REGULATION OF EXCITATION-CONTRACTION COUPLING IN CARDIAC MYOCYTES

Insights from Mathematical Modelling

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“Ko isse tekee, ni saa, mitä tulee.”
(When you do it yourself, you end up with what you get.)
– Äänipää –

3
Abstract

Background – The heart cell is a prime example of a system, in which numerous interconnected regulatory mechanisms affect the dynamic balance of cellular function. The function of the system emerges from the interactions of its components rather than from their individual properties. Thus, it is a challenging task to understand the causal relations within such a system, based on the analysis of experimental results. Facing this complexity, the systems biological approach has gained interest during recent years, since with using it we can make an effort to observe, quantitatively and simultaneously, multiple components and their interdependencies in biological networks.

Methods and aims – One of the most important tools in systems biology is mathematical modelling. In this thesis, novel model components have been developed and existing components integrated to describe mathematically the calcium dynamics in cardiac myocytes with improved physiological accuracy. Special attention was paid to both the activity-
dependent and automatic regulation of the dynamics. This enabled the quantitative analysis of the regulation’s role in both physiological and pathophysiological conditions.

**Results** – Validation of the novel model components that describe the calcium transport mechanisms indicates that the developed schemes are accurate and applicable also beyond the normal physiological state of the cardiac myocyte. Results also highlight the importance of autoregulation of calcium dynamics in the excitation-contraction coupling. Furthermore, the analysis indicates that the CaMK-dependent regulation of the calcium uptake to and release from the sarcoplasmic reticulum calcium stores could have substantial roles as downstream effectors in beta-adrenergic stimulation.

**Conclusions** – Results emphasize mathematical modelling as a valuable complement to experiments in understanding causal relations within complex biological systems such as the cardiac myocytes. That is, rigorous data integration with mathematical models can provide significant insight to the quantitative role of both the individual model components and the interconnected regulatory loops. This is especially true for the analysis of genetically engineered animal models, in which the intended modification is always accompanied by compensatory changes that can mask to a varying degree the actual phenomenon of interest.

**Keywords:** calcium, cardiac myocytes, computational physiology, genetic engineering, signal transduction, systems biology
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Oulu, October 2009

Jussi Koivumäki
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
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<tr>
<td>$[\text{Ca}^{2+}]_{XX}$</td>
<td>calcium concentration in compartment ‘XX’</td>
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<tr>
<td>CaMK</td>
<td>calcium/calmodulin-dependent kinase II; cardiac isoform CaMKIIδC</td>
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<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>CaN</td>
<td>calcineurin; protein phosphatase 2B</td>
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<td>CICR</td>
<td>calcium-induced calcium release</td>
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<tr>
<td>CSQN</td>
<td>calsequestrin; calcium binding protein in the SR</td>
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<tr>
<td>ECC</td>
<td>excitation-contraction coupling</td>
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<tr>
<td>FDAR</td>
<td>(pacing) frequency-dependent acceleration of relaxation</td>
</tr>
<tr>
<td>I₁</td>
<td>(protein phosphatase) inhibitor 1</td>
</tr>
<tr>
<td>$I_{\text{Ca,L}}$</td>
<td>L-type calcium current</td>
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in silico a phrase for "performed on computer"
in vitro a phrase for "within the glass" (in a test tube)
in vivo a phrase for "within the living"
K⁺ potassium ion
LCC L-type calcium channel
Na⁺ sodium ion
NCX Na⁺/Ca²⁺ exchanger
NKA Na⁺/K⁺-ATPase
PKA protein kinase A
PLB Phospholamban
PLB-KO PLB knockout
PLM Phospholemman
PMCA plasmamembrane Ca²⁺-ATPase; sarcolemmal calcium pump
PP1 protein phosphatase 1
RyR Ryanodine receptor; SR calcium release channel; cardiac isoform RyR2
SERCA SR reticulum Ca²⁺-ATPase
SR sarcoplasmic reticulum; intracellular calcium store
TnI Troponin I
T-tubules transverse tubuli
Contents

Abstract .............................................................................................................3
Acknowledgements ...........................................................................................7
Abbreviations.....................................................................................................9
Contents..........................................................................................................11
List of original articles......................................................................................13
1  Introduction ....................................................................................................17
   1.1  Background and significance..............................................................17
   1.2  Aims of the thesis ..............................................................................19
   1.3  Outline of the thesis ...........................................................................20
2  The heart basics ............................................................................................23
   2.1  Anatomy and physiology of the heart ..............................................23
   2.2  Supply and demand ...........................................................................25
   2.3  Cardiac myocyte ................................................................................26
3  Excitation-contraction coupling in cardiac myocytes .................................29
List of original articles

This thesis is based on the following publications that are referred to in the text as Publication I, Publication II, and so on.


The author of this thesis (JTK) contributed to the publications as follows. As the first author of Publication I, JTK integrated the reaction chain of calcium/calmodulin-dependent kinase II (CaMK) into a model of mouse cardiac myocytes, performed the simulations, and drafted the manuscript together with MW. In Publication II, JTK designed the study, implemented and validated the CaMK-dependent regulation mechanisms, performed the simulations, and drafted the manuscript. The novel model component presented in Publication III was implemented by JTK, JT and TK. JT and JK validated the model of cardiac myocyte with simplified ion dynamics. JTK performed the simulations, and drafted the manuscript together with MW. The study reported in Publication IV, was designed by PT. TK implemented the electrophysiology part of the model, JTK implemented the regulatory mechanisms of the model, and JT implemented the contractile element part of the model. JTK carried out the *in silico* experiments and data analysis, and drafted the manuscript together with PT, TK and MW. As the first author of
Publication V, JTK designed the study. The novel model component was implemented by JTK together with JT. Drafting of the manuscript was a joint effort of JTK, TK, MW and PT.
1 Introduction

1.1 Background and significance

Various problems related to the heart are the most common reasons for sickness and mortality in the western society. This has motivated a substantial investment of resources to the field of cardiovascular research and, thus, an explosive increase of experimental data. Currently, the two main challenges for the elucidation of unknown disease mechanisms and the development of novel therapeutic approaches are:

1) shortage of experimental data measured from patients with physiological and pathological conditions, as well as
2) integration of the fragmented experimental data across various experimental protocols and diverse species of animal models.

Due to the necessarily large variability of measurement data and to the species-dependent differences in cardiac physiology, it is not a
straightforward task to relate the experimental findings to the context of human cardiovascular system. The challenges become even more evident if one considers the fact that even a single heart muscle cell (cardiac myocyte) is an almost inconceivably complex system with numerous interrelated regulatory signalling connections. The heart is thus a prime example of biological system, in which “everything affects everything”. Therefore it is challenging to draw conclusions of the function of the system as whole with intuitive deductions based on experimental data alone (Matthaei 2007). Furthermore, while genetic interventions provide a powerful technique for, e.g. deleting or overexpressing certain functional component inside the cardiac machinery, the results are often not as unambiguous as they may have been presented (Cook et al. 2009). That is, even though the approach provides accurate information about the physiology at the endpoint, it provides little or no information about the processes that have lead to the endpoint phenotype. One major reason for this is the existence of physiological compensatory mechanisms that try to main the homestasis of the system despite the inflicted changes.

Facing these challenges the systems biological approach has gained much interest during recent years. Combined with methods of mathematical modelling this strategy can help to bridge the knowledge gaps between data retrieved from humans and animal models, as well as to integrate data from various experimental settings. Essentially, systems biology treats biological entities as a collection of inter-connected components, which are of reasonable complexity as research subjects. Mathematical modelling builds upon this by describing the function of components with mathematical equations and thus enabling a detailed analysis of the causalities within the system (Figure 1.1).
Figure 1.1 The role of mathematical modelling as a complement to the experimental approach. By implementing a mathematical model of the biological research subject it is possible to analyse separately the quantitative role of regulatory systems. This provides significant complementary information for the data analysis. In addition, it is possible to simulate experiments that are not feasible with current measurement technology.

There are a number of on-going large-scale international research projects that aim to use mathematical modelling to describe the physiology of the human as a whole; from molecules and cells all the way to the level of organs and whole organisms (Hunter et al. 2002, Hunter & Borg 2003). While these projects have already resulted, for example, in elegant models that describe the electrophysiology of the human heart, there are still many challenges to be solved even at the cellular level. Therefore, novel mathematical models need to be developed so that the cellular ion dynamics, including the calcium transport, so essential in cardiac myocytes, can be presented accurately in line with recent experimental findings.

1.2 Aims of the thesis

The purpose of the present study was to develop and implement mathematical models of the mammalian cardiac myocytes, including the various regulation mechanisms that are present in the cells. Main focus was
on the description of cellular calcium transport and its activity-dependent regulation. The specific goal and issues in this study were to:

1) Integrate the model of calcium/calmodulin-dependent kinase II (CaMK), an enzyme that is thought to be stimulated by the activity-dependent changes of intracellular calcium dynamics, into a model of mouse cardiac myocytes.

2) Describe the regulation of the targets of CaMK in such detail that the phenomena found in corresponding experiments could be reproduced in simulations.

3) Develop novel deterministic models of calcium transporters that represent the intracellular ion flow as simply and robustly as possible, yet in a physiologically accurate manner.

4) Study the usability of the developed mathematical models in the analysis of genetically engineered experimental animal models.

5) Elucidate the role of CaMK in the modulation of the cellular mechanisms that are involved in sympathetic stimulation (beta-adrenergic regulation) of the cardiac function.

The timeline for execution of these tasks is presented in the Appendix.

1.3 Outline of the thesis

This thesis is organised as follows. §2 introduces briefly the electrophysiology and the contractile function of the heart. This is followed, in §3, by a review of 1) the fundamental mechanisms that define the cardiac contractile function at the cellular level and 2) the recent advances that have been achieved in understanding the regulation of those mechanisms. The applicability of mathematical modelling as a complementary approach to experimental methods is evaluated in §4, together with a brief review of the history of
mathematical models relevant for the cardiac myocyte. The methods used in this study are presented in §5. The results of Publications I – V are summarised in §6 and discussed further in §7. A summary with conclusions is presented in §8. In all sections, issues that have been well documented in recent reviews by prominent authors, have been covered by only short explanations of the findings or major controversies, with references.
2 The heart basics

2.1 Anatomy and physiology of the heart

The heart is a muscular organ, whose weight in relation to the body weight varies between 0.2–1 percent for mammalian species. Heart is separated by the septum into two separate but functionally and anatomically similar subsystems: the left and the right half, which represent the division of the circulatory system into two separate systems (Figure 2.1). These halves are further divided into upper and lower parts, which are referred to as the atrium and the ventricle, respectively.

The main function of the heart is to supply the body with oxygen and nutrients by pumping blood through the blood vessels. Deoxygenated blood returns from the body to the right atrium during the relaxation phase, which is called diastole. Blood is then transported to the right ventricle by the contraction of the right atrium in the first phase of contraction, which is referred to as systole. In the second phase, the contraction of the right ventricle pumps the blood to the lungs where the blood is oxygenated in
pulmonary capillaries. From the lungs blood is collected by the four pulmonary veins into the left atrium. During the first phase of contraction, blood flows to the left ventricle, assisted by the contraction of the left atrium. The following contraction of the left ventricle then ejects the blood via the aorta into the systemic circulation. Thus, during the alternating cycles of systole and diastole, blood is continuously pumped from the systemic veins through the heart, the lungs, the heart again, and finally into the systemic circulation.

The coordinated contraction of the heart is orchestrated by the specialised electrical conduction system (Figure 2.1). The sinus node is the primary pacemaker of the heart, automatically generating electrical pulses. These electrical pulses spread as waves of electrical excitation over the atria, leading to atrial contraction. The atria and ventricles are electrically isolated from each other except for the region of the atrio-ventricular (A-V) node, via which the excitation passes to ventricles. The speed of propagation in the A-V node is slow, causing a delay in the passage of the electrical wave from the atria to the ventricles, ensuring that the ventricles contract after the atria. In the ventricles the excitation spreads fast through the A-V bundle, left and right bundle branches and Purkinje fibres, leading to ventricular excitation and contraction.
Figure 2.1 Diagram of the heart. On the left, the heart consists of four chambers that are formed by division in the left-right and upper-lower (atrium-ventricle) halves. Deoxygenated blood returns from the body to the right atrium through the Vena cava inferior and the Vena cava superior. During the relaxation phase, blood is then transported through the tricuspid valve to the right ventricle. The filling of the right ventricle is augmented by the contraction of the right atrium in the first phase of contraction. In the second phase, the contraction of the right ventricle pumps the blood through the pulmonary valve into the truncus pulmonalis and further to the lungs where the blood is oxygenated. From the lungs blood is collected by the four pulmonary veins in to the left atrium. During diastole and the first phase of contraction, blood flows through the mitral valve to the left ventricle. The following contraction of the ventricle then ejects the blood via the aorta into the body circulation. The valves prevent backward flow of blood inside the heart. On the right, the heartbeat is controlled by a unique nodal tissue located in the sinus node and atrioventricular (A-V) node. The sinus node initiates the heartbeat with impulses spreading out over the atria, causing them to contract. In the A-V node, there is a slight delay of signal conduction, which allows the atria to finish their contraction before ventricles start their contraction. From the A-V node, the impulse travels first via the A-V bundle (also known as His-bundle) then via left and right bundle branches (also known as Tawara branches) and finally spreads out to the ventricles via the Purkinje fibers. (Modified from wikipedia.org)

2.2 Supply and demand

The amount of blood that needs to be circulated in the body varies greatly depending on physical activity. For example, the cardiac output (volume of blood pumped per minute) of the human heart can increase from the resting
value of ~5 l/min to ~20 l/min during intense physical activity. The supply is made to meet the demand by three mechanisms:

1) changed heart rate (chronotropy),
2) adjusted stroke volume (inotropy) and
3) modified relaxation (lusitropy).

All these are subject to neuro-humoral regulation, acting through the vegetative nervous system, but also involve intrinsic mechanisms inside the myocytes. The first mechanism is linked essentially to the cardiac excitation initiation and conduction system. The other two mechanisms are based on the regulation of contractile force, i.e. the amplitude and relaxation rate of the force. In the heart, the myocytes are all activated for every contraction and the force produced by the whole heart is defined by adjusting the contractile function of individual cardiac myocytes. Therefore it is a matter of utmost importance to understand the principles of the excitation-contraction coupling of cardiac myocytes if we are trying elucidate the function of the whole heart (see §3).

2.3 Cardiac myocyte

The basic work units of the myocardium are the cardiac myocytes. They have commonly a cylindrical shape, the length ranging from 50 to 120 µm and the diameter between 5 and 25 µm. Each myocyte is linked to one or more nearby capillary for the support of oxygen and nutrients. The cell’s interior is surrounded by the cell membrane to separate the intracellular space from the extracellular space, between which ions, metabolites etc. are transported by specific membrane proteins. The intracellular space of cardiac myocytes consists mainly of the nucleus, mitochondria, the sarcoplasmic reticulum and protein networks of the contractile elements.

The heart consists of different cardiac myocyte types that can be divided into two categories: 1) myocytes of the sinoatrial (SA) node,
atrioventricular (AV) node and Purkinje fibers as well as 2) myocytes of the main bulk of the atria and ventricles. The myocytes of the first group are characterised by the capability of generating spontaneous electrical activation, whereas myocytes of the latter group, i.e. the working myocardium, remain normally quiescent without an external electrical stimulus. Furthermore, there are some differences in the electrophysiology of cardiomyocytes between the atria and ventricles, as well as some regional heterogeneity especially within the ventricles (for a review, see e.g. (Roden et al. 2002, Nerbonne & Kass 2005, Grant 2009)).

Compared to myocytes from other regions of the heart, the ventricular myocytes have a unique structural feature, the transverse tubuli (T-tubules) (for a review, see (Brette & Orchard 2003)). The T-tubules are invaginations of the cell membrane that create small subspaces between the membrane and intracellular calcium stores (see §3.1 and §5.1 for further details). Thus, the T-tubules shorten the diffusion distance and enable a more uniform intracellular calcium diffusion compared to the atrial, AV, SA and Purkinje fiber myocytes that have less or completely lack the T-tubules (Ayettey & Navaratnam 1978). The uniform diffusion of calcium from outside is needed to synchronise calcium release from internal calcium stores. Hence, the role of intracellular calcium stores is most significant in ventricular myocytes. However, the basic principle of contractile function is the same in the atrial and ventricular myocytes (including the cells of the conduction system): electrical activation triggers the rise of intracellular calcium concentration, which leads to calcium binding to the contractile element and further on to contraction.

For the efficient pumping function of the whole heart, it is essential that the cardiac myocytes contract in coordinated fashion. The cardiac conduction system promotes this strongly on the level of the whole organ (Figure 2.1), and at the cellular level the electrical contacts (gap junctions) between cardiac myocytes are of great importance. Together these two mechanism guarantee that electrical stimulus originating from the SA node
leads to an elegantly orchestrated contraction, which is fine-tuned in cardiac myocytes in the process referred to as excitation-contraction coupling.
3 Excitation-contraction coupling in cardiac myocytes

The periodic changes of intracellular calcium concentration, which take place in the cardiac myocytes, form the basis of the contractile function of the heart. The contractile cycle is initiated by a transient change of transmembrane electrical potential, and the whole process is thus referred to as the excitation-contraction coupling (ECC) (for a review, see e.g. (Bers 2001)). Calcium is the secondary messenger that drives the process of transforming the chemical energy available in cardiac myocytes (in the form of adenosine triphosphate; ATP) into mechanical work, i.e. shortening of the cell. The ionised form of calcium (Ca$^{2+}$) is the most common signal transduction element in a variety of cell types regulating functions important for both life and death (Clapham 1995).

When the electrical signal propagates along the cardiac tissue, it triggers the initiator of ECC in individual cells: the action potential (AP), which includes roughly two phases: depolarisation (loss of electrical potential
difference over the cell membrane, and a small “overshoot”) and repolarisation (restoration of the potential difference). In a cardiomyocyte, this process relies on low sodium concentration \([\text{Na}^+]\) and high potassium concentration \([\text{K}^+]\) inside the cell compared to extracellular space, the maintained actively by the \(\text{Na}^+/\text{K}^-\)-ATPase (NKA). As depolarisation promotes opening of fast ion channels that are permeable for \(\text{Na}^+\), a large positive inward current is produced, which causes the normally negative transmembrane voltage to become positive. Due to their intrinsic properties, the fast \(\text{Na}^+\) channels are closed rapidly after depolarisation. At the same time, the membrane \(\text{K}^+\) conductance increases due to the voltage-dependent activation of several types of \(\text{K}^-\) channels, which results in large positive outward current. Thus, the transmembrane voltage returns to the resting level (Figure 3.1). See e.g. (Bers 2001) and (Nerbonne & Kass 2005) for a reviews of the ion dynamics and channel kinetics during the AP, respectively.

As the cytosolic calcium concentration rises, more calcium binds to the myofilaments, which tap into the ATP pool to produce conformational changes, thus causing the myocyte to contract. This is a result of two types of myofilaments, which consist mainly of actin and myosin, sliding relative to each other (for a review, see e.g. (Huxley 2000)). In the relaxation phase, this process is reversed: as calcium is extruded from the cytosol to the extracellular space and to intracellular stores, the amount of calcium bound to the myofilaments is decreased in the same time, reversing the conformational changes and resulting in re-lengthening of the cell due to intrinsic elastic forces (Figure 3.1).
3.1 Calcium dynamics in cardiac myocytes

The rise of intracellular calcium concentration \([\text{Ca}^{2+}]_i\) is caused initially by the calcium influx from the extracellular space and followed by further release from the sarcoplasmic reticulum (SR), through the voltage-activated L-type calcium channels (LCCs) and the SR calcium release channels (ryanodine receptors; RyRs), respectively. During one contraction cycle, the calcium influx has to be balanced with an efflux to the same compartments, in order that calcium does not start to accumulate and impede contraction. In cardiac myocytes, majority of calcium is re-circulated back to the SR by the SR \(\text{Ca}^{2+}\)-ATPase (SERCA), leaving a smaller fraction of calcium to be extruded from the cell by the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (NCX) and sarcolemmal calcium pump (plasmamembrane \(\text{Ca}^{2+}\)-ATPase; PMCA). The relative contribution of SERCA
and NCX is species-specific. In the cardiomyocytes of large mammals that have a long AP (e.g., the human and the dog), the calcium removal fraction of SERCA and NCX are approximately 50–80 and 20–50 percent, respectively. Whereas for the small mammals that have a short AP (e.g. the mouse and the rat) the corresponding values are 85–95 and 5–15 percent, respectively. Across all mammalian species, the contribution of PMCA is rather small; approximately a one percent fraction of the removal, but its role is important in defining the resting level of intracellular calcium, because it has very high affinity. The relative role of these mechanisms is illustrated in Figure 3.2 and reviewed for example in (Bers 2001).

The depolarisation phase of the AP activates L-type calcium channels at the plasmamembrane, which results in calcium flow to the cell; the L-type calcium current ($I_{Ca,L}$). A secondary calcium influx route is the reverse mode of NCX ($I_{NCX}$); in the normal mode, NCX extrudes one calcium ion for three imported sodium ions. The contribution of these two influx mechanisms to the transient changes of intracellular calcium concentration is more significant in animals with long APs, whereas, in animals with short APs, their role is more
like a trigger for the intracellular calcium release (Figure 3.2). For a review of the physiological roles of $I_{Ca,L}$ and $I_{NCX}$ in cardiac myocytes, see e.g. (Brette et al. 2006) and (Bers & Despa 2006), respectively.

In ventricular myocytes, the main releasable pool of calcium is the SR; an intracellular store where part of the calcium is bound to the calsequestrin protein (CSQN). The calcium content of the SR is maintained by the SERCA pump that is able to produce a 1:7000 calcium gradient between the cytoplasm and SR (Shannon & Bers 1997). The opening of RyRs is sensitive to $[Ca^{2+}]_i$, which enables the $I_{Ca,L}$ to trigger calcium release via RyR from the SR. This event is commonly referred to as calcium-induced calcium release (CICR) (for reviews, see e.g. (Bers 2001, Diaz et al. 2005)).

In addition to the beat-to-beat regulation, $[Ca^{2+}]_i$ is also affected by the background calcium current across the cell membrane and the mitochondrial calcium uptake. Earlier studies have indicated that the role of mitochondria is rather small (Bassani et al. 1992). However, more recent studies suggest that mitochondrial calcium transport might have a significant role in the cellular calcium homeostasis even in short timescale (Duchen et al. 1998, Trollinger et al. 2000). For a review of this controversial issue see e.g. (Dedkova & Blatter 2008).

### 3.2 Regulation of ECC

Since the process of ECC is at the focal point of adapting the pumping function of heart to meet needs of the body (see also §2.2), it appears rather obvious that it is regulated with a number of partially overlapping signalling cascades operating inside the cardiac myocytes to assure homeostasis on both beat-to-beat basis and on the time scale of hours, days, etc. That is, in addition to obvious mechanisms related to the Frank-Starling regulation: the greater the preload, the greater the force generated by the cardiac muscle. At the level of individual cells, the modulation is realised by changing either one or more of these four features 1) the morphology of the AP, 2) influx of
calcium through the LCC, 3) functional state of intracellular calcium transport mechanisms and 4) sensitivity of the contractile elements to calcium. There are, of course, a number of further ways of categorising the regulation mechanisms, but for the purpose of highlighting the role calcium, the following division is used in the following sub-chapters:

1) activity-dependent regulation and
2) autoregulation.

The first mechanism originates from the autonomic nervous system. The sympathetic transmitters, epinephrine and norepinephrine, bind to and stimulate adrenergic receptors in the membrane of cardiac myocytes. This leads to increased levels of cyclic adenosine monophosphate (cAMP) and the subsequent activation of PKA. The increased contractile force of the myocytes under sympathetic stimulation is a result of larger calcium transients due to the activation (phosphorylation) of SERCA, RyR and LCC by PKA. These three targets, as regulators of ECC, are assessed in the §3.2.1, §3.2.2 and §3.2.3, respectively; see also Figure 3.3.

In addition, beta-stimulation regulates the contractile elements by two PKA-dependent phosphorylation mechanisms: 1) decreased myofibrillar calcium sensitivity of Troponin I (TnI) (Pi et al. 2002, Kentish et al. 2001, Pan et al. 1999) and 2) accelerated crossbridge cycle kinetics of the contractile element (Kentish et al. 2001, McClellan et al. 2001, Winegrad 1999, Robertson et al. 1982) (for a review, see e.g. (Layland et al. 2005). The contribution of troponin phosphorylation vs. enhanced calcium uptake by SERCA to lusitropy is a somewhat controversial issue. Li et al. (2000) suggested that the enhanced relaxation was mainly due to phospholamban (PLB) phosphorylation and the consequent stimulation of SERCA. However, a more significant role for troponin phosphorylation has been proposed in considerable number of studies (Pi et al. 2002, Pan et al. 1999, Fentzke et al. 1999).
The intracellular calcium dynamics, and thus the contractile force, are also modulated indirectly via NKA, which is crucial regulator of the intracellular sodium concentration. According to current understanding NKA is inhibited by Phospholemman (PLM) in an analogous way to the interaction between SERCA and PLB (see §3.2.1). Experimental findings (Han et al. 2006, Despa et al. 2005) indicate that PKA stimulates NKA function by relieving the inhibitory effect of PLM, thus reducing the K_m-value for cytosolic sodium. Increased activation of NKA leads to a decreased intracellular sodium concentration, which further promotes larger calcium extrusion by NCX.

Finally, multiple cardiac ion channels are regulated by PKA-dependent phosphorylation (for a review, see e.g. (Hartzell 1988)). For one of the main determinants of AP morphology the fast sodium current function, it appears that the effects of beta-adrenergic stimulation may be species-specific. For the rat and the mouse, which have fairly similar AP kinetics, both an increase (Schreibmayer et al. 1994) and a decrease (Schubert et al. 1989, Ahmmed et al. 2001) in sodium current have been reported. So, even though the cAMP-dependent phosphorylation targets in the alpha subunit of the mammalian cardiac sodium channel have been decisively documented (Murphy et al. 1996), the role of the regulation in the mouse cardiac myocyte is still an open issue. Additionally, the PKA-dependent changes in the I_{Na} involve channel trafficking (Hallaq et al. 2006), thus making it possibly relevant for long term regulation as well.

In addition to PKA, another dynamic regulator of ECC is the multifunctional serine/threonine protein kinase CaMK that phosphorylates numerous target proteins including SERCA, RyR and LCC (see §3.2.1, §3.2.2 and §3.2.3 for details). The major cardiac isoform of CaMK is CaMKIIδ, and the cytosolic splice variant is CaMKIIδC. It has been shown that CaMK can decode the frequency of calcium spikes into distinct amounts of kinase activity (De Koninck & Schulman 1998). Thus, it is a prominent candidate for mediating the activity-dependent regulation of calcium transport machinery. In addition, there exists evidence that sodium channels (Nav1.5) are dynamically
regulated by CaMK phosphorylation in rabbit cardiac myocytes, however, the current experimental findings are not conclusive for the mouse (Wagner et al. 2006). Furthermore, the channels underlying the transient outward potassium current ($I_{to,f}$), which are heteromultimers of Kv4.2 and Kv4.3, have been shown to be phosphorylated by CaMK in mammalian cells, e.g. in rat cardiac myocytes (Colinas et al. 2006). However, in mouse cardiac myocytes this mechanism appears to be either missing or of a physiologically irrelevant magnitude. Based on experiments with specific inhibitor of CaMK, Li et al. (2006) concluded that CaMK activity does not have an acute effect on either $I_{to,f}$ or $I_{K1}$ (inward rectifier $K^+$ current). According to the results of Roeper et al. (1997) the mammalian slow type transient outward potassium current ($I_{to,s}$) that is mediated primarily by Kv1.4 is regulated by CaMK. However, this channel type is absent from most regions of the mouse heart, excluding the septum (Xu et al. 1999).

**Figure 3.3 Phosphorylation targets of PKA and CaMK.** The schematic presentation includes those targets that are thought to be the most relevant for regulating the contractile force in ventricular myocytes of adult mice. PKA phosphorylates the ryanodine receptor (RyR) channel, L-type calcium channel, Na"+K" ATPase ($I_{NKA}$), phospholamban (PLB) and the contractile element. The phosphorylation targets of CaMK are partly the same: RyR channel, L-type calcium channel and PLB, with addition of direct phosphorylation of sarcoplasmic reticulum (SR) $Ca^{2+}$ ATPase (SERCA).
The principal difference between activation of PKA by beta-adrenergic stimulation and activation of CaMK is that the first one depends on an exogenous (extracellular) stimulus (epinephrine and norepinephrine), while the latter one depends on an endogenous (intracellular) stimulus ([Ca^{2+}]). The activation of PKA and CaMK are not completely separate, since activated PKA supports the activation of CaMK both directly via the interaction between PKA, protein phosphatase inhibitor 1 (I1), protein phosphatase 1 (PP1) and CaMK (Figure 5.2) and indirectly via enhanced cytosolic calcium transients. See e.g. (Hudmon & Schulman 2002) and (Maier & Bers 2007) for reviews of the function and the role in cardiac ECC, respectively. The targets and effects of PKA- and CaMK-dependent regulation are summarised in Figure 3.3 and Table 3.1.

Besides the overlapping and interconnecting nature of PKA and CaMK signalling pathways, the dynamics of the regulation become even more complicated when the compartmentalisation of the signalling domains is considered (Steinberg & Brunton 2001). Recent studies indicate that both PKA (Jones et al. 2006, Iancu et al. 2007, Iancu et al. 2008, Leroy et al. 2008) and CaMK (Saucerman & Bers 2008) experience differences in activation, depending on in which part of the cell they are located.

In addition to PKA and CaMK, there are other important players in the game of activity-dependent regulation of ECC, such as protein kinase C

<table>
<thead>
<tr>
<th>Target</th>
<th>Effect</th>
<th>PKA</th>
<th>CaMK</th>
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<tbody>
<tr>
<td>Tnl contractile element</td>
<td>Myofibrillar Ca^{2+} sensitivity</td>
<td>↓</td>
<td></td>
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<tr>
<td>LCC</td>
<td>Crossbridge cycling kinetics</td>
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<td></td>
<td>Activation</td>
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<td>Recovery from inactivation</td>
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<tr>
<td>SERCA</td>
<td>Forward affinity</td>
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<td></td>
<td>Maximal forward pump rate</td>
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<td>Reverse affinity</td>
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<tr>
<td>RyR</td>
<td>Calcium sensitivity</td>
<td></td>
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</tr>
<tr>
<td>NKA</td>
<td>Sodium affinity</td>
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(Kamp & Hell 2000, Walsh & Kass 1988), protein kinase D (Haworth et al. 2004) and protein kinase G (Layland et al. 2005). Since these signalling pathways are beyond the scope of this thesis, the reader is kindly asked to refer to respective publications for further information.

For maintaining the dynamic calcium homeostasis on beat-to-beat basis, the activity-dependent regulation by the enzymes presented above is too slow. Instead, the amount of calcium stored in and the fraction of calcium releasable from the SR, which are the fundamental factors regulating the ECC, are modulated by process a that is termed autoregulation due to its fast dynamics and straightforward mechanisms (Eisner et al. 2000, Dibb et al. 2007). This phenomenon is reviewed in the following subchapters.

An epitome for the co-operative nature of the activity-dependent and the “automatic” regulation of ECC is the frequency-dependent acceleration relaxation (FDAR); as the pace of heartbeat increases, the relaxation of contraction become faster (for a review, see e.g. (Bers 2001)). In beta-adrenergic stimulation, e.g. the PKA-dependent phosphorylation of the SERCA enhances the FDAR, but in the same time, the increased amplitude of the calcium transient directly stimulates pumping function of SERCA (via the normal substrate concentration related manner). Thus, these two loops of regulation jointly promote accelerated decay of the calcium transient and relaxation of contraction.

3.2.1 The SR calcium pump

The function of SERCA is inherently regulated by the dynamic changes of calcium concentration both in the cytosol and in the SR. With a stoichiometry of two Ca\(^{2+}\) ions moved per ATP hydrolysed, SERCA is able to produce a 1 : 7000 calcium gradient, cytoplasm : SR, correspondingly (Shannon & Bers 1997). It has been shown that these can be reversed: ATP is synthesised when calcium is transported from the SR to cytosol (Makinose & Hasselbach 1971). However, under physiological conditions the reverse rate is thought be
negligible (Tran et al. 2009). One further important feature of SERCA is its role as the second largest calcium buffer after the Troponin C (Bers 2001).

The importance of SERCA in the regulation of ECC is emphasized by the fact that the amount of calcium stored in SR depends solely on the balancing action of SERCA, where by it restores the calcium released during each cycle. Enhancing the pumping function of SERCA promotes faster decay of the calcium transient and thus faster relaxation of myocyte contraction. The pumping activity of SERCA is matched to the prevailing circumstances via the inhibitory protein, PLB (Simmerman & Jones 1998). Phosphorylation of PLB releases this inhibition. The phosphorylation can take place either at the Ser16 site by PKA (Chu et al. 2000) or at the Thr17 site by CaMK (Hagemann et al. 2000). Furthermore, the phosphorylation takes place additively by PKA and CaMK. That is, the total phosphorylation state of PLB is the sum of the effect of PKA and CaMK and the maximal phosphorylation by either kinase is enough to fully relieve the inhibitory effect of PLB on SERCA (Chu et al. 2000, Hagemann et al. 2000, Frank et al. 2000).

The activity of the SERCA can be modulated by CaMK via direct phosphorylation at the Ser38 site, thus increasing its maximum calcium transport capacity (Toyofuku et al. 1994, Xu & Narayanan 1999). This direct mechanism remains controversial, since some studies have not found any CaMK-dependent phosphorylation effects that would be uncoupled from PLB (Hagemann et al. 2000, Odermatt et al. 1996). However, analysis of murine PLB knockout (PLB-KO) myocytes has indicated that there indeed exists a CaMK-dependent stimulation of SR calcium transport, which does not require phosphorylation of PLB (DeSantiago et al. 2002). On the other hand, it has also been suggested that the FDAR would be totally decoupled from the CaMK-dependent modulation of the SERCA and the PLB (Valverde et al. 2005). For a review on this controversy, see e.g. (Maier & Bers 2002).

In addition to the phosphorylation state of the PLB, the molar ratio of PLB to SERCA is also a critical determinant of the regulation of the SERCA (Koss et al. 1997, Meyer et al. 1999). One important reason why PLB has
been studied so extensively during recent year, besides the decisive role SERCA has in setting the releasable pool of calcium in the SR, is that changes of the relative expression level of PLB to SERCA have been found in various heart failure conditions (MacLennan & Kranias 2003).

### 3.2.2 The RyR channel

The RyR channel is multimolecular complex whose main auxiliary proteins are CSQN, triadin and junctin, which impose a variety of modulatory effects on the channel (Bers 2004, Gyorke et al. 2004). For example, it has been shown that 1) overexpression of CSQN depresses cardiovascular function (Sato et al. 1998) and reduced expression increases calcium leak (Chopra et al. 2007), 2) triadin overexpression stimulates ECC and increases predisposition to cellular arrhythmia (Terentyev et al. 2005) and 3) partial downregulation of junctin enhances cardiac calcium cycling (Yuan et al. 2009).

The open probability of the cardiac RyR is regulated by cytosolic calcium (Schiefer et al. 1995) with strong modulatory effect by the SR calcium as well (Shannon et al. 2000, Gyorke & Terentyev 2008, Terentyev et al. 2008, Zima et al. 2008). The mechanism of termination of the SR calcium release is controversial. It has been suggested, for example, that RyR inactivation and the depletion of calcium in the junctional SR would contribute to it (Gyorke & Terentyev 2008, Stern et al. 1999).

The sensitivity of the RyR to the cytosolic calcium activation is regulated by the PKA and the CaMK via multiple phosphorylation sites; however, currently no consensus exists on their exact functional effects (Bers 2006). Based on lipid bi-layer experiments, it is known that CaMK phosphorylation enhances channel open probability (Wehrens et al. 2004), which is seen as both increased release and diastolic calcium leak at the whole-cell level (Maier et al. 2003, Kohlhaas et al. 2006, Curran et al. 2007, Ai et al. 2005). One earlier study even reported that CaMK desensitises the RyR to the calcium trigger (Wu et al. 2001), but this finding has not been supported...
by other reports. Some studies have also linked hyperphosphorylation of the RyR to PKA-dependent mechanisms (Marx et al. 2000, Gaburjakova et al. 2001). However, according to recent in vivo findings, the PKA-mediated effects appear to be indirect (Curran et al. 2007, Li et al. 2002, Guo et al. 2006, Benkusky et al. 2007, Ferrero et al. 2007), and rely predominantly on elevation of SR calcium load, whereas CaMK seems to be able to activate RyR directly via phosphorylation (Curran et al. 2007, Guo et al. 2006).

At the cellular level, the release seems to be governed by the SR calcium content rather than by modulation of the gating properties of the channel (Shannon et al. 2000, Lindegger & Niggli 2005). The physiological significance of this observation is highlighted by the studies of Trafford et al. (2000), which indicate that the modulation of RyR sensitivity to calcium has no maintained effect on the amplitude of cytosolic calcium transients.

In addition to the regulation of RyR by CaMK and PKA in normal physiological conditions, a number of studies have linked the abnormal phosphorylation status of their targets, and the consequent diastolic calcium leak, to various heart failure conditions (Maier et al. 2003, Netticadan et al. 2000, Wu et al. 2002, Xiao et al. 2005, Xiao et al. 2006, Terentyev et al. 2009). However, a lot of controversy is still related to these issues and the functional relevance of the calcium leak via RyR remains to be elucidated (Sipido 2007, George 2008).

3.2.3 The L-type calcium channel

The LCC is also under the dual regulation of the PKA and the CaMK, but by mechanisms that are far less controversial than the ones related to RyR channel. It has been shown with single channel measurements that both PKA (Yue et al. 1990) and CaMK (Dzhura et al. 2000) promote longer openings of LCC, i.e. gating mode 2. At the whole-cell level, this has been reported as enhanced activation and availability of the channel (Sako et al. 1997, Schroder et al. 2007), as well as slowed inactivation (Hashambhoy et al. 2009).
2009) and accelerated recovery from inactivation (Guo & Henry 2006), for the PKA and the CaMK, respectively. The overall effect of these two partially overlapping modulation mechanisms by the two enzymes is to enhance the trigger for calcium release from the intracellular stores. This phenomenon is generally referred to as facilitation; a term that is more commonly linked to CaMK, since it serves as a link for $I_{Ca,L}$ to facilitate itself (Yuan & Bers 1994). For reviews of PKA- and CaMK-dependent regulation of the LCC, see e.g. (Kamp & Hell 2000) and (Maier & Bers 2002), respectively.

Faulty regulation of LCC by CaMK has been related to a variety of heart failure models, including e.g. arrhythmia (Wu et al. 2002, Anderson et al. 1998, Thiel et al. 2008) and defective ECC (Maier et al. 2003) (for reviews, see e.g. (Anderson 2004, Zhang & Brown 2004)). Furthermore, some studies have reported that inhibition of CaMK can provide protection against e.g. heart failure caused by pressure overload (Wang et al. 2008) and structural heart disease caused by excessive beta-adrenergic stimulation and myocardial infarction (Zhang et al. 2005)

### 3.3 Genetically engineered animals in studies of ECC

Due to the inherent complexity of the cardiomyocyte, alternative approaches have been deployed to study the ECC process in addition to the normal variety of electrophysiological measurements. One of the most powerful and widely used tools developed for this purpose is the genetic engineering of animal models (Franz et al. 1997). In those studies the expression level of a certain protein is first modified and then the induced change in the expression level of the protein and the consequent changes in the ECC parameters are measured. The phenotype studies of the animal, the heart, and the isolated myocytes with altered gene expression can also include analysis of the morphology, heart rate, viability, etc. Several previously characterised, genetically engineered animal models have provided a large amount of information about the ECC.
The analysis of the functional roles of the targeted proteins, however, is far from being a straightforward procedure due to the complexity of the system (Matthaei 2007, Cook et al. 2009). Firstly, since the ECC is a highly non-linear process, the change in the expression level of a protein does not correlate linearly with the output of the system, such as contractile force. Secondly, the activity of the ECC system has been shown to have a feedback effect on the transcription of genes (Molkentin et al. 1998, Tavi et al. 2004). For example, the changes in ECC induced by genetic alteration to a single protein may induce further changes in the expression levels of other proteins (Maier et al. 2003).

The novel technique of inducible transgenesis, in which the expression of targeted protein can be changed at a given time point with an external stimulus, such as heat or a drug, has the potential to overcome the latter obstacle (Bockamp et al. 2002). That is, if the change in the expression of the target protein is fast and reversible the animal model can be studied without, or at least with very little, compensatory changes. This technique has already been used to study, e.g., the role of the SERCA (Andersson et al. 2009) and the NCX (Wang et al. 2009) in ECC and the results have been quite different from the earlier experiments with constitutive transgenesis of SERCA (Baker et al. 1998, Meyer & Dillmann 1998, Hiranandani et al. 2007) and NCX (Terracciano et al. 1998, Yao et al. 1998, Reuter et al. 2004, Pott et al. 2005).
4 Mathematical modelling of ECC

A multitude of models of cell electrophysiology for different species and regions of the heart have been developed over the last two decades (for a review, see e.g. (Fenton & Cherry 2008, Fink et al. 2009)). These include models of ventricular myocytes, e.g. for the human (Priebe & Beuckelmann 1998, ten Tusscher et al. 2004, ten Tusscher & Panfilov 2006, Iyer et al. 2004), the guinea pig (Luo & Rudy 1994, Noble et al. 1998, Matsuoka et al. 2003), the dog (Winslow et al. 1999, Greenstein & Winslow 2002, Hund & Rudy 2004), the rabbit (Puglisi & Bers 2001, Shannon et al. 2004, Mahajan et al. 2008), the rat (Pandit et al. 2001) and the mouse (Bondarenko et al. 2004).

The first step towards mathematical modelling of cardiac ECC was originally made in the area of neural AP modelling when Hodgkin & Huxley (1952) presented the mathematical description of ion currents in the squid giant axon. That formalism was later introduced to the field of cardiac AP models (Noble 1962). The first cardiomyocyte models with intracellular calcium dynamics (Beeler & Reuter 1977) and with intracellular release and uptake
mechanisms (DiFrancesco & Noble 1985) were introduced in the 1970s and 1980s. After the mid-1990s modelling moved from general models towards species-dependent models, which is reasonable due to the large differences in ECC between different species (for a review, see e.g. (Puglisi et al. 2004)).

4.1 Modelling approaches

A principal division can be made for the models of intracellular calcium dynamics as lumped vs. spatially distributed approaches. In the first case, compartments represent functional intracellular elements; e.g. the bulk cytosolic volume corresponds to one entity that has certain influxes and effluxes whose time constants are related to diffusion properties. There exist also a subgroup for this approach, in which the myocyte model have been simplified drastically to reduce the computational load so that a large number of them can put together to simulate AP propagation in tissue (ten Tusscher & Panfilov 2006). In the second group of models, the intracellular space is explicitly resolved, which allows e.g. for a detailed description of diffusional transport. Although the latter approach offers the advantage of including subcellular detail, it comes at a significantly higher computational cost in comparison to lumped models. For a review on the justification of these approaches, see e.g. (Fink et al. 2009). The compartmental model developed and employed in this thesis work is presented in §5.1.

A somewhat constant subject for debate is the validity of deterministic ECC models in comparison to stochastic schemes. This issue has been considered, for example, in the context of calcium oscillations (Kummer et al. 2005) and molecular reactions (Bhalla 2004). Specifically for the ECC models, it was, however, demonstrated in the elegant study of Hake & Lines (Hake & Lines 2008) that fully stochastic simulations are not necessary for even the detailed description of calcium dynamics in the vicinity of RyR. That is, deterministic diffusion within that compartment combined with stochastic
calcium binding to RyR is a valid approach to describe the stochasticity and discreteness of the calcium signalling.

### 4.2 Limitations of mathematical modelling

A mathematical model, as defined, is a representation of the system of interest with a set amount of detail. Thus, it is always a simplification of the real system. For the compartmental modelling approach in cardiac myocytes, this limitation can be seen as a lack of distinct anatomical structures inside cells that would correspond one-to-one to the elements in the model. Thus, there is no straightforward way of choosing the appropriate selection of compartments for a specific research question.

A fundamental challenge in developing an ECC model is the large variability of experimental data, to which the model behaviour has to be compared or matched. Therefore, it is essential that the outputs of the model agree qualitatively with the majority of the measured results rather than fitting perfectly to one single set of *in vivo* or *in vitro* data. Naturally, critical evaluation is needed if some set of data differs substantially from the average behaviour found in other studies. In such a situation, it is perhaps better to exclude the diverging data altogether so that it does not add error to the whole analysis.

The second major challenge in ECC modelling is that for most of the model parameters there is no corresponding measurement data, to which the parameter values could be directly fitted. Selecting appropriate parameter values is a procedure that can also quite easily become ambiguous due to species-specific differences as was demonstrated in the recent meta-analysis by Niederer et al. (2009). Often, the lack of proper theory or experimental data is compensated with approximations and even guesses. This strategy is inherent in mathematical modelling; however, it becomes a problem only if these limitations are not transparent in the research reports. The situation becomes very confusing if the assumptions, which affect the conclusions
drawn from the models, are not taken into account. The information requirements in modelling have been studied e.g. in the context of ion channels by Fink & Noble (2009).

Even though the development of community standards, such as CellML (Cuellar et al. 2003) and SBML (Hucka et al. 2004), enable efficient and accurate re-use of previously published model components or sub-models, there are significant challenges (Niederer et al. 2009). For example, the assumptions and data, on which the modelling work is based, are not transparently inherited to the next generation of models. For an in-depth review of the state-of-the-art of cardiac cell modelling, see (Fink et al. 2009).

Naturally, mathematical models also inherit, in a way, the limitations of the current experimental techniques and protocols. Firstly, the cardiac myocytes are typically isolated from the tissue, thus they are not functioning in their natural environment that would include, for example, neuronal and humoral stimulation, and their interconnections via gap junctions. Secondly, due to viability and stability issues, the measurements are often carried out in temperatures that are below the physiological range. Thirdly, it is a rather standard approach to use unphysiological bath solutions to enable easier measurement of e.g. the kinetics and magnitude of ion currents. These issues and many more were recently reviewed by Fink and colleagues (2009).

However, when these limitations are kept in mind and the models are validated to the best available knowledge and on all hierarchical levels (component, sub-model and whole model), mathematical modelling can be claimed to be a useful complement to experimental work. Consequently, it is nowadays applied in several fields from engineering to economics and biology. In ECC studies, mathematical modelling has become an essential part of the research methodology with an increasing trend. For reviews of ECC models specifically (Puglisi et al. 2004) and in the context including measurements (Fink et al. 2009), as well as, the whole heart modelling (Rudy et al. 2008), see the respective references.
4.3 Integrated models of activity-dependent regulation

As the kinase-dependent dynamic regulation of ECC is essential for the myocytes when adapting to the prevailing circumstances, a number of studies have sought to elucidate the underlying mechanisms with mathematical modelling. These schemes include PKA-dependent regulation of the following targets:

- LCC and PLB (Saucerman et al. 2003),
- LCC, PLB, RyR and Tnl (Saucerman & McCulloch 2004, Saucerman et al. 2006),
- LCC, PLB, RyR, Tnl, PMCA and NKA (Kuzumoto et al. 2008),

In addition, some recent modelling studies have addressed the effect of signalling sub-domains in the cAMP-PKA activation chain (Iancu et al. 2007, Iancu et al. 2008), but these more detailed models have yet to be integrated to cardiac myocyte-level ECC models. In addition to the beta-adrenergic modulation, there are three models with CaMK-dependent regulation of the following targets:

- LCC (Hashambhoy et al. 2009),
- RyR and PLB (Iribe et al. 2006) and

Of these, only the first one employs a detailed model of the chemical reactions involved in the complex chain from calcium input to CaMK activation, while the others use a phenomenological description (with one differential equation). In addition to the above mentioned CaMK models, a number of detailed schemes have been published (Saucerman & Bers 2008, Bhalla & Iyengar 1999, Chiba et al. 2008), but they have not been integrated to ECC models to describe the regulation by CaMK. Given the importance of other components, such as CaM, CaN and PP1, in this enzyme network (see
Figure 5.2), it appears essential to include these components as well, in order to gain more mechanistic information of the calcium-dependent feedback regulation by CaMK.
5 Methods

All the results presented in this thesis were obtained by executing experiments in silico, i.e. using mathematical models to compute the simulation results. The specific simulation methods mimic to as much detail as possible the corresponding in vivo or in vitro experiments (§5.2). In addition to novel model components developed within this project, the modelling platform employs extensively the concept of model reuse, as described below.

5.1 Platform for mathematical modelling

The basis for the modelling work presented in thesis is a compartmental description of intracellular calcium dynamics for representing ECC; see §4.1 for general review of modelling approaches. The origin of cardiomyocyte models that describe calcium dynamics with separate pools is the first model by DiFrancesco & Noble (1985), which divided the calcium concentration to cytosolic, non-junctional SR and junctional SR. Currently, a number of
approaches exist with the number of calcium compartments reaching from two (Fox et al. 2002) to six (Mahajan et al. 2008). The justification of separate compartments for the cytosolic, junctional and sub-sarcolemmal space arises from experimental findings, which indicate that during a normal action potential cycle, the calcium concentration in the vicinity of RyR channels sites reaches peak values 10–100 times (Langer & Peskoff 1996) and close to the sarcolemma 2–3 times (Weber et al. 2002) higher than in the bulk cytosol.

The division of SR into two compartments, network and junctional represents the experimental observations that close to the calcium release sites there are parts of the SR that are depleted of calcium during each release to a much larger extent than the SR in other areas of the cell (Shannon et al. 2003). The junctional SR compartment thus refers to a releasable calcium pool, which is “separated” from the network SR due to a significant diffusional resistance (Wussling & Szymanski 1986).

The schematics of the modelling platform, representing ECC in ventricular myocytes of adult mouse, as used in the present work are shown in Figure 5.1. This model was used in all the original studies, i.e. Publications I–V. The scheme was originally published by Bondarenko et al. (2004) and it has been modified extensively to improve the accuracy of the model, especially in relation to the calcium dynamics, as described in Publication IV and (Korhonen 2006). The description of the contractile element used in Publication IV is based originally on the work of Cortassa et al. (2006). That scheme was adjusted so that it replicates the characteristics of the contractile force that are typical for mouse cardiac myocytes, as described in Publication IV and (Takalo 2008). The enzyme network scheme of CaMK activation was originally developed by Bhalla & Iyengar (1999); Publications I, II, IV, V. The applicability of this scheme in prediction of CaMK activation has been documented in previous studies from our group for both skeletal (Tavi et al. 2003, Aydin et al. 2007) and cardiac (Tavi et al. 2004) myocytes.

The software code for the CaMK activation model was kindly provided by Dr. Perttu Niemelä.
Figure 5.1 Schematic presentation of the components and features of the model. The model accounts for processes that regulate intracellular concentration changes of sodium, potassium and calcium ions. The Ca\textsuperscript{2+} transport mechanisms are the L-type Ca\textsuperscript{2+} current (ICa,L), ryanodine receptor (RyR), SR Ca\textsuperscript{2+} ATPase (SERCA), sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX), sarcolemmal Ca\textsuperscript{2+} ATPase (PMCA), and background Ca\textsuperscript{2+} current (ICa,b). The Ca\textsuperscript{2+} fluxes within the cell are the uptake of Ca\textsuperscript{2+} (Jup) from the cytosol to the network sarcoplasmic reticulum (NSR), Ca\textsuperscript{2+} release (Jrel) from the junctional SR (JSR), Ca\textsuperscript{2+} flux (Jtr) from the NSR to JSR, Ca\textsuperscript{2+} leak (Jleak) from the NSR to the cytosol, Ca\textsuperscript{2+} flux from the subspace (SS) volume to the bulk myoplasm (Jxfer) and from the cytosol to Troponin (JTRPN). The model also includes the following transmembrane currents: the Ca\textsuperscript{2+}-activated chloride (Cl\textsuperscript{–}) current (I_{Cl,Ca}), the rapidly recovering transient outward K\textsuperscript{+} current (I_{Kto,f}), the slow delayed rectifier K\textsuperscript{+} current (I_{Ks}), the rapid delayed rectifier K\textsuperscript{+} current (I_{Kr}), the ultrarapidly activating delayed rectifier K\textsuperscript{+} current (I_{Kur}), noninactivating steady-state voltage activated K\textsuperscript{+} current (I_{Kss}), the time-independent K\textsuperscript{+} current (I_{K1}), fast Na\textsuperscript{+} current (I_{Na}), Na\textsuperscript{+} background current (I_{Na,b}), and the Na\textsuperscript{+}/K\textsuperscript{+} pump (I_{NKAT}).
Figure 5.2 Schematic presentation of the enzyme network model. The input for the enzyme reactions is the intracellular cytosolic Ca\(^{2+}\) concentration, [Ca\(^{2+}\)]. A rise in the [Ca\(^{2+}\)] level increases Ca\(^{2+}\) binding to calmodulin (CaM), which in turn phosphorylates more Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMK) and calcineurin (CaN). Phosphorylation of the latter induces phosphorylation of protein phosphatase 1 (PP1) by inhibiting protein phosphatase inhibitor 1 (I1), which itself is activated by protein kinase A (PKA). The autophosphorylation of CaMK is presented as a positive feedback loop and PP1 inhibition as a negative feedback (arrows indicate positive, activity-increasing effect, and stops negative, activity-decreasing effect).

The mathematical models of the myocyte function used here, including the ECC model and the subcomponents of these models, e.g. the ion channel and chemical reaction models, are systems of ordinary differential equations (ODEs) that have been implemented to the Matlab™ environment of technical programming. An example of the Matlab™ code files (m-files) used to present the models is shown in the Appendix. The same code structure was used in all models: the ‘main’ file solves the problem implemented in the ‘dy’ file and stores “solved” data to a file. Since the models consist of both rapidly and slowly changing components depending on the dynamics of variables, the simulation results were obtained by numerically integrating the model equations with a stiff ODE solver method (ode15s in Matlab™). To simulate the models, numerical integration is applied with a normal desktop computer or with a multi-CPU server designated for numerical computation.
5.2 Simulation protocols

The simulation results of the ECC model were obtained with three basic schemes: 1) quiescent, 2) current injection and 3) voltage clamp, of which the last two were adapted for specific purposes as explained below. The first protocol is rather simple: the myocyte is first stimulated with one current pulse and then left undisturbed for as long as is needed for the model to reach a quiescent steady state. This protocol can be used, e.g. to verify that those ion transport mechanisms of the model that are not AP-dependent have been presented correctly (for numerical details, see Table 1 in Publication IV).

The second protocol is the standard method for executing pacing experiments in silico. The stimulus current is carried by K⁺ ions; a general scheme suggested by Hund et al. (2001). To obtain a required stimulus sequence, the current pulse (amplitude –80 pA/pF and length of 0.5 ms) is repeated according to the pacing frequency for the desired length of the whole pulse train. This protocol was used in all the original works (Publications I-V). See Figure 5.3 for variations of the voltage clamp protocol employed either as such or combined with other specific protocols.

A commonly used experimental intervention, which provides information about the SR calcium content and the contribution of NCX to the cellular calcium dynamics, is the caffeine pulse protocol. A rapid application of caffeine onto the myocyte causes abrupt and prolonged openings of the RyR channels, while at the same time blocking the SERCAs. Thus, the SR is emptied from calcium and this calcium is then extruded from the cytosol mainly by NCX. Naturally, the caffeine pulse can be used also as starting point for SR loading experiments, e.g. to study the calcium release via RyR at
different SR calcium loads (Figure 5.3). The caffeine pulse experiment was executed *in silico* by setting the SERCA closed and the RyR constantly open. The calcium released from the SR is thus extruded from the cell mainly by NCX and secondly by PMCA. The caffeine pulse protocol was used in *Publications IV, V*. 
Figure 5.3 Examples of stimulation protocols. A) The standard voltage clamp protocol that can be used to study the voltage-dependence of L-type calcium current ($I_{Ca,L}$). B) The SR of the myocyte was first emptied (caffeine application), then loaded to increasing $[Ca^{2+}]_{SR}$ values, while external $[Na^+]$ was set to zero, and followed by one pulse. This protocol can be used to estimate the fractional release as function of SR calcium content. C) The myocyte was clamped to varying voltages after conditioning pulses. The trigger of the release, $I_{Ca,L}$, is proportional to the clamp voltage and thus the resulting calcium transient amplitude increases as a function of the applied voltage. In all protocols, the actual clamp pulse is preceded by a pre-pulse to inactivate the Na$^+$ channel.

The potential of mathematical modelling lies in the ability to observe multiple, possibly experimentally inaccessible, variables simultaneously, while applying acute interventions to the digital myocyte. As an example, it is possible to study the CaMK-dependent modulation of the RyR channel during one AP in such a way that the simulation is started from the same initial state.
for varying phosphorylation levels of CaMK, thus eliminating the effect of secondary changes in calcium dynamics (see Figure 3A in Publication V). Furthermore, “super” acute interventions, in which all the variables have been clamped to normal or otherwise desired time traces, enable “what if” type of in silico experiments. This approach was used in Publication IV (see Figure 6F) to study the effect of autoregulation in ECC.

In addition to all those advantages, mathematical modelling is also a clever method to study genetically engineered animal models, due to the possibility of dissecting the contributing process to a level of quantitative detail that is difficult or impossible to reach with in vivo or in vitro measurements. Commonly, the genetic interventions result also in compensatory changes in addition to the initial modifications (see §3.3. for a review). In mathematical models, the effect of these parallel mechanisms on the phenotype can be studied separately (Publication IV). The genetically engineered animal models were also used to validate the model components in Publications II-V.
6 Summary of results

In this thesis, novel model components describing the calcium transport mechanisms were develop in order to study the ECC in as simple as possible, yet physiologically accurate manner in both normal and genetically engineered animal models. Furthermore, the activity-dependent regulation of these mechanisms was integrated to the myocyte model. The studies elucidate the relative contribution of the various regulation loops of ECC to intracellular calcium dynamics and evaluate the applicability of mathematical modelling as complementary tool for the experimental approach.

Publication I presents the integration of a model of CaMK enzyme reaction chain to ECC model of a mouse cardiac ventricular myocyte. Importantly, the CaMK model replicates the fundamental characteristics of the enzyme: 1) decoding of the frequency of calcium spikes into distinct amounts of kinase activity (De Koninck & Schulman 1998) and 2) a saturation of the CaMK activation as the calcium input signal approaches the concentration of 10µM (Bradshaw et al. 2003). Results also indicate that the CaMK-dependent
modulation of the targets (LCC, RyR and SERCA) can be implemented accurately with the scheme adapted from Hund & Rudy (2004).

Publication II describes the implementation of CaMK- and PKA-dependent regulation into a bidirectional model of the SERCA. Results indicate that the model can faithfully replicate the phenotypic effect of SERCA-PLB interaction in genetically engineered myocytes that have been analysed experimentally in the case of both PLB knockout (Luo et al. 1994, Chu et al. 1996, Huser et al. 1998) and PLB overexpression (Kadambi et al. 1996, Chu et al. 1997). Furthermore, the results shed light on the roles of CaMK and PKA in the regulation of the SERCA under dynamic beta-adrenergic stimulation. That is, separate “time windows” could exist for the dual regulation: PKA taking the lead and CaMK having an increasingly important role as the PKA activity attenuates.

Publication III presents a systematic review of the principal solutions used for the modelling of the SERCA. The results indicate that while the unidirectional pump model may be sufficient for replicating normal physiological function, the bidirectional nature and calcium-buffering aspect of the SERCA should be taken into account, especially when modelling pathophysiological conditions.

Publication IV demonstrates the potential of mathematical modelling as complementary approach to experiments in the analysis of genetically engineered animal models and complex physiological phenomena. The results show that the compensatory changes observed in some genetically engineered animal models can mask the effects of original intervention and thus render that causal chain of events ambiguous, based on which the original conclusions were reached. In addition, the results highlight the power and the complexity of the autoregulatory phenomena in intracellular calcium dynamics.

Publication V describes the implementation of CaMK-dependent regulation into a mathematical model of the RyR channel that includes the modulatory effect of CSQN on the channel opening. Results suggest that the
model describes, with good accuracy, the fundamental features of SR calcium release: 1) the link between the calcium trigger and release, 2) the effect of SR calcium content on the release and 3) the regulation of the release by CaMK phosphorylation. In line with measurements, the results indicate that 1) direct phosphorylation of RyR is not necessary to increase the diastolic calcium leak through the RyR channel (Curran et al. 2007) and 2) even subtle increase or decrease in the expression level of CSQN causes substantial changes in the intracellular calcium dynamics (Chopra et al. 2007). Thus, the analysis suggests that the RyR-CSQN interaction is an important mechanism to be included in computational studies of ECC.
7 Discussion

This chapter assesses 1) the significance of the modelling work presented in this thesis in comparison to recent published developments, 2) the role of the CaMK in the activity-dependent regulation of the ECC, 3) the advantages and disadvantages of genetically engineer animal models in dissecting the mechanisms important to CICR and signalling pathways, and 4) the applicability of mathematical modelling to the analysis of the ECC.

7.1 The novel model components

As a part of this thesis work, some novel model components important for the ECC and its regulation have been developed. Most importantly, extensive work has been done to better describe the calcium dynamics of the SERCA and its phosphorylation by the CaMK and the PKA (Publications II and III). Motivation came from the fact that at the time there were no comprehensive models of SERCA available that would have included all the principal
features: 1) reversible pumping function, 2) calcium buffering and 3) modulatory phosphorylation by CaMK and PKA, although all the aspects had been considered and described separately by Shannon et al. (2004), Higgins et al. (2006), Hund & Rudy (2004) and Saucerman et al. (2003), respectively. Recently, also Tran et al. (2009) presented a Markov model describing elegantly the calcium dynamics and energetics of the SERCA. However, they focused on building a model that is thermodynamically based, an utterly important aspect for evaluating the energy-dependencies, and thus disregarded some of the features mentioned above.

The first step in the present endeavour was to merge the concept of reversible pumping function (Shannon et al. 2004) with the regulatory phosphorylation by the CaMK (Hund & Rudy 2004) and the PKA (Saucerman et al. 2003). Building on these previously existing schemes, the developed model (Publication II) describes the CaMK- and PKA-dependent regulation as well as the bidirectional function of SERCA in a way that is both very intuitive — the fundamental parameters are directly linked to in vitro observations — and computationally efficient. Furthermore, model validation indicates that it can replicate the SERCA-PLB interaction quantitatively in both extremes: PLB knockout (Luo et al. 1994, Chu et al. 1996, Huser et al. 1998) and PLB overexpression (Kadambi et al. 1996, Chu et al. 1997). The ability of the model to extrapolate beyond the normal physiological conditions is an important feature, for example, in the analysis of various heart failure conditions, in which the PLB-SERCA interaction is disturbed (see §3.2.1 and §7.2).

The second step extended the work by including the calcium-buffering aspect of the SERCA in modelling scheme (Publication III); an important feature of the pump (§3.2.1). Despite the added complexity, the model fulfils the original goal of developing a scheme that should be robust and intuitively linked to the in vitro results. Furthermore, the model complies with the same important validation criteria as the more simplified implementation (Publication II).
The RyR model presented in Publication V was adapted from the scheme published by Restrepo et al. (2008) that describes the important regulatory effect of CSQN on channel openings (§3.2.2). This formulation was complemented with the CaMK-dependent modulation of the opening probability, along with the principles that were presented in preliminary form earlier (Koivumäki et al. 2008). The developed model (Publication V) agrees nicely with the experimental findings regarding the fundamental features of SR calcium release: 1) the dependence on the SR calcium content (Shannon et al. 2000), 2) the modulation of the kinetics by CaMK phosphorylation (Wehrens et al. 2004) and 3) the CaMK-induced diastolic calcium leak (Curran et al. 2007). In addition, the model was validated in the situation of CSQN overexpression and underexpression, and the results agree qualitatively with the experimental findings of Miller et al. (2005) and Chopra et al. (2007), respectively.

A limitation of the RyR model is that it can not describe the graded release. Instead, there is a certain threshold for the calcium trigger, above which the release is nearly maximal (Publication V, Figure 2D); a feature that is rather characteristic for deterministic RyR models. For example, in the original ECC model by Bondarenko et al. (2004) this problem was solved by introducing a phenomenological dependence of the RyR channel opening on $I_{\text{Ca,L}}$. While this approach can reproduce the graded release in a voltage clamp protocol, it also creates an unphysiological mechanism, with which $I_{\text{Ca,L}}$ can trigger calcium release from the SR even if the calcium content were very low.

Of the recently published RyR models the most comprehensive ones are perhaps the four-state models introduced by Shannon et al. (2004) and Hinch et al. (2004), as well as the six-state model of Laver (2007). Currently, there is no “golden standard” for the RyR implementation in deterministic cardiomyocyte models, but rather, a vast number of approaches have been used, in which the model structure and features seem to depend quite heavily on the application. Thus, the situation differs substantially from e.g. the one of...
SERCA, for which unidirectional description has been used almost without exceptions (Publication III).

7.2 Role of CaMK in regulation of ECC

Since CaMK is an important regulator of the L-type calcium current, the uptake of cytosolic calcium to the SR and the calcium release from the SR, which are the key elements of the CICR, it has gained a lot of research interest during the last two decades. Despite extensive efforts, the exact quantitative role of the CaMK in the regulation of ECC still remains to be elucidated (Maier & Bers 2007). This might be, at least partly, due to the emphasis of these studies, which has been to a rather large extent in the various heart failure conditions, while normal physiological behaviour has not been such a “hot topic”. Perhaps this approach is in some sense justified, since a fair portion of the studies have been driven by medical investigators that might have a tendency to be more interested in diseases, and their diagnosis, treatment and prevention, rather than normal physiology. Since CaMK has multiple phosphorylation targets in the ECC machinery, it is a challenging task to try grasping the “big picture”, which has to be based on deductions made from comparatively small details of the analysis of experimental results. Therefore, utilizing the work presented in this thesis, the recently reported advances in mathematical modelling of the dynamics of CaMK activation (Saucerman & Bers 2008, Bhalla & Iyengar 1999, Chiba et al. 2008) and integration of the CaMK-dependent feedback into ECC models (Hashambhoy et al. 2009, Hund & Rudy 2004, Iribe et al. 2006), could contribute significantly in elucidating the role of CaMK in regulation of ECC.

One of the most important phenomena in the dynamics of the ECC, to which the CaMK has been linked, is the FDAR (§3.2). Numerous studies have suggested an affirmative and significant role for the CaMK in that process (Hagemann et al. 2000, Odermatt et al. 1996, DeSantiago et al. 2002, Li et al. 1997, Li et al. 1998, Bluhm et al. 2000, Ji et al. 2006). However, more recent
experimental results have blurred this previously clear picture by indicating that the CaMK-dependent phosphorylation of the SERCA might not be essential for the FDAR (Valverde et al. 2005, Huke & Bers 2007, Picht et al. 2007). Furthermore, the latter view was supported by the recent modelling study by Saucerman & Bers (2008). Together, these results, obtained with complementary approaches, might appear rather conclusive if there were not a substantial unknown factor in the analysis: the intracellular phosphatase activity. Specifically, the activity of the PP1 is a crucial regulator of CaMK dynamics (Chiba et al. 2008, El-Armouche et al. 2003, Pathak et al. 2005, Nicolaou et al. 2009); see Figure 5.2. The main modulator of the PP1 activity is the PKA and recent in vivo (lancu et al. 2008) and in silico (lancu et al. 2007) studies indicate that the PKA has a substantial baseline activation level. This factor was not considered in the earlier studies of Saucerman et al. (2006), and it appears to be ignored in the recent study as well (Saucerman & Bers 2008). Thus, it appears to be an essential further development to integrate the beta-adrenergic and CaMK-dependent regulation networks to the ECC models. First steps to that direction were taken in this thesis, and the results indicate that CaMK is a significant downstream effector in beta-adrenergic stimulation of the ECC via regulation of SERCA (Publication II). This view is supported by recent in vivo findings (Yurukova et al. 2007) and also by our preliminary in silico analysis, which took advantage of a more comprehensive modelling approach, including also e.g. the LCC regulation by PKA (Koivumäki et al. 2009a).

While the role of the CaMK in regulation of SERCA, and thus in the FDAR, has been mainly of physiological interest, the CaMK-mediated effects on the RyR channel have been studied largely in relation to clinical problems like heart failure or with animal models relevant for studies of those conditions. A commonly reported finding is the diastolic calcium leak via the RyR that was initially linked to hyperphosphorylation by the PKA (Marx et al. 2000, Gaburjakova et al. 2001). Based on more recent studies, it appears, however, that CaMK might be the dominant twin in the phosphorylation of the
RyR (Curran et al. 2007, Guo et al. 2006); for a review, see e.g. (Kockskamper & Pieske 2006).

The functional relevance of the calcium leak via the RyR remains to be elucidated (George 2008). Since evidence does not exist that would justify a more complex scheme, a conservative model of RyR regulation by CaMK is that the same phosphorylation target modulates both the systolic and the diastolic opening probability. While modulation of the RyR sensitivity appears to have no maintained effect on the CICR (Trafford et al. 2000), the CaMK-dependent stimulation of RyR and the consequent leak might be relevant in avoiding excess accumulation of calcium in the SR during sympathetic activation. If there is a calcium overload in the SR, which increases the RyR open probability, calcium would also be released spontaneously during the diastole (Lukyanenko et al. 1999). Since CaMK-dependent phosphorylation of the RyR has been shown to take place downstream of beta-adrenergic regulation (Curran et al. 2007, Ferrero et al. 2007), this could be an important physiological mechanism. In that, it is very important to avoid spontaneous releases, which would depress the pumping function of whole heart due to asynchronous contraction of individual cardiomyocytes and thus hamper the initial ‘goal’ of sympathetic activation: the enhanced ECC. Naturally, this is a very delicate balance: an overly large RyR leak would tend to reduce the SR calcium content too much and thus abolish the positive inotropic effect of sympathetic activation.

The physiological role of the CaMK in regulation of its third target, the L-type calcium channel, is to enhance the triggering of CICR. The term ‘facilitation’ is somewhat misleading, but it fits well to the idea behind the experiments: in voltage clamp, with myocytes initially quiescent, the consecutive voltage pulses are used to produce an $I_{Ca,L}$ train with gradually increasing amplitude; an enhancement (or “facilitation”) that is CaMK-dependent (§3.2.3). However, working (healthy) cardiac myocytes never experience this kind of sequence of events. In physiological settings, the role of the CaMK might rather be to partly compensate for the reduced LCC
availability, which is caused by the calcium-dependent inactivation of the LCC at high heart rates (Maier & Bers 2002). The role of the CaMK as a downstream effector in the beta-adrenergic regulation of LCC has not been studied in such a quantitative detail as that of SERCA (Yurukova et al. 2007) and RyR (Ferrero et al. 2007). However, the preliminary results of our in silico studies indicate that CaMK might have an important role in this signalling pathway as well (Koivumäki et al. 2009a, Koivumäki et al. 2009b). Clearly, much more experimental and modelling work is needed to elucidate this regulation in sufficient mechanistic detail.

One problem of a general nature that might be rather significant in the analysis of experimental results is related to the commonly used inhibitory compound for CaMK, KN-93. Studies have shown that KN-93 has direct inhibitory effects on \( I_{Ca,L} \), independent of CaMK (Gao et al. 2006). The decreased intrusion of calcium to the cell changes the dynamic state of the cardiac myocyte, in which the ECC takes place. Therefore it is a rather ambiguous starting point for analysing the role of CaMK. The situation is further complicated by the fact that unspecific effects of KN-93 depend both on the concentration and the length of drug application, as well as the direct inhibitory effect on potassium currents (Ledoux et al. 1999, Rezazadeh et al. 2006) that will then change APs and thereby the whole ECC.

In spite of the above mentioned controversy, the following scheme can be suggested for the physiological role of CaMK in the regulation of ECC. In ventricular myocytes, the overall effect of beta-adrenergic regulation of ECC via the PKA activation, and the subsequent downstream activation of the CaMK, is to increase the peak force of contraction and to accelerate relaxation. Both these phenomena are linked strongly to the stimulation of SERCA, which increases the SR calcium content and accelerates the decay of cytosolic calcium transient after the systolic peak. In this scheme, the role of regulation of the RyR and the LCC could be to fine-tune the dynamic balance, by promoting RyR leak and increasing the triggering of CICR. These features are important in the sense that the myocyte does not become
autonomic due to excess accumulation of calcium in the SR but rather remains under the control of external electrical stimuli. Supporting this view, DeSantiago et al. (2008) reported that, at least in the failing heart, arrhythmogenic effects of beta-adrenergic stimulation are attributable to enhanced SR calcium load. In principle, the CaMK-dependent modulation of the LCC and the RyR might appear sufficient. However, for avoiding arrhythmogenic effects, it is important to include the SERCA in this modulatory loop. For example, the complexity of intracellular calcium alternans was elegantly demonstrated by the recent in-depth studies of Xie et al. (2008), who proposed that the phenomenon depends, in a cooperative fashion, on SR calcium release, uptake and passive leak.

7.3 Role of CaMK in faulty regulation of ECC

In addition to the physiological role of CaMK, it has been linked to the faulty regulation of ECC in various types of heart failure conditions. In these abnormal conditions, the above mentioned balance of CaMK-dependent regulation of the intracellular calcium dynamics could be off the mark (for a review, see e.g. (Zhang & Brown 2004)). Faulty regulation of ECC by CaMK has been related to a variety of heart failure models (and phosphorylation targets): arrhythmia (LCC) (Wu et al. 2002, Anderson et al. 1998, Thiel et al. 2008), a defective ECC (LCC) (Maier et al. 2003), defective ECC (RyR) (Ai et al. 2005) and a limited FDAR (SERCA) (Antoons et al. 2006). Supporting this, it has been shown that a sustained beta-adrenergic stimulation, the underlying phenomenon in many heart failure situations, modulates the ECC via the CaMK signalling pathway (Wang et al. 2004). For a review of the role of CaMK in hypertrophy and heart failure, see e.g. (Maier 2005).

Complementing the above findings, some studies have reported that the suppression of CaMK activity can provide protection against: 1) heart failure caused by pressure overload (Wang et al. 2008), 2) structural heart disease caused by excessive beta-adrenergic stimulation and myocardial
infarction (Zhang et al. 2005), 3) development of the arrhythmogenic transient inward current during AP prolongation (Wu et al. 1999), and 4) arrhythmias after acidosis (Said et al. 2008).

Considering the above findings, cardiac-specific interventions of CaMK activity might offer some potential therapeutic solutions. For example, a targeted inhibition of CaMK-dependent regulation of the RyR might be promising: decreased diastolic calcium leak would enhance the ECC. However, if the intervention would also decrease the systolic opening probability, the end-effect might not be as expected: while a reduction of the RyR opening probability has been shown to abolish spontaneous calcium release and to increase the amplitude of the calcium transient in myocytes (Venetucci et al. 2006), it has also been shown to increase the variability of calcium transients (Diaz et al. 2002). Furthermore, experimental findings indicate that increasing the RyR opening probability alone does not produce arrhythmogenic calcium waves, but in addition a threshold SR calcium content is required (Venetucci et al. 2007).

In addition to decreasing the RyR leak as a means to improve cardiac function in heart failure, numerous studies have tried, with contradictory results, to enhance the defective ECC with genetic interventions related to SERCA (Vinge et al. 2008). For example, PLB ablation, a condition corresponding to maximal phosphorylation of PLB by CaMK, has been shown to improve intracellular calcium transients but not the cardiac function in a mouse model, in which heart failure arises from cardiac-specific overexpression of the tumour necrosis factor (Janczewski et al. 2004). The underlying mechanisms of this controversy might be related to the energy metabolism, since in cardiac myocytes the SERCA is among the most energy-demanding components, in addition to NKA and the contractile machinery. That is, if cells are under energetic restrictions, ECC can not be improved by stimulating a mechanism that increases energy consumption.

Thus, it appears that an inhibition of a single CaMK target is not a suitable approach, at least in the case of the RyR. There is strong evidence
for the view that arrhythmogenic phenomena are a product of the interplay of the SERCA, the RyR and the LCC. A straightforward overall inhibition of the CaMK is not feasible, because this has been shown to suppress the dynamic responses of the calcium transient to changing pacing (Wu et al. 2006). Therefore, the possible usefulness of the CaMK as a therapeutic target warrants further research work.

7.4 Genetically engineered animals in analysis of ECC

The ECC process appears to be rather robust against small interventions, which is due, at least partly, to its autoregulation. On the other hand, because of the non-linearity of the system (Qu et al. 2007), large disturbances, for example several fold overexpression of some protein important for the ECC, can cause dramatic changes to its behaviour. The non-linearity of the cellular mechanisms governing the ECC is highlighted, for example, by the overexpression studies of the CSQN. Dramatic interventions, which resulted in a 20-fold (Sato et al. 1998) and a 10-fold (Jones et al. 1998) increase in the expression of the CSQN over the normal level, resulted in a drastically depressed cardiovascular function and hypertrophy that were associated with a number of secondary changes in the expression of the RyR and the SERCA, among others. Whereas a subtle (58%) overexpression of the CSQN enhanced cellular calcium dynamics: the amplitude of the calcium transient and $I_{Ca,L}$ increased (Miller et al. 2005).

The fundamental challenges in the analysis of genetically engineered animal models are the compensatory mechanisms that can mask to a varying degree the effect of the original intervention. This problem was highlighted in the comparisons made between the chronic PLB-KO and three-fold CaMK overexpression models in Publication IV. Intuitively, the end-point phenotypes of these two cases should be rather similar: an enhanced ECC. This discrepancy between the expected and actual findings might be explained by the different roles of the PLB and the CaMK in the regulatory
system. That is, knocking-out PLB affects only one target of the machinery, while overexpression of the CaMK disrupts, or least alters dramatically, the whole regulatory signalling pathway (Publication IV).

In spite of the above described challenges in characterisation of the ECC with genetic engineering, a number of models exist of e.g. increased or reduced expression of SERCA, NCX and CSQN. Also, the effect of the altered PLB to SERCA ratio has been studied extensively, since it has been linked to various heart failure conditions (MacLennan & Kranias 2003). Furthermore, a multitude of animal models of heart failure have been induced by some physical impairment, such as aortic constriction (for a review, see e.g. (Hasenfuss 1998)).

A promising novel modality is the induced transgenesis, in which the genetic intervention can be inflicted acutely with some external stimulus, thus avoiding long-term compensatory changes in the system. This technique can provide significant new data for the elucidation of ECC, as has been already demonstrated with studies focusing on the SERCA and the NCX (see §3.3). Furthermore, that data can be utilised to better validate the mathematical models in conditions reaching beyond normal variation of the physiological state; an important aspect for the predictive ability of the models (see §4.2 and §7.1).

7.5 Mathematical modelling in analysis of ECC

Elucidation of the underlying mechanisms of ECC has already benefitted substantially from mathematical modelling and there is an increasing trend to use this methodology (Fink et al. 2009, Puglisi et al. 2004, Winslow et al. 2005). Especially, the understanding of the complex interaction of LCCs and RyRs in the CICR has gained a lot of detail from the ECC models. For example, in studies of the controversial mechanisms that define the termination of calcium release via the RyRs, highly detailed stochastic models have shed light to the complex processes in a way not possible with the
experimental approach alone (Izu et al. 2006, Williams et al. 2007, Tao et al. 2008). However, such an amount of detail in the model is not always necessary to resolve the role of interconnected regulatory loops of the CICR (Sobie & Ramay 2009). Even a compartmental description of the deterministic ECC models can provide significant insight to e.g. the autoregulation of calcium dynamics in the vicinity of RyRs and LCCs (Publication IV).

The potential of in silico studies lays in the ability to simultaneously observe multiple variables and to compare the underlying processes of cellular function in both physiological and pathophysiological situations. Furthermore, it is possible to estimate such outputs that cannot be measured in experiments. Combining these advantages with in vivo and in vitro analysis of genetically engineered animal models can provide quantitative information about the causalities between myocyte signalling cascades in such mechanistic detail, which would be challenging to reach without the integrative approach (Publication IV).

Finally, for realising the full potential of mathematical modelling in the analysis of ECC, it is essential that the availability and user-friendliness of the models and simulation software reaches such a level that they could be exploited by researchers who are not experts of mathematical modelling themselves but rather involved in experimental work.
8 Conclusions

In this thesis, novel model components have been developed and existing schemes have been adapted to create an integrated cardiomyocyte model in order to describe the excitation-contraction coupling with unprecedented accuracy in adult ventricular myocytes of the mice. The fulfilment of this general goal required: 1) integration of the CaMK-related enzyme network into an ECC model of mouse cardiac myocytes, 2) description of the regulation of CaMK’s targets and 3) development of novel models of calcium transport mechanisms. Successful realisation of these objectives enabled further studies that 4) evaluated the usability of the developed mathematical models in the analysis of genetically engineered animal models and 5) elucidated the role of CaMK in the regulation of the ECC.

Results indicate that the CaMK could have a substantial role as a downstream effector in beta-adrenergic stimulation. In a broader context, the results emphasize mathematical modelling as a valuable complement to experimental work in understanding causal relations within complex biological
systems such as the cardiac myocytes. This is especially true for the analysis of genetically engineered animal models, in which the intended modification is always accompanied by compensatory changes. Naturally, this application imposes demands on the modelling in the sense that the model components should be able to describe accurately the function, even though the system is operating in a state that might deviate significantly from normal physiological conditions.
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Appendix
Appendix I: An example of the Matlab™ code files (m-files).

```matlab
function dy = dy_buffering_SERCA(t, y)

%*******************************************************************************
% DY_BUFFERING_SERCA
% % This model calculates the calcium buffering and uptake by the SR % calcium ATPase as reported in:
% % Koivumäki, J., Takalo J., Korhonen T., Tavi P. and Weckström M. (2008) % Modelling Sarcoplasmic Reticulum Calcium ATPase and Its Regulation in % Cardiac Myocytes. Phil Trans R Soc A.
% % Version number, author and a short description of the changes.
% %-----------------------------------------------------------------------
% % VERSION NUMBER: AUTHOR: DATE:
% %-----------------------------------------------------------------------
% 1.0 Jussi Koivumäki 02.03.2009
% % First published version.
% %*******************************************************************************

%% Get index numbers and create empty vector for equations.
i_Ca_serca  = 1;
i_SIZE      = 1;
dy          = zeros(i_SIZE,1);

%% Global variables for inputs and outputs.
global Ca_i Ca_nsr J_cyt_serca J_serca_sr;

%% Set values for general parameters.
CAcyt       = Ca_i;
CAnsr       = Ca_nsr;
CaMKII_reg  = 0;

%% Set values for SERCA parameters.
SRBUFtot    = 47;
PSR         = 1;
Kmf_PLBKO   = 0.15;
Kmf_PLB     = 0.15;
Kmr_PLBKO   = 2500;
```

97
\[
\begin{align*}
\text{Kmr}_{\text{PLB}} & = 1110; \\
\text{Kmf} & = (\text{Kmf}_{\text{PLBKO}} + \text{Kmf}_{\text{PLB}} \times \text{PSR}) \times (1 + 0.27 \times \text{CaMKII}_{\text{reg}}); \\
\text{Kmr} & = \text{Kmr}_{\text{PLBKO}} - \text{Kmr}_{\text{PLB}} \times \text{PSR};
\end{align*}
\]

%% Calculate transition parameters for SERCA.
\[
\begin{align*}
\text{k}_{\text{cyt_serca}} & = 1000 \times 0.00625 \times (1 + 0.7 \times \text{CaMKII}_{\text{reg}}); \\
\text{k}_{\text{serca_cyt}} & = \text{Kmf}^2 \times 1000 \times 0.00625; \\
\text{k}_{\text{serca_sr}} & = 0.00625 \times (1 + 0.7 \times \text{CaMKII}_{\text{reg}}); \\
\text{k}_{\text{sr_serca}} & = 0.00625 / \text{Kmr}^2;
\end{align*}
\]

%% Calculate calcium fluxes for calcium binding in the cytosol and release in the SR.
\[
\begin{align*}
\text{J}_{\text{cyt_serca}} & = \text{k}_{\text{cyt_serca}} \times \text{CAcyt}^2 \times (\text{SRBUFtot} - \text{y(i_Ca_serca)}) - \ldots \\
& \quad \text{k}_{\text{serca_cyt}} \times \text{y(i_Ca_serca)}; \\
\text{J}_{\text{serca_sr}} & = \text{k}_{\text{serca_sr}} \times \text{y(i_Ca_serca)} - \text{k}_{\text{sr_serca}} \times \text{CAnsr}^2 \times \ldots \\
& \quad (\text{SRBUFtot} - \text{y(i_Ca_serca)});
\end{align*}
\]

%% Calculate concentration of calcium bound to the SERCA.
\[
\text{dy(i_Ca_serca)} = \text{J}_{\text{cyt_serca}} - \text{J}_{\text{serca_sr}};
\]
Appendix II: The timeline of the execution of tasks included in this thesis work.

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<tr>
<th>TASK</th>
<th>2004</th>
<th>2005</th>
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Khan et al. (2008)
Original articles