224.94 ± 112.0*</td>
</tr>
<tr>
<td>2 Hz</td>
<td>1754.32 ± 28.4</td>
<td>1576.98 ± 52.7</td>
<td>1517.83 ± 93.6*</td>
<td>1251.76 ± 124.9*</td>
</tr>
<tr>
<td>5 Hz</td>
<td>1759.15 ± 32.7</td>
<td>1202.31 ± 48.5*</td>
<td>1273.29 ± 67.5*</td>
<td>912.12 ± 81.8*</td>
</tr>
<tr>
<td>Amplitude (nM)</td>
<td>1521.14 ± 32.1</td>
<td>1324.69 ± 35.6</td>
<td>1273.29 ± 67.5*</td>
<td>912.12 ± 81.8*</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>1521.63 ± 35.4</td>
<td>1300.50 ± 43.2</td>
<td>1263.34 ± 71.5*</td>
<td>842.95 ± 82.7*</td>
</tr>
<tr>
<td>1 Hz</td>
<td>1527.12 ± 32.5</td>
<td>1276.54 ± 43.1*</td>
<td>1241.79 ± 75.4*</td>
<td>716.94 ± 111.0*(\dagger)</td>
</tr>
<tr>
<td>2 Hz</td>
<td>1529.15 ± 32.7</td>
<td>1202.31 ± 48.5*</td>
<td>1018.36 ± 80.8*(\dagger)</td>
<td>526.00 ± 127.4*(\dagger)</td>
</tr>
</tbody>
</table>

Data are mean±S.E.M., *P<0.05 vs. previous frequency, †P<0.05 vs. Sham 1 week.
3. Results

Figure 3-18: Stimulation frequency-dependent changes of nucleoplasmic and cytoplasmic CaTs after pressure overload-induced hypertrophy in adult mouse CMs. (A) Original recordings of electrically stimulated [Ca^{2+}] transients in the nucleus (red) versus cytoplasm (black) of ventricular CMs from sham (up) and TAC-operated mice (after 1 week-middle and after 7 weeks-down) during gradual increases of stimulation frequency from 0.5 Hz to 5 Hz. Average values of diastolic and systolic [Ca^{2+}] as well as CaTs amplitude from a total of 10-12 mouse ventricular myocytes without and with isoprenaline application are shown in (B) and (C), respectively. The frequency-dependent increase in diastolic [Ca^{2+}] in the nucleus was significantly larger (P<0.05) than in the cytoplasm.
In similar experiments we investigated if β-adrenergic stimulation can diminish or prevent the altered response of hypertrophied and failing CMs to increased stimulation frequencies (Fig. 3-18A, right). Application of isoprenaline resulted in a robust increase of CaTs in control CMs (Fig. 3-18A, right). In contrast, in failing myocytes, whilst there was a response to isoprenaline, it was significantly reduced. Similar to what was observed in the absence of isoprenaline, alterations of CaTs in response to increased stimulation frequency in the nucleoplasmic compartment preceded, and by far exceeded the ones in the cytoplasm, and they persisted at higher stimulation rates in both compartments. Average data for all three groups with and without isoprenaline and the corresponding statistical analyses are summarized in the Table 3.
3.4. Structural and biochemical alterations of nuclear envelope in hypertrophy and failure in mouse model of pressure overload and in failing human hearts

3.4.1. Perinuclear Ca stores and nuclear size in hypertrophy and failure in mouse model of pressure overload and in failing human hearts

As we observed that changes in nucleoplasmic CaTs preceded the cytoplasmic ones, we characterized nuclear envelope structure and function and nuclear dimensions during development and progression of hypertrophy. In CMs from Sham mice, staining of perinuclear Ca$^{2+}$ stores with the low affinity Ca$^{2+}$ dye Mag-Fluo-4 revealed a nuclear envelope and tubular structures traversing the nucleus (Fig. 3-19A, left). A significant increase in number of tubules per nucleus was observed during physiological growth (not shown). Nuclear dimensions remained unaltered (n=90; Fig. 3-19C). In TAC CMs, the number of tubules per nucleus progressively decreased with time after TAC, whereas length and width of nuclei increased (n=90; Fig. 3-19C). A strong positive correlation was found between the length of the nucleus and the number of tubules in CM isolated from Sham animals both 1 week and 7 weeks after the Sham surgery. A weaker, but significant positive correlation was found in CMs 1 week post-TAC, while weak negative correlation was observed in CMs 7 weeks post-TAC (Fig. 3-20).

Similar results were obtained in CMs from failing and non-failing human hearts and staining of the nuclear envelope with either an anti-nuclear pore complex (NPC) proteins antibody (n=20; Fig. 3-19B, top) or Mag-Fluo-4 (n=8; Fig. 3-19B, bottom). The length of the nuclei was significantly increased and the number of tubules was significantly decreased in the failing versus non-failing groups (Fig. 3-19C; not shown). Width of the nuclei was unchanged (Fig. 3-19C).

In summary, the data indicate that perinuclear Ca$^{2+}$ stores undergo significant structural changes during hypertrophy and heart failure. With the development of
hypertrophy and heart failure the size of the nuclei increases, whereas, at the same time, the number of tubules decreases.

Figure 3-19: Perinuclear Ca stores and nuclei size after pressure overload-induced hypertrophy in mouse CMs and in CMs from non-failing and failing human hearts. (A) Original 2D images of Mag-Fluo-4 fluorescence of nuclei from sham-operated mice (left), isolated 1 (up) and 7 (down) weeks after the intervention and nuclei from post-intervention time matched TAC-operated mice (right). (B) Original 2D images of nuclei from non-failing (left) and failing (right) human hearts stained with either nuclear pore complex (NPC) antibody (up) or Mag-Fluo-4 (down). (C) Dimensions of nuclei from mouse (left) and human (right) hearts. Data from a total of 90 mouse and 20 human ventricular myocytes. Asterisks indicate P<0.05 versus sham 1 week or non-failing control group.
3. Results

3.4.2. Subcellular localization and expression of Ca\(^{2+}\) regulating proteins in control and failing mouse and human CMs

The immunocytostaining of isolated CMs from control and failing mouse and human hearts showed the changes in the expression of Ca\(^{2+}\) release channels, RyR and IP\(_3\)R, as well as SERCA pump. In sham-operated mice as well in non-failing human CMs, IP\(_3\)R was found in the striated pattern throughout the cell, on the cell membrane and on the nuclear envelope. In TAC-operated mice and failing human CMs, accumulation of IP\(_3\)R in the perinuclear region was observed (n=15; Fig. 3-21A and B, up).
3. Results

Figure 3-21: Subcellular localization and expression of Ca^{2+} regulating proteins in control and failing mouse and human CMs. (A) Original 2D images of CMs isolated from sham and TAC-operated mice 7 weeks after the intervention and immunostaining with IP$_3$R (up), RyR (middle) and SERCA2a (down). (B) Original 2D images of CMs isolated from non-failing and failing human CMs and immunostaining with IP$_3$R (up), RyR (middle) and SERCA2a (down). (C) Original 2D electron microscopy images of nuclei from human left ventricular trabelulae (left), zoom on the NucE invaginations (middle and right).

A punctuate pattern with a striated organisation was observed when cells were stained with an antibody directed against RyR. Interestingly, a complete absence of RyR staining in perinuclear regions was observed in TAC-operated animals, while a decrease in the expression around the cell nucleus was noticed in CMs from failing human hearts (n= 15; Fig. 3-21A and B, middle).
3. Results

SERCA2 was present on the nuclear envelope and in a characteristic network-like pattern throughout the cell resembling the SR. A network of nuclear tubules network was clearly visible in CMs from sham-operated murine hearts and non-failing human hearts. Such a tubular network, however, was absent in TAC-operated mice and terminally failing human hearts (n= 15; Fig. 3-21A and B, down).

Additional confirmation of the existence of NucE invaginations observed with Ca\(^{2+}\) indicators and antibodies against NPC and SERCA was done by electron microscopy. Figure 3-21C shows that the invaginations are formed from both, inner and outer nuclear membrane, interrupted by NPCs (Fig. 3-21C).
4. Discussion
4.1. **In situ** calibration of Ca$^{2+}$ inhibitors in adult CMs

The *in situ* calibration procedure described in the present study represents a suitable, newly developed method for the quantification of $[\text{Ca}^{2+}]_{\text{nuc}}$ vs. $[\text{Ca}^{2+}]_{\text{cyto}}$ changes in cardiomyocytes for both Fluo-4 and ACaR. It has been shown previously that the fluorescent properties of the Ca$^{2+}$ indicator Fluo-4 are altered by the nuclear environment of various cell types (72,75). We have thus evaluated two sets of Fluo-4 parameters, cytosolic and nucleoplasmic, in mouse and rat cardiac myocytes. Our results for mouse and rat ventricular cardiac myocytes are in good agreement with previous findings in HeLa cells, where the $K_{d,\text{app}}$ amounted to $\sim$1000 nM in the cytoplasm (vs. $\sim$1100 nM in our study) and to $\sim$1260 nM in the nucleoplasm (vs. $\sim$1200-1300 nM in our study) (72). However, the opposite was observed in rat atrial myocytes where the $K_{d,\text{app}}$ was higher in the cytoplasm ($\sim$1400 nM) than in the nucleoplasm ($\sim$1100 nM). The reason for this difference between atrial and ventricular myocytes is unknown at present. It is probably related to the fact that the $K_{d,\text{app}}$ for Ca$^{2+}$ binding of the fluorescent probe can be drastically affected by environmental conditions. This implies that there may be substantial differences between atrial and ventricular myocytes in terms of the nuclear environment – at least with respect to the physico-chemical properties of Fluo-4.

Our calibration method also relies on estimates of the dynamic range, $R_f$, and $[\text{Ca}^{2+}]_{\text{rest}}$. Dynamic range was reported as the property of the indicator that does not need to be determined for every experiment (94). However, previously obtained values for the dynamic range of Fluo-4 varied. Thomas and colleagues found it to be 6 and 15 for the cytoplasm and nucleoplasm of HeLa cells, respectively (72), while values of 85-100 have also been observed (94). In the context of this study – more important than the exact value of $R_f$, which can vary depending on the cell type and experimental conditions – it is important to note that the results were uniform within a given cell type, independent of dye loading and gain settings. This justifies the use of the dynamic range as a parameter for transformation of fluorescence into calibrated $[\text{Ca}^{2+}]$. The dynamic range in the
nucleoplasm was consistently higher than in the cytoplasm, and this was true for all types of cardiac myocytes studied. This is in perfect agreement with previous studies on non-cardiac myocytes (72,75). Cellular resting [Ca$^{2+}$] may also vary (20-200 nM) depending on the state and type of the cell (95). In cardiac myocytes, it is usually assumed to be in the range of 100 nM or below. Here, we found that [Ca$^{2+}$]$_{\text{rest}}$ values varied only in a narrow range in each cell type studied as well as between cell types. [Ca$^{2+}$]$_{\text{rest}}$ values amounted to ~80 nM in the cytoplasm and ~100 nM in the nucleoplasm. This suggests that their mean is a valid parameter for calibration.

We further compared [Ca$^{2+}$] values obtained using different forms of the Grynkiewicz equation. No significant difference was observed using either equation [1] or [4]. The frequently used self ratio equation (96):

\[
\text{[Ca}^{2+}\text{]} = \frac{K_d R}{(K_d/[\text{Ca}^{2+}]_{\text{rest}} - R + 1)} \tag{7}
\]

is simpler and provides a good estimate of [Ca$^{2+}$] changes without aiming at precise calibration. It assumes that $F_{\text{min}}$ is 0. With bright indicators like Fluo-4, however, we found that $F_{\text{min}}$ is considerably larger than 0, thus necessitating the use of a different approach for calibration of [Ca$^{2+}$]. The advantage of the newly developed method is that all parameters needed for the transformation of Fluo-4 fluorescence signals into calibrated [Ca$^{2+}$] can be pre-determined, making this process very convenient for practical use with mouse and rat cardiac myocytes.

We also determined – for the first time in cardiomyocytes – the in situ properties of the ratiometric Ca$^{2+}$ dye ACaR. Similar to Fluo-4, ACaR exhibited different fluorescent properties in the nucleoplasm vs. cytoplasm with larger Ca$^{2+}$-dependent fluorescence changes and a higher $K_d,\text{app}$ in the nucleoplasm. Also similar to most fluorescent Ca$^{2+}$ dyes tested so far, the in situ determination of $K_d,\text{app}$ revealed considerably higher values (~2200 nM in the nucleoplasm and ~1340 nM in the cytoplasm) than in vitro (according to manufacturer's information: $K_d$ ~300-400 nM). Ca$^{2+}$-dependent fluorescence changes could be
easily detected at both wavelengths during calibration as well as electrically stimulated [Ca$^{2+}$] transients. Thus, ACaR represents a new Ca$^{2+}$ indicator – with all the advantages of a ratiometric dye – that is well suited for quantifying [Ca$^{2+}$] in the cytoplasm and nucleoplasm of cardiac myocytes. Using ratiometric imaging with ACaR yielded almost identical results to those obtained with the non-ratiometric dye Fluo-4 (Fig. 3-5 and 8) thus complementing and validating the calibration approach and the results obtained with Fluo-4.

4.2. Quantification and characterisation of subcellular Ca$^{2+}$ handling in resting and electrically stimulated CMs

4.2.1. The nuclear CaT consists of two components

An important finding of the current study is that the nuclear CaT exhibits distinct kinetics and that the nucleoplasmic systolic [Ca$^{2+}$] peak consists of two components, a passive and an active component. The passive component is most probably mediated by cytoplasmic Ca$^{2+}$ diffusion through nuclear pore complexes to increase [Ca$^{2+}$]$_{\text{nuc}}$. Several lines of evidence support this notion. First, nuclear pore complexes are readily permeable to Ca$^{2+}$ ions (53). Second, the nuclear CaT lags behind the cytoplasmic CaT (34). This delay is most likely caused by the time needed for diffusion of cytoplasmic Ca$^{2+}$ through the nuclear pore complexes. In addition, the effective diffusion of Ca$^{2+}$ within the nucleus is significantly slower than in the cytoplasm (97). Third, the amplitude of the nuclear CaT is proportional to the amplitude of the cytoplasmic CaT providing further evidence for the passive nature of nuclear CaT (Fig. 3-7). On the other hand, some cells exhibited prominently higher nucleoplasmic diastolic [Ca$^{2+}$] and higher nucleoplasmic systolic peak [Ca$^{2+}$]. This implies that there is an additional source of Ca$^{2+}$ in the nucleus. This observation is in line with previous findings that endothelin-1 can induce an independent increase in [Ca$^{2+}$]$_{\text{nuc}}$, without changing [Ca$^{2+}$]$_{\text{cyto}}$, and that this Ca$^{2+}$ release originates from perinuclear stores (34). The exact mechanism by which cardiac myocytes can regulate their [Ca$^{2+}$]$_{\text{nuc}}$
independently from $[\text{Ca}^{2+}]_{\text{cyto}}$ and the reason why some cells show prominently higher nucleoplasmic diastolic $[\text{Ca}^{2+}]$ than others is not known at present. However, it is clear that the nucleus is a cellular compartment with its own perinuclear $\text{Ca}^{2+}$ stores capable of actively storing and releasing $\text{Ca}^{2+}$ (61). This supports the idea of an active role of nuclear envelope $\text{Ca}^{2+}$ release in nuclear $\text{Ca}^{2+}$ signaling. It is expected that nuclear envelope $\text{Ca}^{2+}$ release varies depending on different physiological and pathophysiological processes taking place in the nucleus.

4.2.2. Perinuclear $\text{Ca}^{2+}$ store content and $\text{Ca}^{2+}$ leak determine the resting nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient

Although investigations into the regulation of nuclear $\text{Ca}^{2+}$ have been pursued for well over two decades by many laboratories using different technical approaches, there is still no consensus on the existence of resting $[\text{Ca}^{2+}]$ gradients between the nucleus and the cytoplasm. We consistently observed – following careful calibration with both Fluo-4 and ACaR accounting for all the major technical problems with fluorescent indicators – a nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient in all types of cardiac myocytes studied and further sought to address the origin of the higher $[\text{Ca}^{2+}]_{\text{nuc}}$ during rest. Both the nuclear envelope and the SR act as $\text{Ca}^{2+}$ stores in cardiac myocytes (98). Furthermore, many lines of evidence suggest a direct connection of the nuclear envelope to the SR $\text{Ca}^{2+}$ store. Because of the high mobility of $\text{Ca}^{2+}$ inside this $\text{Ca}^{2+}$ store (35), the luminal continuity of the entire nuclear envelope-SR store complex (35), and the existence of $\text{Ca}^{2+}$ release channels in the nuclear envelope (99,100), $\text{Ca}^{2+}$ can – in principle – be effectively mobilized from the nuclear envelope into the nucleoplasm. To test whether the nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient is related to the $\text{Ca}^{2+}$ load of the perinuclear $\text{Ca}^{2+}$ stores, we applied CPA to deplete these stores. Depletion of $\text{Ca}^{2+}$ led to the complete loss of the nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient. This finding indicates that the $\text{Ca}^{2+}$ load of the perinuclear stores is responsible for the nucleo-to-
cytoplasmic $[\text{Ca}^{2+}]$ gradient and suggests that a $\text{Ca}^{2+}$ leak through nuclear envelope $\text{Ca}^{2+}$ release channels facing the nucleoplasm causes the higher $[\text{Ca}^{2+}]$ in the nucleoplasm. A mismatch of SR $\text{Ca}^{2+}$ pumps, which may be located primarily at the outer face of the nuclear envelope, could contribute to or even increase the nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient (61).

Experiments with 2-APB and tetracaine clearly identified a 2-APB-sensitive $\text{Ca}^{2+}$ leak pathway as the cause of the nucleo-to-cytoplasmic $[\text{Ca}^{2+}]$ gradient, suggesting that tonic $\text{Ca}^{2+}$ release through perinuclear IP$_3$Rs is responsible for it. Furthermore, in electrically stimulated myocytes, 2-APB caused a selective decrease of nucleoplasmic $[\text{Ca}^{2+}]$ in diastole. These findings are in line with previous observations that perinuclear IP$_3$Rs are involved in regulation of $[\text{Ca}^{2+}]_{\text{nuc}}$ and excitation-transcription coupling (34,35,98,100). The results also provide further evidence for a segregation of $\text{Ca}^{2+}$ release channels, suggesting that IP$_3$Rs are predominantly involved in nuclear $\text{Ca}^{2+}$ regulation, whereas RyR2s are predominantly involved in cytoplasmic $\text{Ca}^{2+}$ regulation in cardiomyocytes (99).

### 4.2.3. Stimulation frequency affects $[\text{Ca}^{2+}]_{\text{nuc}}$ and $[\text{Ca}^{2+}]_{\text{cyto}}$ differentially

Heart rate is an important determinant of cardiac function. In cardiac myocytes, stimulation frequency increases intracellular CaTs (2). This effect is largely caused by an increase in $\text{Ca}^{2+}$ influx per unit time followed by increased uptake of $\text{Ca}^{2+}$ into the SR and subsequently elevated SR $\text{Ca}^{2+}$ load, which in turn increases the CaT. Because of the mostly passive nature of the nucleoplasmic CaT – being governed largely by $\text{Ca}^{2+}$ diffusion from the cytoplasm – it might be assumed that there is a similar frequency-dependent increase in the nucleoplasmic CaT. Indeed, systolic $[\text{Ca}^{2+}]_{\text{nuc}}$ increased in parallel with systolic $[\text{Ca}^{2+}]_{\text{cyto}}$ following an increase in stimulation frequency. Diastolic $[\text{Ca}^{2+}]_{\text{nuc}}$, on the other hand, increased with frequency to a much larger extent than diastolic $[\text{Ca}^{2+}]_{\text{cyto}}$. There was an about eightfold increase in nucleoplasmic but only a
~fourfold increase in cytoplasmic diastolic \([\text{Ca}^{2+}]\) following an increase in stimulation frequency from 0.5 to 4 Hz. At 4 Hz stimulation, diastolic \([\text{Ca}^{2+}]\) reached \(~800\) nM in the nucleus but only \(~400\) nM in the cytoplasm. The reason for this large increase in diastolic \([\text{Ca}^{2+}]\) in the nucleus is the slow decay of the nucleoplasmic CaT, which is considerably slower than the cytoplasmic decay thus leading to a much higher build-up of \([\text{Ca}^{2+}]_{\text{nuc}}\) when diastole is shortened. The results exemplify that a rather simple physiological maneuver, i.e. an increase in stimulation frequency, may lead to profound differences in the regulation of \([\text{Ca}^{2+}]_{\text{nuc}}\) vs. \([\text{Ca}^{2+}]_{\text{cyto}}\) with important implications for frequency-dependent excitation-transcription coupling.

4.3. Alterations in cytoplasmic vs. nucleoplasmic \(\text{Ca}^{2+}\) handling in hypertrophy and failure in an animal model of pressure overload and in failing human hearts

4.3.1. Changes of electrically stimulated CaTs during the development and progression of hypertrophy in mouse and human hearts occur first in the nucleus

Altered \(\text{Ca}^{2+}\) homeostasis is a hallmark of heart failure. In common with the findings in many experimental models of HF and in human heart failure (101-104) the amplitude of the cytoplasmic CaT was reduced and the kinetics were markedly slowed down. The reduction in the amplitude of the intracellular CaT in the end-stage failing hearts was mainly due to the increased diastolic \([\text{Ca}^{2+}]\). The increase of diastolic \([\text{Ca}^{2+}]\) causes elevated diastolic tension and, if excessive, may hamper ventricular filling (105) and, thereby, contribute to diastolic dysfunction.

Altered \(\text{Ca}^{2+}\) homeostasis may not simply be an epiphenomenon observed in end-stage failing hearts. Accumulating evidence suggests that it may be causally involved in the remodelling process leading to heart failure. Recently, it was proposed that alterations in nuclear \(\text{Ca}^{2+}\) – via activation of nuclear CaMKII –
may be a key event in the regulation of Ca$_{\text{2+}}$-dependent transcription. Thus, it is important to understand not only how cytoplasmic Ca$_{\text{2+}}$ is altered during hypertrophy and heart failure, but even more so how nucleoplasmic Ca$_{\text{2+}}$ is changed. Therefore, we have studied, for the first time, alterations in nuclear Ca$_{\text{2+}}$ homeostasis in cardiac myocytes from hypertrophied and failing hearts. The most important finding of this study is that the progressive alterations in Ca$_{\text{2+}}$ homeostasis that occur in the cytoplasm are present also in the nucleus. Even more important, most of the nuclear Ca$_{\text{2+}}$ changes occurred in the early stage of compensated hypertrophy and preceded the cytoplasmic Ca$_{\text{2+}}$ changes. For example, the CaTs amplitude and the kinetics parameters, TTP and DT$_{50}$, were selectively altered in the nucleus in the early stage of remodelling. We also found that the velocity of the spread of the CaT through the nucleus was slowed down, while the spread through the cytoplasm was preserved at this stage. The slower removal of the Ca$_{\text{2+}}$ from the nucleus leads to an even larger build-up of [Ca$_{\text{2+}}$]$_{\text{nuc}}$ as compared to [Ca$_{\text{2+}}$]$_{\text{cyto}}$ during diastole. Prolonged, increased [Ca$_{\text{2+}}$]$_{\text{nuc}}$ will lead to the alterations in key cellular processes, including gene expression, which are regulated by nucleoplasmic [Ca$_{\text{2+}}$] (1,50,51,53,106). At higher stimulation frequencies, when [Ca$_{\text{2+}}$]$_{\text{nuc}}$ is significantly more affected than [Ca$_{\text{2+}}$]$_{\text{cyto}}$ due to its slower kinetics (55), this may have an even more profound effect on [Ca$_{\text{2+}}$]$_{\text{nuc}}$ with important implications for frequency-dependent excitation-transcription coupling. Taken together, the functional data on subcellular Ca$_{\text{2+}}$ regulation during hypertrophy and heart failure suggest that the nuclear Ca$_{\text{2+}}$ changes occur first, before cytoplasmic Ca$_{\text{2+}}$ is affected, with an onset so early that they are likely involved in the further progression of hypertrophy and heart failure.

4.3.2. [Ca$^{2+}$]$_{\text{nuc}}$ is affected more than [Ca$^{2+}$]$_{\text{cyto}}$ by increased stimulation frequency after pressure overload-induced hypertrophy in electrically stimulated mouse CMs

Our previous work has shown that an increase in stimulation frequency leads to profound differences in the regulation of [Ca$^{2+}$]$_{\text{nuc}}$ versus [Ca$^{2+}$]$_{\text{cyto}}$ (55). Due to
the much slower kinetics of the nucleoplasmic CaT, diastolic \([\text{Ca}^{2+}]_{\text{nuc}}\) increased to a much larger extent than diastolic \([\text{Ca}^{2+}]_{\text{cyto}}\) when stimulation frequency was increased from 0.5 to 4 Hz. In the present study, we confirmed this finding in non-failing CMs. Although the increase of diastolic \([\text{Ca}^{2+}]\) was previously shown to be important for maintaining the \([\text{Ca}^{2+}]\) flux balance during the cardiac cycle at higher stimulation frequencies (105), it can cause elevated diastolic tension and consequently impair ventricular filling. Several recent reports have shown that under conditions of tachycardia, the high frequency of action potentials can also cause a net intracellular \([\text{Ca}^{2+}]\) gain that can activate the \(\text{CaN/NFAT}\) pathway (107,108). As expected, due to the slower decay of CaTs in CMs from failing hearts there was a much higher build-up of \([\text{Ca}^{2+}]\) when diastole was shortened. Prolonged, increased \([\text{Ca}^{2+}]\) as observed in CMs from failing hearts at higher stimulation frequencies may, therefore, cause stronger activation of key cellular processes which are regulated by intracellular \([\text{Ca}^{2+}]\) than in control CMs.

The increase of diastolic \([\text{Ca}^{2+}]_{\text{nuc}}\) was markedly higher than the increase of diastolic \([\text{Ca}^{2+}]_{\text{cyto}}\) at any frequency studied. At higher stimulation frequencies, diastolic \([\text{Ca}^{2+}]_{\text{nuc}}\) was found to be greatly increased in CMs from either early or late-stage hypertrophy groups as compared to the control. Some processes were shown to specifically require nuclear rather than cytoplasmic increases in calcium (reviewed in (109)). An example of nuclear calcium signalling is the modulation of the transcription factor \(\text{CRE-binding protein (CREB)}\) and its coactivator, \(\text{CREB-binding protein (CBP)}\), by changes in nuclear calcium (110). Nuclear calcium has also been implicated in modulating NPC function (111) and in controlling apoptosis (51). A profound increase in \([\text{Ca}^{2+}]_{\text{nuc}}\) may therefore influence these processes and contribute to the development of myocardial remodelling.

Exposure of CMs from failing hearts to isoprenaline produced blunted response in CaT amplitude as compared to control CMs. This result suggest that the defect in excitation-contraction coupling that develops in heart failure can not be compensated by \(\beta\)-adrenergic stimulation and may explain in part why these hearts fail to cope with increased heart rates.
4.4. Structural and biochemical alterations of nuclear envelope in hypertrophy and failure in mouse model of pressure overload and in failing human hearts

4.4.1. Calcium perinuclear stores suffer progressive loss of nucleoplasmic tubular network during hypertrophy and heart failure

Serial section transmission electron microscopy (TEM), 3D confocal microscopy and other powerful imaging techniques confirmed that the NucE of a wide variety of cell types contains invaginations including deep, branching tubular structures (for review see (56)). Recently, the existence of NucE invaginations was also observed in adult CMs (106,112,113). This study, however, is the first to investigate the remodeling of perinuclear Ca\textsuperscript{2+} stores, including invaginations of the NucE, in hypertrophy and heart failure in murine and human hearts.

It might be argued that NucE invaginations are simply an artifact, caused by nuclear envelope folding due to the shorter sarcomere length in isolated cells (113). However, the same study also reported that such infoldings still exist in myocytes fixed in situ at longer sarcomere length, albeit occurring less frequently and penetrating less deep into the nucleus. Our observation of NucE invaginations in nuclei from left ventricular trabeculae, other intact tissues (57,58,106,114,115) and cells grown in 3D cell culture (35) represent additional evidence in favor of the notion that such invaginations are a naturally occurring structures. Furthermore, the fact that sarcomeres in the cardiac myocytes shorten during systole suggests that the NucE invaginations might increase in number during contraction of the heart. This even suggests that the number of nuclear tubuli might change during the cardiac cycle.

There are several important functional consequences of on the NucE invaginations penetrating deep into the nucleoplasm. First, they will contribute to and facilitate the increase in nuclear Ca\textsuperscript{2+} during electrically stimulated CaTs, in particular in deep nuclear regions, both by decreasing the diffusion distance and
by increasing the membrane surface area, which may be critical for the regulation of gene transcription by local subnuclear Ca$^{2+}$. Second, the presence of nuclear pore complexes ensures facilitation of nucleo-to-cytoplasmic ion diffusion and transport of cargo in regions that would otherwise be remote from the nuclear periphery. Third, expression of the SERCA pumps on the NucE invaginations provides sites capable of Ca$^{2+}$ removal deep within the nucleus, which may play an important role in shaping nucleoplasmic CaTs kinetics.

The progressive reduction of the number of invaginations per nucleus length that we observed during the progression of hypertrophy in the TAC model, as well as in CMs from failing human hearts, may therefore contribute to both the slower upstroke (absence of NPC and thus Ca$^{2+}$ diffusion from the cytoplasm to nucleoplasm in deep nuclear regions) and decay (absence of SERCA pumps) of the electrically stimulated nuclear CaTs. The altered nuclear CaTs, in turn, might contribute to altered Ca$^{2+}$-dependent regulation of transcription and, thereby, the progression of hypertrophy.

4.4.2. Remodelling of Ca$^{2+}$ release channels expression pattern in perinuclear region during hypertrophy and heart failure

It was previously shown that endothelin-1, via inositol-1,4,5-trisphosphate receptor (IP$_3$R)-mediated Ca$^{2+}$ release from the nuclear envelope, may increase [$Ca^{2+}]_{\text{nuc}}$ independently from [Ca$^{2+}]_{\text{cyto}}$ (34). This process plays a key role in cardiac excitation-transcription coupling (35) and has been implicated in the development of cardiac hypertrophy and the progression of heart failure (65-67). Elevated IP$_3$R2 expression was detected in hearts from spontaneously hypertensive rats, human patients with heart failure, as well as in hypertrophic mouse hearts (116,117). Hence, increased IP$_3$R expression was proposed as a general mechanism that underlies remodelling of Ca$^{2+}$ signalling during hypertrophy and heart failure (116). In line with this notion, we observed increased IP$_3$R expression in perinuclear regions in CMs from failing mouse and human hearts. At the same time, perinuclear RyR expression was reduced.
suggesting a shift in perinuclear Ca$^{2+}$ signalling in favour of IP$_3$R-mediated Ca$^{2+}$ release during the progression from hypertrophy to heart failure. Previously, a strong accumulation of NFATc3 around the nucleus and especially in perinuclear regions was observed in isolated ventricular CMs. It was speculated that the perinuclear region could represent a local reserve of NFAT that is poised for shuttling in and out of the nucleus when local [Ca$^{2+}$] is elevated via IP$_3$-mediated Ca$^{2+}$ release (107). Accumulation of IP$_3$Rs in this region in CMs from failing hearts would support the idea of enhanced NFAT signalling and activation of a hypertrophic genes program.
5. Conclusion
During the past two decades a lot of attention was driven to the alterations of ion homeostasis in CMs during the development of heart failure. However, this is the first study (to our knowledge) to quantify the alterations in nucloplasmic vs. cytoplasmic calcium homeostasis during the development of heart failure and to provide the evidence for structural and functional remodelling of the nuclear envelope as hypertrophy progresses. In fact, I could demonstrate that the nuclear envelope is not just an ion-permeable membrane allowing passive diffusion of ions into and out the nucleus, but it is a functional Ca$^{2+}$ store containing a set of Ca$^{2+}$ regulatory proteins and actively controlling nucleoplasmic Ca$^{2+}$ handling. My findings that alterations of electrically stimulated CaTs in the nucleus precede and exceed cytoplasmic ones during the progression of hypertrophy in adult mouse and human hearts led to the conclusion that [$\text{Ca}^{2+}]_{\text{nuc}}$ is an important determinant of cardiac remodeling which may contribute to the development and/or progression of heart failure. Not only that nucleoplasmic Ca$^{2+}$ handling needs further experimental attention, but targeting nucleoplasmic Ca$^{2+}$ handling may provide powerful means to prevent maladaptive cardiac remodeling and its progression to heart failure.
6. References
6. References


(5) Klabunde RE. Cardiovascular Physiology Concepts. : Lippincott Williams & Wilkins; 2011.


(9) Adelmann GA. Cardiology Essentials in Clinical Practice. 2010.


(14) Survey Results to the General Public, Annual Congress of the European Society of Cardiology in Vienna. ; September; ; 2003.


(34) Kockskamper J, Seidlmayer L, Walther S, Hellenkamp K, Maier LS, Pieske B. Endothelin-1 enhances nuclear Ca\textsuperscript{2+} transients in atrial myocytes through Ins(1,4,5)P\textsubscript{3}-dependent Ca\textsuperscript{2+} release from perinuclear Ca\textsuperscript{2+} stores. J Cell Sci 2008 01/15;121(0021-9533; 0021-9533):186-195.


6. References


(76) Giovannardi S, Peres A. Nuclear and cytosolic calcium levels in NIH 3T3 fibroblasts. Exp Biol Online 1997 07/01;2(9):1-9.


(80) Kockskaemper J, Sheehan KA, Bare DJ, Lipsius SL, Mignery GA, Blatter LA. Activation and propagation of Ca\textsuperscript{2+} release during excitation-contraction coupling in atrial myocytes. Biophys J 2001 11;81(0006-3495; 0006-3495; 5):2590-2605.


(89) M. Mittler. Die Struktur der Kernhülle von Herzmuskelzellen. Graz: Medical University of Graz; 2009.


(92) Haugland RP. The Molecular Probes Handbook, Fluorescent Ca\textsuperscript{2+} Indicators Excited with Visible Light-Section 19.3. : Molecular Probes; 2008.

(93) Heinzel FR, MacQuaide N, Biesmans L, Sipido K. Dyssynchrony of Ca\textsuperscript{2+} release from the sarcoplasmic reticulum as subcellular mechanism of cardiac contractile dysfunction. J Mol Cell Cardiol 2011 3;50(3):390-400.


(97) Soeller C, Jacobs MD, Jones KT, Ellis-Davies GC, Donaldson PJ, Cannell MB. Application of two-photon flash photolysis to reveal intercellular communication and

(98) Kockskaemper J, Zima AV, Roderick HL, Pieske B, Blatter LA, Bootman MD. Emerging roles of inositol 1,4,5-trisphosphate signaling in cardiac myocytes. J Mol Cell Cardiol 2008 08;45(1095-8584; 0022-2828; 2):128-147.


(100) Zima AV, Bare DJ, Mignery GA, Blatter LA. IP\(_3\)-dependent nuclear Ca\(^{2+}\) signalling in the mammalian heart. J Physiol 2007 10/15;584(0022-3751; 0022-3751):601-611.


