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The Molecular Mechanisms of the β-Adrenergic Stimulation:

Activation of CaMKII by Nitric Oxide during β_1-Adrenergic Stimulation of Cardiomyocytes Increases the Frequency of Ca^{2+} Sparks

PhD Thesis submitted by

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1.ABBREVIATIONS

$[Ca^{2+}]_i$	Cytosolic Ca^{2+} Concentration
$[Ca^{2+}]_o$	Extracellular Ca^{2+} Concentration
$[Ca^{2+}]_{SR}$	Intra-Sarcoplasmic Reticulum Ca^{2+} Concentration
AC	Adenylate Cyclase
AIP	Autocamtide-2-Related Inhibitory Peptide; CaMKII Inhibitor
AP	Action Potential
CaM	Calmodulin
CaMKII	Calcium/Calmodulin-Dependent Protein Kinase
CICR	Calcium-Induce Calcium Release
EC-Coupling	Excitation Contraction Coupling
eNOS	Endothelial Nitric Oxide Synthase
Forsk	Forskolin
HF	Heart Failure
I_{Ca}	L-type Ca^{2+} Current
iNOS	Inducible Nitric Oxide Synthase
Iso	Isoproterenol
JSR	Junctional SR
L-NIO	L-N(5)-(1-Iminoethyl)Ornithine. e-NOS Inhibitor
LTCC	L-type Ca^{2+} Channel
MI	Myocardial Infarction
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
PKA	Protein Kinase A
P_o	Open Probability
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RyRs	Ryanodine Receptors
SR	Sarcoplasmic Reticulum
V_h	Holding Potential
WT	Wild Type
β-AR	Beta Adrenergic Receptor

2.ACKNOWLEDGMENTS

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3.OVERVIEW

3.1.Abstract

Aims: During β-adrenergic receptor (β-AR) stimulation, phosphorylation of cardiomyocyte ryanodine receptors (RyRs) by protein kinases may contribute to an increased diastolic Ca^{2+} spark frequency. Regardless of prompt activation of PKA during β-AR stimulation, this appears to rely more on activation of CaMKII, by a not yet identified signaling pathway. The goal of the present study was to identify and characterize the mechanisms which lead to CaMKII activation and elevated Ca^{2+} spark frequencies during β-AR stimulation in single cardiomyocytes in diastolic conditions.

Methods and results: Confocal imaging revealed that β-AR stimulation increases endogenous NO production in cardiomyocytes, resulting in NO-dependent activation of CaMKII and a subsequent increase of diastolic Ca^{2+} spark frequency. These changes of spark frequency could be mimicked by exposure to the NO donor GSNO and were sensitive to the CaMKII inhibitors KN-93 and AIP. In-vitro, CaMKII became nitrosated and its activity remained increased independent of Ca^{2+} in the presence of GSNO, as assessed with biochemical assays.

Conclusions: β-AR stimulation of cardiomyocytes may activate CaMKII by a novel direct pathway involving NO, without requiring Ca^{2+} transients. This crosstalk between two established signaling pathways may contribute to arrhythmogenic diastolic Ca^{2+} release and Ca^{2+} waves during adrenergic stress, particularly in combination with cardiac diseases. In addition, NO dependent activation of CaMKII is likely to have repercussions in many cellular signaling systems and cell types.

3.2.Rationale

EC-coupling is the electro-physiological process that governs the contractile functioning of each single cardiomyocyte in the heart. Upon a depolarization of the cell membrane, a small amount of Ca^{2+} from the extracellular medium enters the cell through the L-type Ca^{2+} channels (I_{Ca}) and subsequently triggers a large Ca^{2+} release (which is needed for contraction) from intracellular Ca^{2+} stores, the sarcoplasmic reticulum (SR).

During physical exercise and emotional stress, subsequent β-adrenergic receptor (β-AR) stimulation is enhanced (Bers, 2002), whereby the intracellular Ca^{2+} handling is boosted in response to a signaling pathway which is triggered downstream the β-AR, towards making active a multitude of relevant Ca^{2+} signaling and regulatory proteins (Curran *et al.*, 2007; 2009; Shan *et al.*, 2010; Bovo *et al.*, 2012).

In cardiac myocytes a well organized system of channels and receptors exist designed to deliver the exact amount of Ca^{2+} that is needed to induce an optimal cell contractility and consequently optimal cardiac output. One of these channels is the ryanodine receptor (RyR), which is a tetrameric macromolecular complex that opens in response to high Ca^{2+} concentrations (i.e arising from I_{Ca}) in a process that has been termed "Ca^{2+}-induced Ca^{2+} release" (CICR) (Fabiato, 1983). This is the mechanism which amplifies the small amount of inward membrane Ca^{2+} current by substantial SR Ca^{2+} release to initiate contraction during systole. The resulting global cytosolic Ca^{2+} transient is composed of the summation of synchronized local Ca^{2+} release events commonly referred to as "Ca^{2+} sparks" (Cheng *et al.*, 1993). Ca^{2+} sparks arise from the Ca^{2+} flux from the SR to the cytosol via a few open RyRs.

In recent cardiac physiology research, the Ca^{2+} sparks have been extensively studied. During diastole the frequency of spontaneous Ca^{2+} sparks is considered a read-out of the open probability (P_o) of the RyRs. Correct and synchronized gating of the RyRs between open and closed states is what drives the CICR process and the Ca^{2+} transients (high P_o) and also what determines the differences between systole and diastole (low P_o).
It has been reported in several experimental studies that during beta adrenergic receptor (β-AR) stimulation of cardiac myocytes, the RyRs increase their open probability, (Hussain & Orchard, 1997; Zucchi & Ronca-Testoni, 1997; Curran *et al.*, 2007; Ogrodnik & Niggli, 2010) as observed by the increase in the frequency of diastolic Ca^{2+} sparks, waves and amplitude of the Ca^{2+} transients. For these observations, several mechanisms could be responsible. Interesting for the present study, this could be explained as a result of

activation of cytosolic protein kinases, such as PKA and CaMKII, that target specific sites, as suggested by many studies, including (Hain *et al.*, 1995; Chelu *et al.*, 2009; Grimm & Brown, 2010).

The pathways for and consequences of such post-translational modifications have been extensively studied but remain highly controversial (for review see (Niggli *et al.*, 2012)). A detailed characterization of the machinery which is activated during β-adrenergic signaling towards affecting the RyRs is of great interest, not only for the normal function of the cardiac muscle, but also for the suspected role of the RyRs in various cardiac diseases, such as arrhythmias and heart failure.

An increased open probability of the RyRs, which is observed in some cases of cardiac diseases under acute or sustained catecholaminergic stimulation, has been suggested to be triggered by several different pathological entities. For example, RyRs mutations have been identified in some patients suffering life-threatening catecholaminergic polymorphic ventricular tachycardias (CPVTs) (Yano *et al.*, 2006). Other types of arrhythmias involving RyRs are the delayed afterdepolarization (DADs), which have been linked to an abnormal SR Ca^{2+} leak and Ca^{2+} waves after the repolarization of the action potential (AP) (Kass *et al.*, 1978). Another important condition is heart failure (HF) resulting from a decreased cardiac contractility (Bers *et al.*, 2003). It has been proposed that RyR hyperphosphorylation by the aforementioned protein kinases may lead to abnormally high P_o of the RyRs, elevated SR Ca^{2+} leak and thus low SR Ca^{2+} content. This would ultimately affect intracellular Ca^{2+} cycling and contribute to a weak heart beat (Marx *et al.*, 2000; Bers, 2012; Swaminathan *et al.*, 2012).

Taken together, it is very important to characterize and understand the signaling pathways underlying the changes of RyR P_o during β-AR receptor stimulation. Considering the physiological importance and disease relevance of this burning question, the present study was conceived, designed and carried out.

3.3.Aims

In this study the overarching goal was to determine the molecular mechanisms and signaling pathways that underlie the changes of RyR function and RyR-dependent Ca^{2+} signals during acute β-AR stimulation. To reach this goal, we aimed to study the frequency of openings of clusters of RyRs in-situ, visible as Ca^{2+} sparks, as a readout of the behavior of the RyRs during β-AR stimulation. Further, we wanted to clarify the role and mode of activation of the two most prominent protein kinases implicated in RyR phosphorylation during β-AR stimulation, the PKA and CaMKII and their respective second messengers.

Finally, we wanted to investigate whether the functional changes of the RyRs resulting from β-AR stimulation may contribute to Ca^{2+} induced cardiac arrhythmias.

3.4.Summary

As detailed above, the main goal of the present thesis was to clarify the signaling pathways between β-AR receptor stimulation and changes of SR Ca^{2+} release via RyRs in cardiac muscle. This thesis project was motivated by recent work of Ogrodnik & Niggli (Ogrodnik & Niggli, 2010) which suggested a CaMKII involvement in the increase of Ca^{2+} spark frequency, upon acute β-AR stimulation of voltage clamped guinea-pig ventricular cardiomyocytes. Since these experiments were carried out in resting cells, where the Ca^{2+} concentrations is low, it remained entirely unclear how CaMKII would become activated under those conditions. CaMKII is a protein kinase that is usually modulated by cytosolic Ca^{2+} both by elevated concentrations and frequencies (De Koninck & Schulman, 1998).

In this thesis and the included published paper we present the solution to this interesting question and will detail the experimental steps that lead us to propose an entirely novel intracellular mechanism involved in the activation of CaMKII during β-AR stimulation

To reach our aims we applied electrophysiological methods such as the patch-clamp technique in the whole-cell mode, in tandem with laser-scanning confocal microscopic imaging of fluorescent indicators (e.g. for Ca^{2+}). Using isolated single cardiomyocytes, these techniques allowed us to have precise control of the membrane potential and intracellular ionic conditions (i.e. the Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_i$) and the Ca^{2+} load of the SR ($[Ca^{2+}]_{SR}$). The analysis of Ca^{2+} sparks allowed us to assess the function of the RyRs in situ. Further, Ca^{2+} spark analysis enabled us to examine relevant changes of RyR function arising from their post-translational modification resulting from our pathway of interest. In addition, we used pharmacological tools, to identify involved mechanisms and possible cross-talks between signaling pathways.

In initial control experiments we reproduced the increase in Ca^{2+} spark frequency during β-AR stimulation in resting cardiomyocytes and provided further support for the involvement of CaMKII, using the specific CaMKII inhibitor autocamtide-2-related inhibitory peptide (AIP). These experiments put the previous findings on a firmer foundation, but did not yet answer the question on how CaMKII becomes activated in resting (without evident Ca^{2+} transients) cardiomyocytes.

Further experiments using the nonspecific NO synthase (NOS) inhibitor, L-NAME, suggested that during β-AR stimulation of resting guinea-pig ventricular cardiomyocytes, a pathway that relies on the production of NO becomes activated, since after unspecific nitric

oxide synthase (NOS) inhibition, the increase in Ca^{2+} spark frequency observed in Iso was prevented. Furthermore, similar experiments using the specific neuronal nitric oxide synthase (nNOS) inhibitor AAAN showed that most likely the NO was derived from this synthase isoform. However, a possible involvement of eNOS cannot be discarded, as the compound itself that was used for eNOS inhibition (L-NIO), also triggered unspecific effects which did not allow for correct control conditions (see chapter: unpublished results, the eNOS inhibitor L-NIO).

By using a fluorescent NO indicator (DAF-2), we could provide additional evidence for a NO-dependent mechanism, by showing that NO was endogenously produced in real time by the cardiomyocyte, upon β-AR stimulation with Isoproterenol (Iso).

Experimentally NO can be generated by various nitrosothiols acting as NO-donors (e.g. SNAP and GSNO). If our NO hypothesis were correct, acute application of the NO-donor GSNO would then be expected to elevate the frequency of Ca^{2+} sparks, in the same way as during β-AR stimulation with Iso. This was indeed observed after following exactly the same experimental protocol. Importantly, this spark stimulation was inhibited by AIP, suggesting a link between NO and CaMKII. As a further complication, NO is known to affect RyRs in various ways (e.g. via cGMP and protein kinase G-dependent pathway, but also by direct nitrosation of cysteins on the RyRs) (Takasago *et al.*, 1991; Espey *et al.*, 2006). These modifications of the RyRs by NO would, however, not involve CaMKII. Thus, our observation that AIP was able to prevent the GSNO-induced elevation of Ca^{2+} spark frequency, indicated that NO released by GSNO somehow leads to activation of CaMKII. Thus, it may be that β-AR stimulation leads to endogenous NO production by the resting cardiomyocytes and that this NO directly activates CaMKII, even without detectable Ca^{2+} transients, which are the typical signals usually turning on CaMKII activity.

To examine the interaction between NO and CaMKII more directly, we carried out a set of biochemical *in-vitro* experiments to asses Ca^{2+}-independent CaMKII activation by NO and CaMKII nitrosation. By using an ELISA test for CaMKII activity, we found that acute application of the NO donor GSNO indeed increased its activity by ~16%, similar to the activity induced by adding a know activator, H_2O_2, as it was shown by (Erickson *et al.*, 2008). Furthermore, using a Western blot assay with an antibody specifically targeting nitrosated cysteines, we directly observed that CaMKII becomes more strongly nitrosated by exposure to the NO donor.

Taken together, the main findings of this study provide compelling evidence that during β-AR stimulation of cardiac myocytes, a significant production of NO occurs and directly activates CaMKII to increase the Ca^{2+} spark frequency and the P_o of the RyRs.

Most likely, this occurs by phosphorylation of the RyRs by CaMKII. This novel mechanism of CaMKII activation may play an important role during cardiac physiology and disease, but may also have repercussions reaching far beyond our field of research.

3.5.References

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4.INTRODUCTION

4.1.Architecture of the heart

The principal function of the heart is to pump blood through the body. The four main parts of the cardiac muscle that are involved in this process are the left and right atria and ventricles. The right heart (i.e. right atrium and right ventricle) collects deoxygenated venous blood that has been previously used to provide oxygen, nutrients and other substances the muscles, brain and other organs for their optimal functioning. In contrast, the left heart is involved in delivering freshly oxygenated blood coming from the lungs through the vasculature to repeat the above-mentioned process. To achieve the mechanical pulsatile contractions of the muticellular cardiac muscle in a synchronized way, the heart makes use of an electrical network of specialized muscle cells (a.k.a. the cardiac conduction system) to spread an electrical signal that induces contraction. The pulsatile mechanical activity is composed of the contraction (systole) and relaxation (diastole). The synchronized rhythm is imposed by the sinoatrial node (SA node), which acts as the main pacemaker. Once SA node cells reach an electrical threshold, they fire a membrane depolarization (action potential, AP) that travels from cell to cell to activate the atrial cardiomyocytes, until it reaches the atrioventricular node (AV node). At this part of the heart electrically connecting the atria with the ventricles, the electrical impulse is delayed by the AV node by bout 120 ms, which will allow the atria to have ejected blood into the ventricles before they contract. After passing the AV Node, the electrical impulse reaches the Bundle of His of the conduction system, which transmits the electrical signal to the Purkinje fibers.

The Purkinje fibers, which are specialized cardiomyocytes that transmit the action potentials very quickly, deliver the electrical signal homogeneously to depolarize the cells of the ventricles to provide a synchronized contraction. For a correct functioning of the electrical network of the heart, it is also important that the re-polarization of the ventricles occurs in a regular and well coordinated fashion. Otherwise, this system can easily

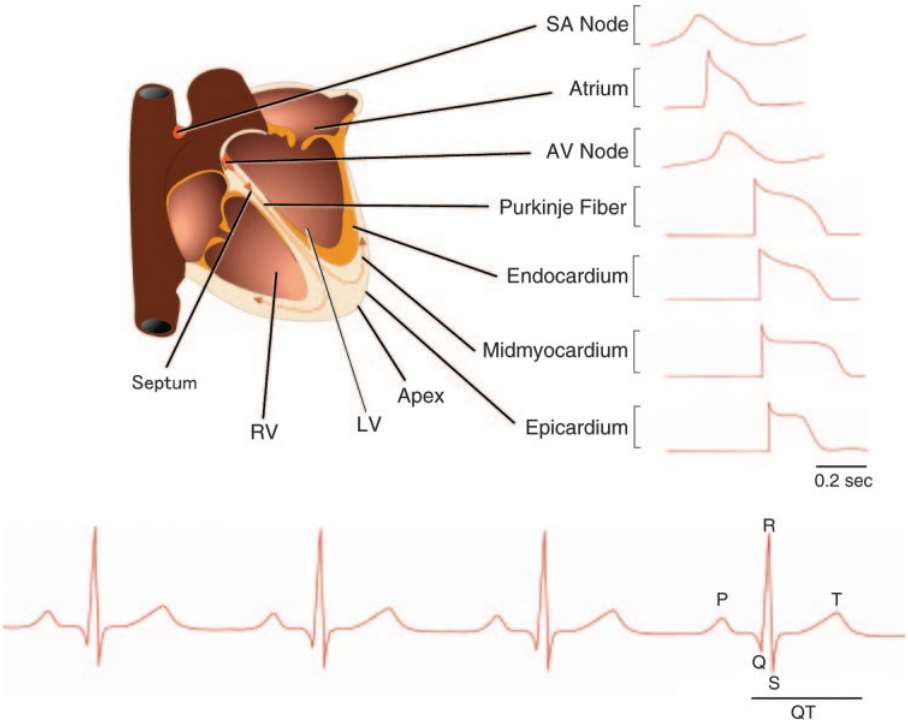


Fig 1: The top panel shows a transversal section of the heart and its 7 regions of the electrical conduction, through which the AP has to go in order to synchronize excitation and contraction. The lower panel shows the electrical activity of the heart when analyzed with electrodes in an electrocardiogram (a.k.a. ECG). This recording shows the depolarizing and re-polarizing phases of the heart between beats (4 beats in this case are shown) and the waves and intervals within a single depolarization are described with the letters P, Q, R, S, T. From (Nerbonne & Kass, 2005).

become de-synchronized and generate arrhythmias.Unfortunately, in many cardiac diseases the coordinated activation of the entire cardiac muscle can be disturbed. Sometimes, the ventricles do not keep up with the heart rhythm and generate unwanted beats (i.e. extrasystoles) which can be harmful. In the following chapters a complete description of the mechanisms that govern the cardiac functioning at the cellular level and how small changes could influence the overall heart behavior will be presented. For a more detailed description please refer to the monograph “Excitation-contraction coupling and cardiac contractile force” by DM. Bers (Bers, 2001)

4.2.Cardiomyocyte ultrastructure and ion channels

4.2.1. Cardiomyocytes: The functional units of the cardiac muscle

The heart muscle tissue is composed of small cardiomyocytes that are electrically and mechanically coupled through gap junctions. In addition, the tissue contains the cells of the vasculature, the connective tissue and immune system.

In order to release the cardiomyocytes from the rest of the connective tissue, it is usually apply a cocktail of enzymes that digest the extracellular matrix; this also helps the cardiomyocytes to separate from each-other in single units. When this happens the gap junctions close and the environment become independent from the rest of the neighboring cells –that at this point rest in a supernatant where survival conditions are allowed. (For a better description of the isolation and solutions see the section 8. “Methodology”)

4.2.2. The cardiomyocyte

In this first part of the introduction (section 4.2.), we will mainly focus on structural features, from the level of cells to the involved molecules. The functions on the cellular and molecular level will be detailed later (sections 4.3.-4.9.).

In most species, the size of a ventricular cardiomyocyte is about 100-120 µm in length, 30-50 μm in width and 20-30 μm in thickens. Cardiomyocytes exhibit a rod-shape and a well defined geometry, which allows them to contract in a symmetrical fashion (see Fig 2). Each isolated cardiomyocyte is fully functional in terms of electrical and mechanical activity and therefore represents a nearly ideal experimental preparation for many studies. The unit of the contractile machinery of this type of cells is the sarcomere. This is a ~2 µm long arrangement of contractile proteins, such as actin and myosin filaments.

The morphology of these cells evolved displaying an arrangement of invaginations (t-tubules, see Fig 3 which are aimed to internalize (but still in the extracellular space) the necessary amount of ions present in the extracellular medium as well as to deliver the action potential (AP) homogeneously throughout the cell.

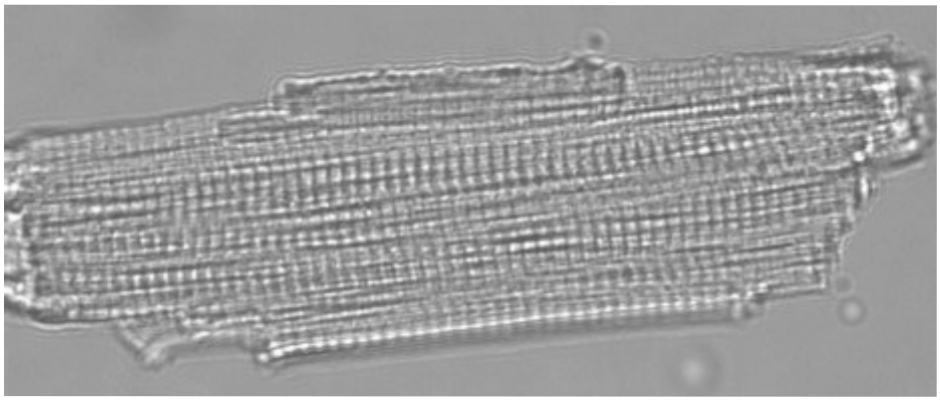


Fig 2: Typical isolated ventricular cardiomyocyte seen under light microscopy. Usually they are rod-shaped and about 100 µm long. Sarcomeres can be identified as striations across the cell. Taken from (Sokolova et al., 2009).

This medium is the interstitial fluid, which by providing an assortment of ions and other constituents, allows for the electrical activity and mechanical contraction of the cardiomyocytes, all by making use of membrane channels, receptors and the electrochemical gradients of the cells.

With the help of different imaging techniques such as electron, atomic force and light microscopy, it has been possible to observe and study the arrangement of the aforementioned structures and depict how they work together (Fawcett & McNutt, 1969).

The cardiomyocyte contains several organelles. One-third of the cell volume is filled with mitochondria, which generate the high amount of ATP consumed by the continuously beating cardiomyocyte. The cells are mono-nucleated, but can become bi-nuclated as a possible indication of terminal differentiation and/or hypertrophy (Anversa & Kajstura, 1998; Jonker *et al.*, 2010). The Ca^{2+} storage organelle, the sarcoplasmic reticulum (SR), contains a large amount of Ca^{2+} which is released to the cytosol for contraction in a process known as Ca^{2+}-induced Ca^{2+} release (CICR). Once the Ca^{2+} is released to the cytosol, it binds to an arrangement of proteins that move upon Ca^{2+} binding, leading to the observed cell twitch contraction (see the section 4.4. “Excitation Contraction Coupling” for details).

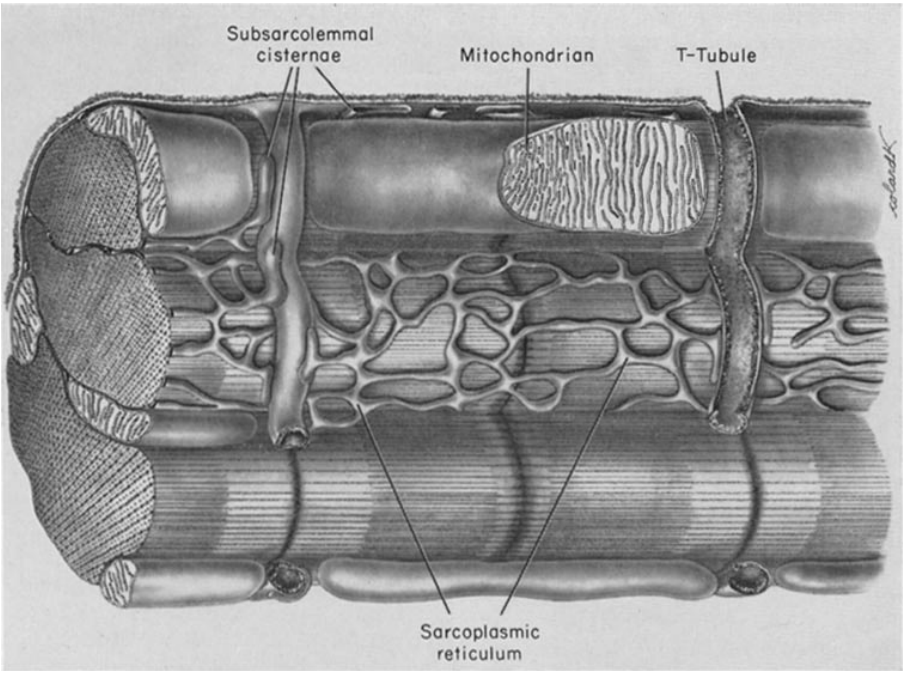


Fig 3: This scheme represents a 3-D reconstruction of the structure of a mammalian cardiomyoctye observed with electron microscopy. Here the internal arrangement of the T-Tubules can be observed which invaginate the cell carrying Ca^{2+} and supplies for physiological processes. In close opposition to them, the sarcoplasmic reticulum (SR) is found (including sub-sarcolemmal cisternae), which act as the Ca^{2+} store of the cardiomyocyte The SR is involved in cell contraction. Promient organelles are the mitochondria which serve as a source of energy. Taken from (Fawcett & McNutt, 1969).

4.2.3.Extracellular medium

The blood serum and the interstitial fluid are rich in both ions that are necessary for the electrical homeostasis of cells and hormones and nutrients (including oxygen), which provide sources of energy and molecules that regulate cell behavior.

For experiments with tissues and isolated cells, a simplified physiological solution is commonly made in an approach to mimic such a vital fluid (for concentrations, see section 8.2. “Solutions” and (Mitra & Morad, 1985*a*)). This extracellular solution provides what the cells need for survival, but most importantly keep the electrical resting potential constant. Unless the cells are under voltage-clamp conditions, the resting potential results from the equilibrium between the ionic concentrations (defining the electrochemical gradients between the internal and external milieu of the cell) and the prevailing membrane conductances, mainly for K^+.

Principally, outside the cell a high concentration of Na^+ (~140 mM) is necessary to induce an acute membrane depolarization, via voltage-dependent Na^+ channels. Extracellular Na^+ and its gradient are also involved in many trans-sarcolemmal transport processes (e.g. Na^+-Ca^{2+} exchange, Na^+-H^+ exchange). Ca^{2+} is present at around 2 mM, 10'000 times more than in the cytosol of the resting cell. Its main task, once inside the cardiomyocytes, is to induce CICR and contraction, but first Ca^{2+} has to cross the cell membrane through the voltage-dependent L-type Ca^{2+} channels.

The extracellular K^+ concentration is low (~5 mM) when compared to the intracellular milieu (~145 mM). This gradient is responsible for the repolarization at the end of the AP (via several types of K^+ channels) and for the resting potential, which is near the Nernst potential for K^+ (~ -80 mV).

Other important substances found in the experimental extracellular medium include glucose, which serves as a source of energy, magnesium and pH buffers. In the following chapters it will be addressed the conditions that are necessary to provide such a homeostasis (see chapter 8.2. "Solutions").

4.2.4.Cellular membrane

This lipid bilayer is formed mainly by amphipathic phospholipids, which spontaneously arrange their hydrophobic tails towards each-other and at the other end the hydrophilic heads, which are in contact with the solution outside and inside the cell, respectively. In cardiomyocytes the cell membrane is usually referred to as sarcolemma or plasmalemma. Its primary function is to provide a separation between the extracellular medium and the inner milieu.

A multitude of transmembrane proteins are located in the lipid bilayer (channels, transporters, receptors, cytoskeletal proteins). These are the tools the cell uses to communicate between the interior and the exterior. Several of them serve as "switches" to modulate cell function.

An important characteristic of the cell membrane is that it works as a biological electrical capacitor, meaning that charges accumulate to either side and since this membrane is very thin (~ 6 nm), the membrane capacitance is quite high (~1 pF/μm^2). A prominent characteristic of the cardiomyocyte sarcolemma is that it is flexible (and

extensible) due to membrane folds (Lorin *et al.*, 2013). This can make the patch-clamp technique somewhat challenging to use.

4.2.5.T-tubules

The transversal tubular structures (T-tubules) correspond to an arrangement of cell membrane invaginations spaced at around 2 μm which aim to reach the cell center so that the electrical depolarization and extracellular Ca^{2+} can be delivered inside the cell in a more homogeneous fashion. Mammalian ventricular cardiomyocytes have ~ 30 - 50% of the sarcolemmal surface as T-tubules (Soeller & Cannell, 1999).

About 75% of the L-type Ca^{2+} channels are located in the T-tubules, in close proximity to the RyRs. The narrow intracellular space (~15 nm) between T-Tubules and SR has been defined as “dyadic cleft”, which forms a signaling micro-domain where the Ca^{2+} levels rise sufficiently during the CICR process, so that RyRs activation can be ensured. In chapter 4.2.6 this topic will be discussed more in detail.

The t-tubules and the high density of L-Type Ca^{2+} channels that are located there permit that the cell membrane can have a homogeneously distributed Ca^{2+} influx. Without T-tubules, the Ca^{2+} signals would spread by Ca^{2+} diffusion from the periphery towards the central axis of the cell, similar to atrial cells, which often lack T-tubules.

T-tubules are also known to become disorganized in several cardiac diseases causing cardiac cellular remodeling. (Heinzel *et al.*, 2008) Remodeling and even complete loss of T-tubules of cardiomyocytes in cell culture occurs because of de-differentiation after 24 hours. This results in a loss of the EC-coupling machinery and therefore studies of this mechanism cannot be performed on cultured adult cardiomyocytes.

4.2.6.The sarcolemmal L-type calcium channel

The L-type Ca^{2+} channels (LTTC, also known as dihydropyridine receptors (DHPRs)), are composed of several subunits (Kamp & Hell, 2000) (see Fig 4). Dihydropyridines are a class of pharmacological inhibitors of these channels. The channel forming subunit (α_1) with a molecular weight of 190 kD has four homologous transmembrane repeats (I - IV).

Each containing six transmembrane segments (S1–S6). The α_1 subunit shapes the Ca^{2+} selective pore.

The S4 segments contain the voltage sensors which control channel gating. They are formed by positively charged arginine and lysine residues. The β subunits are relevant for localizing and targeting the channel complex towards the surface membrane as well as in modifying its gating properties.

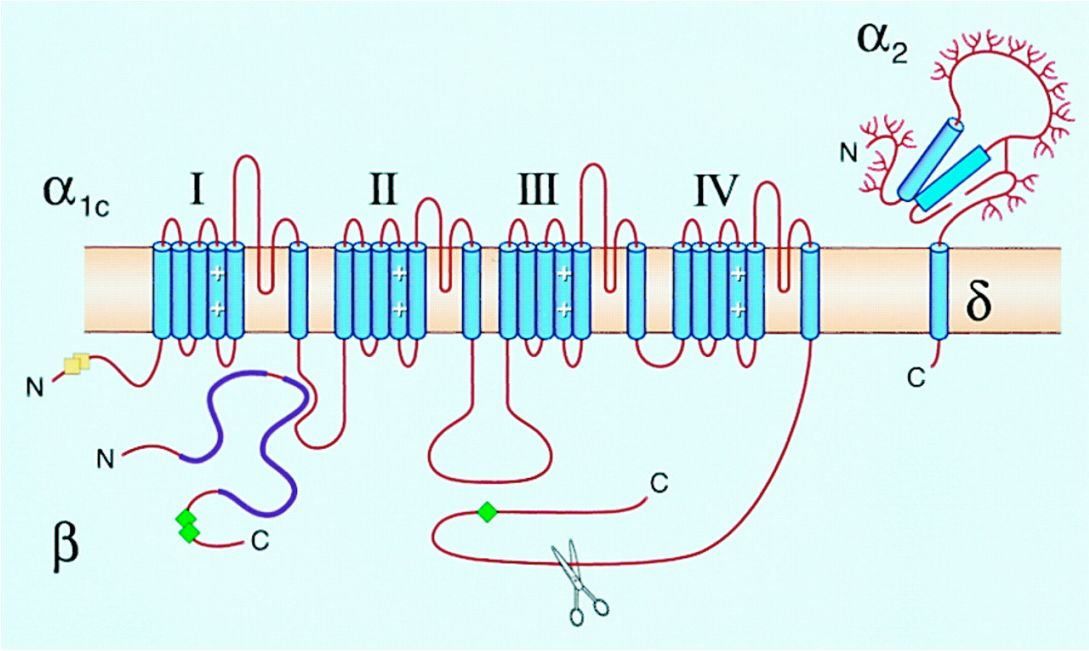


Fig 4: Schematic representation of the transmembrane topology and subunit composition of the cardiac L-type Ca^{2+} channel. From (Kamp & Hell, 2000).

The α_2-δ subunits are linked to each other via a disulfide bond and have a combined molecular weight of 170 kDa. The α_2 subunit is an extracellular glycosylated protein which interacts with the α_1 subunit. The function of the δ subunit is to act as an anchor to the protein in the plasma membrane (Bichet *et al.*, 2000; Kamp & Hell, 2000).

4.2.7.The sarcoplasmic reticulum (SR) and the SERCA

The SR occupies only 3% of the cardiomyocyte volume (Bers, 2001). It is filled with the Ca^{2+} binding protein calsequestrin (CASQ), an intra SR Ca^{2+} buffer, which allows to hold the necessary amount of Ca^{2+} to activate contraction upon release. It has been shown that the SR increases in volume upon deletion of this intrinsic Ca^{2+} buffer (Knollmann *et al.*, 2006). The SR forms a network with several components. Some parts contain clusters of RyRs (for details of their structure see 4.2.6) and embrace the T-tubules (junctional SR). The arrangement of these structures is vital for the EC-coupling mechanism as it supports the homogeneous Ca^{2+} release as the L-type Ca^{2+} channels opens. Other parts of the SR form the commonly known “free” SR, which is a region that is closer to the Z lines and contains a high density of SR Ca^{2+} ATPase (Ca^{2+} pumps, a.k.a. SERCAs). Parts of the SR network close to the release sites are known as the junctional SR and contain clusters of RyRs, to allow a for a fast release of Ca^{2+} (Inui *et al.*, 1988). Besides CSQ, the RyRs and SERCAs, many more proteins have been identified in the SR membrane and lumen. Some of them have been proposed to play a role in Ca^{2+} signaling. Triadin (32 kDa) (Marty *et al.*, 2009) and junctin (26 kDa) (Hong *et al.*, 2002) are small SR transmembrane proteins and have been implicated in the regulation of the RyR by SR luminal Ca^{2+} and by CSQ (Györke *et al.*, 2004).

By using ATP the SERCA is able work against the chemical gradient of Ca^{2+} ions to maintain a high end diastolic concentration of Ca^{2+} inside the SR, which is needed to initiate the next contraction. The SERCA activity is modulated by its natural inhibitor phospholamban (PLB), which is itself inhibited by PKA-dependent phosphorylation, hence contributing to the positive lusitropic effect during adrenergic stimulation.

The SERCA activity is also controlled by a PLB homologous membrane proteins called sarcolipin (SLN). This protein locks the Ca^{2+}-ATPase in a conformation with exposure of the Ca^{2+} sites open towards the cytoplasmic side, this process is stabilized by Mg^{2+}. Fig 5 shows the structure of this proteins and their sites of interaction (Winther *et al.*, 2013). The SERCA is formed by 4 main functional domains, which participate in the regulation of the Ca^{2+} transport: nucleotide-binding (N), phosphorylation (P), actuator (A) and transmembrane (TM) as shown at the right side of Fig 5.

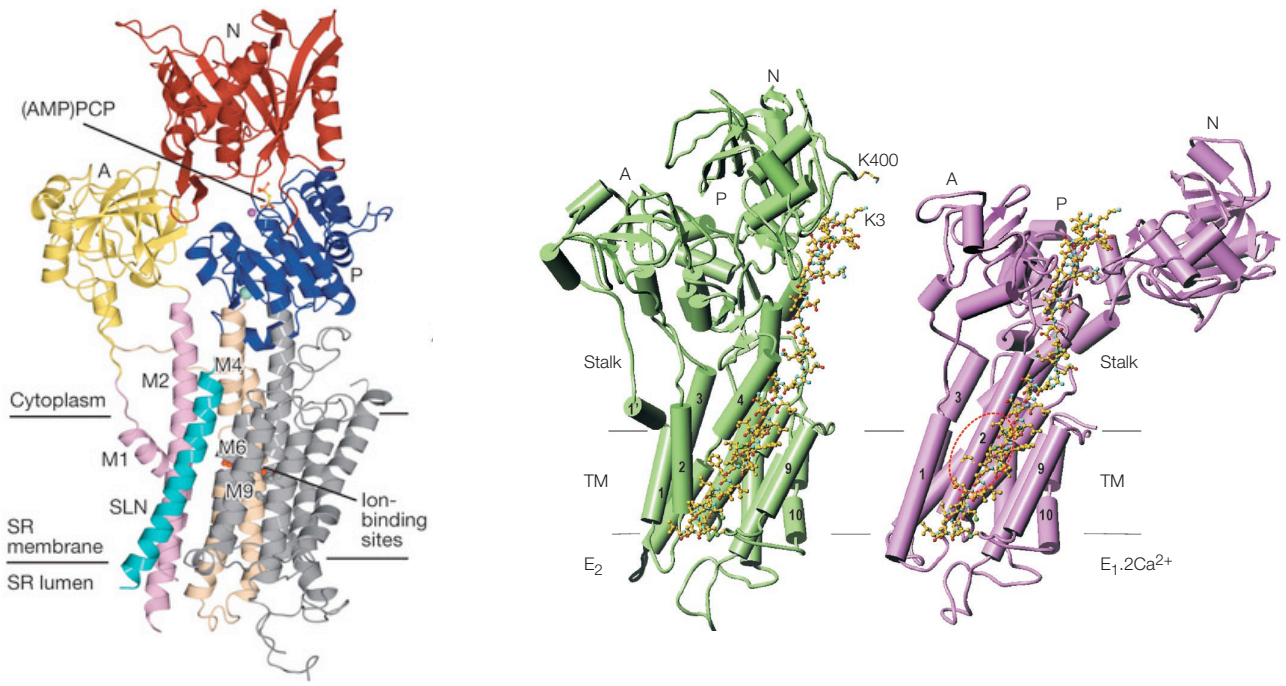


Fig 5: structural model of the sites of interaction between PLB and SERCA. **Left**. Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) which shows the transmembrane helices M2, M6 and M9 of SERCA, Mg^{2+} in magenta and K^{+} as green spheres. The β- and γ-phosphates of AMPPCP and the Ca^{2+}-binding-site residues are shown as ball and stick, adapted from {Winther:2013ve}. **Right**. Structure of SERCA and phospholamban interaction. The domains of SERCA shown are the Ca^{2+}-binding transmembrane domain (TM), the stalk domain connecting cytosolic domains to the transmembrane domain (stalk), the actuator domain (A), the phosphorylation domain (P) and the nucleotide-binding domain (N). K400 is lysine 400 in SERCA1a and K3 is lysine 3 in PLN. (MacLennan & Kranias, 2003).

Two conformations of SERCA1a interacting with phospholamban (PLN) (E_2 and $2Ca^{2+}$ E_1) are also shown in Fig 5. The E_2 conformation of SERCA1a displays a groove, which allows for the carboxy-terminal transmembrane helix of PLN to fit into this groove and increase the probability to form interaction sites with amino acids in transmembrane helices M2, M4, M6 and M9. Unrolling of the carboxy-terminal end of the amino-terminal helix allows the kinases to access the phosphorylation sites at serine 16 and threonine 17.

During the $2Ca^{2+}E_1$ conformation, movement of M2, narrows the transmembrane groove. The PLN–SERCA interaction in this region is gone and PLN remains alone on the surface of the SERCA transmembrane domain, stoping PLN from inhibiting the enzyme (MacLennan & Kranias, 2003).

4.2.8.The ryanodine receptor

The identification of the alkaloid ryanodine and its role in skeletal and cardiac muscle has helped to identify the role of the SR calcium release channel known today as the ryanodine receptor, RyR. The alkaloid ryanodine has a dual control of the RyRs open probability; at low concentrations (1 nM - 10 µM) it locks the channel in a sub-conducting open state and completely blocks the channel at very high concentrations (> 100 µM) (Meissner, 1986).

This macromolecular complex of about 565 kDa per monomer (2.26 MDa per tretramer) can conduct a Ca^{2+} current of ~0.4 pA in periods of about 4 ms at physiological conditions (Mejía-Alvarez *et al.*, 1999).

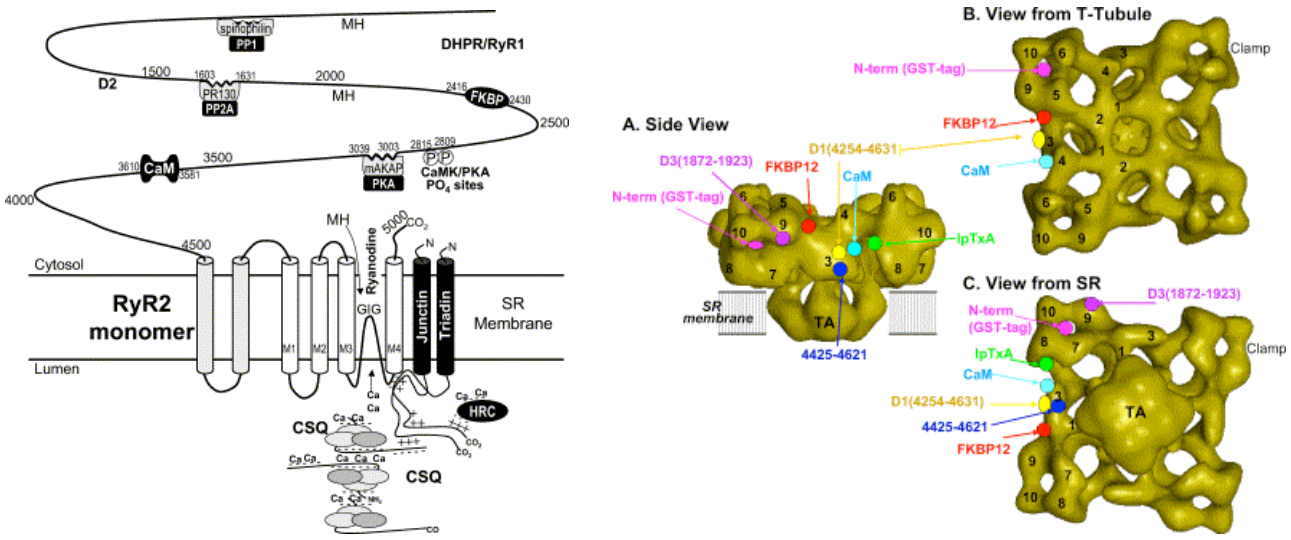


Fig 6: Left: This schemes represent the binding sites and transmembrane domains of the cardiac RyR. We find the scaffolding structure composed by monomers in the SR membrane, the luminal Ca^{2+} buffers and the cytosolic binding sited that regulate the channel. **Right:** 3-Dimensional structure of the WT RyR. TA stands for transmembrane assembly and is located in the SR membrane. The large portion of the molecule spans the dyadic cleft. The numbers represent binding sites of the receptor to other cytosolic proteins. Overall, each RyR tetramer measures 29X29X12 nm (Hayashi *et al.*, 2009). Figure taken from (Bers, 2004).

The regulation of this channel is thought to be mainly driven by the amount of Ca^{2+} in the SR and by various post-translational modifications. The stimulus for its activation is delivered by the L-type Ca^{2+} channel, as I_{Ca}.

There are several accessory proteins associated with the macromolecular complex, partly with modulatory functions. Calstabin-2 (a.k.a. FKBP-12.6) appears to stabilize the channels and couple their gating (Marx *et al.*, 2000). The mAKAP, a PKA anchoring protein complex has the function of binding to the regulatory subunit of the protein kinase A (PKA).

For posttranslational modifications, several sites for PKA- and CaMKII-dependent phosphorylation have been identified on the channel (for reviews see (Valdivia, 2012)). Similarly, a variety of residues can undergo oxidation (Hidalgo *et al.*, 2008; Sun *et al.*, 2008) and/or S-nitrosation. Modification of these sites has recently been considered as the functional mechanisms that are involved in several cardiac diseases (Li *et al.*, 1997; Gonzalez *et al.*, 2010; Anderson, 2011).

In the following sections a more detailed description of this SR membrane Ca^{2+} channel will be discussed.

4.2.9.Na^+ / Ca^{2+} exchanger

The Na^+/Ca^{2+} exchanger is the main Ca^{2+} extruding mechanisms that cardiac cells use to remove the excess of cytosolic Ca^{2+} from beat to beat (Bers, 2002). This electrogenic protein consists of 970 amino acids with a molecular mass of 110 kDa and is located in the sarcolemma, has a relatively low affinity for binding Ca^{2+} (~1 μM) and exchanges 3 Na^+ ions for every Ca^{2+} ion.

The NCX is modeled to be composed of 9 transmembrane helices, with a large intracellular loop (see Fig 7). There is a 20–amino acid segment, designated the XIP region at the N-terminal end of this loop; it contains a regulatory binding site for Ca^{2+} and calmodulin and the also for exchange inhibitory peptide (XIP). The putative loop of the α-1 and α-2 have been proposed to modulate the affinity for Ca^{2+} as mutations in this region down-regulated the transport of the protein therefore it is believed that the cytoplasmic loop regulates the NCX activity.

In the main cytoplasmic loop of the NCX1 structure, there are two 70 amino acid internal repeat motifs, called the β repeats, the β-1 and β-2 domains, which are suggested

to be involved in the regulation of the Ca^{2+} removal from the regulatory site, as a structural change was observed after Ca^{2+} binding to this region. Finally, regulation of the NCX function by phosphorylation and/or oxidation has been reported, but is not yet firmly established (Shigekawa & Iwamoto, 2001).

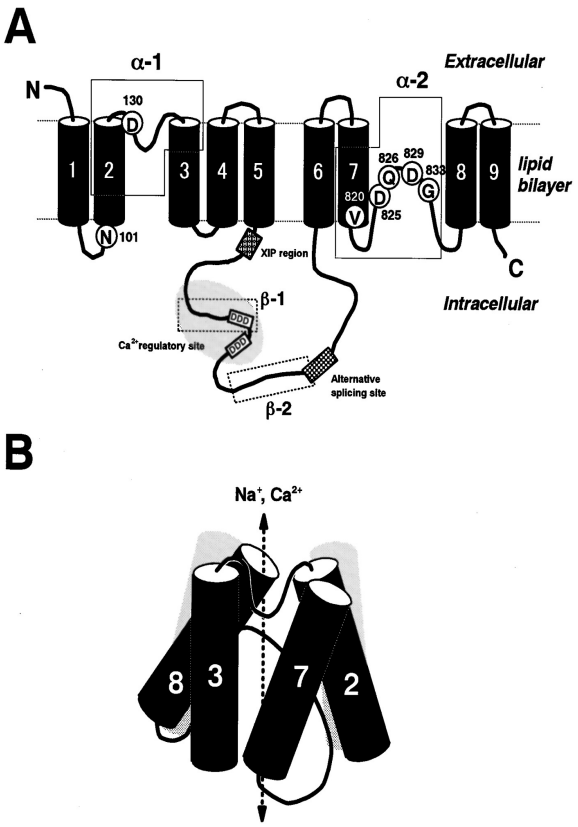


Fig 7: Topological representation of the sodium-calcium exchanger (NCX). A) Nine transmembrane helices indicated by cylinders with numbers from 1 to 9. N and C indicate N- and C-terminals. B) Model for helix packing of the trans membrane domain 2, 3, 7, and 8 suggested by cross-linking data. Taken from (Shigekawa & Iwamoto, 2001).

4.2.10.Contractile Machinery

Upon Ca^{2+} release from the SR to the cytosolic environment, binding to the myofilaments activates cellular contraction. The Ca^{2+} sensor of the myofilaments to trigger contraction is the protein troponin. The contractile complex which is formed by actin, myosin, tropomyosin and troponin I, C and T, displays a less “packed” or more loose

conformation. Upon binding of Ca^{2+} to troponin C, troponin undergoes a conformational change that moves tropomyosin away from the myosin binding sites on actin, which favors the interaction between actin and myosin. Energetically speaking, this interaction between actin and myosin (upon Ca^{2+} binding to troponin C), increases the ability of the myosin ATPase to hydrolyze ATP. This process liberates a phosphate (P_i) and as a consequence a transition called “power stroke” occurs, where a complex of myosin and ADP induces a head rotation and consequent movement of the myosin head and actin filament. The magnitudes observed in filament contraction has been characterized to be around 5-10 nm which corresponds to a 0.25% to a 0.5% of the sarcomere length (Naber *et al.*, 2007; Stern, 1992).

Upon ADP dissociation the affinity of actin and myosin increases, while it becomes very low upon ATP re-association to the complex, which ends up by crating relaxation as the Ca^{2+} levels in the cytosol decline. The filaments and proteins involved in this process are shown in Fig 8.

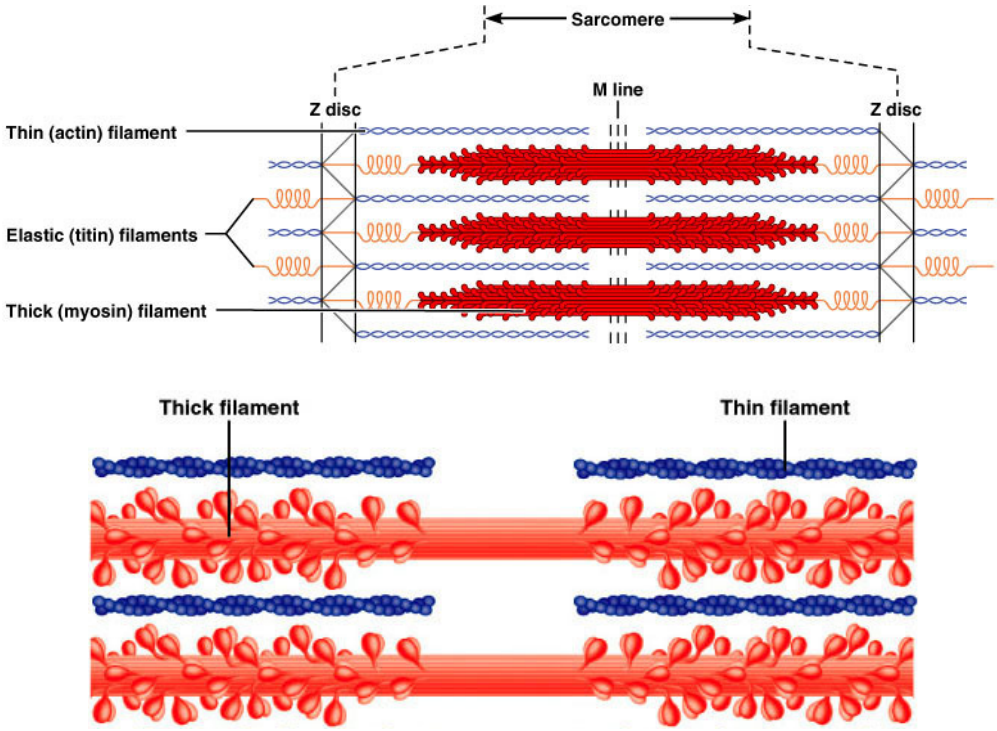


Fig 8: Representation of the composition of the sarcomere as a unit of cardiomyocyte contraction. The M line is the sarcomere center, the Z discs are the sarcomere boundary and the thick and thin filaments interact to produce contraction. Taken from (Marieb & Hoehn, 2007).

4.2.11.Nitric oxide Synthases

Nitric oxide (NO) can be synthesized in the cardiomyocytes by three different nitric oxide synthase isoforms (NOSs). NO is a rapidly diffusing gaseous intracellular reactive signaling molecule. NOSs have a molecular weight of around 110 - 160 kDa (Groves & Wang, 2000), they catalyze the oxidation of the substrate l-arginine to form NO and l-citrulline. In healthy cardiomyocytes two NOSs are present and able to synthesize this molecule, the neuronal NO synthase (nNOS) and the endothelial NO synthase (eNOS). A third synthase, the inducible nitric oxide synthase (iNOS), is mainly expressed upon inflammation or infection (Umar & Laarse, 2009), therefore is not involved in any rapid signaling, but rather active during chronic effects.

The nNOS is located in close proximity to the RyRs on the SR membrane. Some studies have shown that these two proteins could work together forming an interacting functional unit, as they co-immunoprecipipate (Barouch *et al.*, 2002).

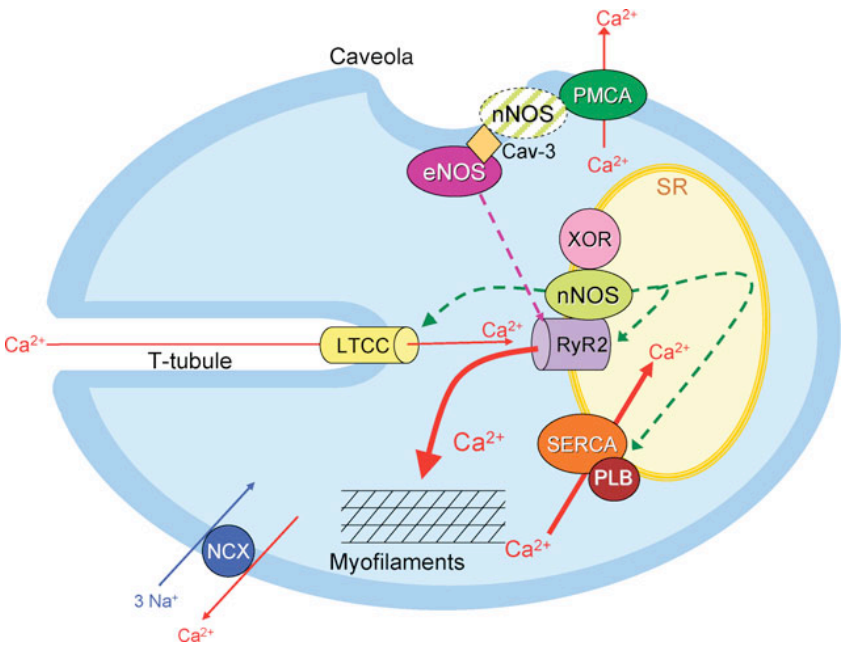


Fig 9: This figure represents the location of nNOS in the state where it is bound to the sarcoplasmic reticulum, or in situations favoring the migration towards the sarcolemmal caveolae, (i.e after myocardial infarction). It also shows the eNOS, another NO synthase. Taken from (Lim *et al.*, 2007)

Recent studies have shown that in certain situations, the nNO synthase is able to migrate to the cell membrane to co-localize with caveolin-3, for example after myocardial infarction (MI) (Bendall *et al.*, 2004). Such a translocation can have serious implications in the regulation of the LTCC by NO-dependent inhibition (Sears *et al.*, 2003; Lim *et al.*, 2007). The nNO synthase in co-localization with the membrane could decrease micro-domain NO release at the SR vicinities and disrupt physiological RyR regulation (Lim *et al.*, 2007; Lima *et al.*, 2010).

Furthermore, it was also proposed that the eNOS is involved in the regulation of the β_2-AR through the activation of the protein kinase AKT (a.k.a. PKB) (Lima *et al.*, 2010). Complementary, other studies demonstrated that eNOS participates in the regulation of enhanced EC-coupling observed when mechanically stretching the cardiomyocytes (Petroff, 2002).

Therefore, the characterization of this molecule and its production upon physiological demands has to be clarified and better understood to extrapolate such experimental results in single cells to the whole organ and especially under conditions of cardiac disease.

4.3.Action potential in Guinea-pig ventricular myocytes

4.3.1.Na+-dependent phase 0

In cardiac muscle cells, the arrival of the AP causes opening of the fast voltage-dependent sodium channels, letting Na^+ ions enter the cell in the form of an inward sodium current, I_{Na}. For the initiation of phase 0 of the AP as it is shown in Fig 10, it is very important that the membrane potential is at resting voltages (around -80 mV) so that the availability of the Na^+ channels to open is high.

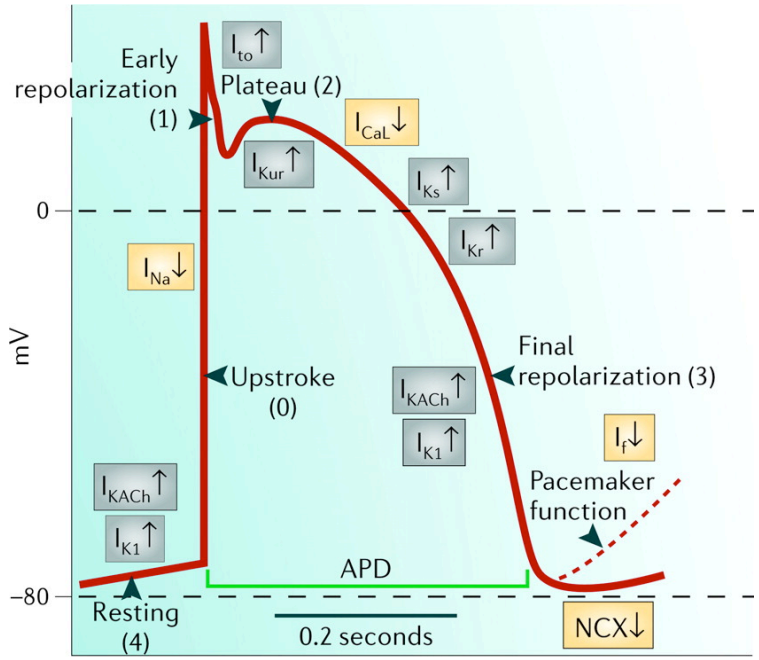


Fig 10: Representation of the cardiac action potential. Resting (phase 4), upstroke (phase 0), early repolarization (phase 1), plateau (phase 2), and final repolarization (phase 3) correspond to the phases of the action potential which take place in about 200 ms. The red doted line represents the behavior of the AP in pacemaker cells. Taken from (Grant, 2009).

In the case of depolarized resting voltages (~ -60 to -50 mV) the cells could have fewer channels available and therefore a smaller I_{Na}. Such a condition is known to produce delayed impulse conduction in the heart and arrhythmogenicity.

As it will be described in the section “8. Methodology” later on, the Na^+ concentration outside the cell is 140 mM, compared to 10 mM in the cytosol. The reason for this difference is that the Na^+ ions need to move rapidly into the cell to induce a change in membrane potential. This electrochemical gradient is a form of energy that is used by the cell to move ions across its membrane.

4.3.2.K^+ and Cl^--dependent phase 1

Upon inactivation and closure of the Na^+ channels due to the depolarization of the membrane potential, in many species a cardiac transient outward potassium current (I_{to1}) activates and contributes to a small membrane re-polarization. This current is accompanied by a Ca^{2+}–dependent Cl^- current (I_{to2}) which is probably more important than the first one, as is recovers faster from inactivation and could play a more significant role in the observed fast re-polarization of the phase 1 of the action potential during physiological cardiac rates (3-4 Hz) observed in rabbit atrial cardiomyocytes (Wang *et al.*, 1995).

4.3.3.Ca^{2+} and K^+-dependent phase 2

At this point of the AP, the L-type Ca^{2+} channels become activated and open to allow Ca^{2+} ions to enter the cell during flow of I_{Ca}, which besides contributing to the CICR process also sustains the depolarizing plateau. The plateau level is governed by the balance of LTCC and by an outward movement of K^+ through the slow delayed rectifier potassium channels I_{Ks}. The (L) in L-type Calcium channels (LTCC) stand for “long lasting” referring to the length of activation.

4.3.4.K^+-dependent phase 3 & 4

During this rapid re-polarization phase, the L-type Ca^{2+} channels finally close due to inactivation, while the slow delayed rectifier K^+ channels (I_{Ks}) still remain open. During the end of the phase 3, the inward rectifying K^+ channels (I_{Kr} and I_{K1}) take over by inducing a net outward positive current which brings the cells back to the negative resting potential (around –80 mV).

Once the cells have reached their resting potential (phase 4) the cell is ready for the arrival of the following depolarization. In some cases of cardiac diseases this phase 4 is altered by delayed afterdepolarizations (or DADs) which would induce an after-contraction usually resulting from an SR Ca^{2+} leak leading to NCX activation and an inward I_{NCX}.

Except for pacemaker cells, this cardiomyocytes (atrial or ventricular) exhibit a flat phase 4 of their action potential. The main mechanism for this relies on the Na^+, K^+-ATPase, the I_{Kr} and the NCX, which keep the electrochemical gradient of the cell constant.

4.4.Excitation contraction coupling (ECC)

In terms of membrane depolarization and triggering of cardiac contraction, EC-coupling is the process that governs contraction and relaxation of the cardiomyocytes and hence the whole heart. It comprises electrical excitation of the cell membrane, activation of I_{Ca}, SR Ca^{2+} release for contractile activation and re-adjustment of resting sytosolic Ca^{2+} levels for relaxation. Following, these processes are presented in more detail.

4.4.1.The SR, couplon, dyadic cleft

The Ca^{2+} concentration inside the SR, the cellular organelle storing Ca^{2+}, is estimated to be around 1 mM. This organelle only takes few milliseconds during systolic Ca^{2+} releases to partially deplete its content to about a 50% and release Ca^{2+} to the cytosol to initiate contraction (Guo *et al.*, 2007). Inside the SR, calsequestrin (CSQ) participates in the buffering and possibly also sensing of SR Ca^{2+} content. The proteins triadin and junctin have been proposed to modulate the function of the RyRs depending on the extent to which CSQ buffers or senses Ca^{2+} (Györke *et al.*, 2009).

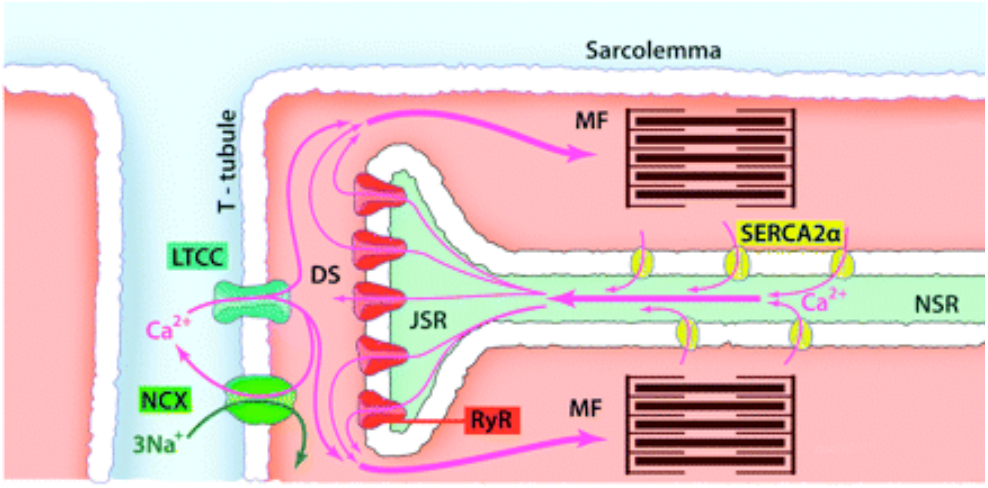


Fig 11: Model of the cardiac couplon. It is represented by the "unlinked" colocalization of the LTCC on the T-tubular membrane together with the opposed location of RyRs on the SR membrane at the JSR. Extracellular Ca^{2+} accessing as I_{Ca} through LTCC induce RyRs to open (CICR), releasing SR Ca^{2+} into the dyadic cleft, to activate contraction. Ca^{2+} is then pumped back into the SR by the SERCA or eliminated out of the cell by the NCX. Taken from (Weiss et al., 2011).

Using luminal Ca^{2+} indicators has also helped to visualize the Ca^{2+} handling inside the SR during local Ca^{2+} releases, as it reflects the depletion of this ion inside the SR (a.k.a Ca^{2+} blink) while the spark (at the cytosol) is visualized by another Ca^{2+} indicator, usually rhod-2 (Brochet, 2005) for reviews see (Cheng & Lederer, 2008).

In terms of excitation contraction coupling, the SR membrane hosts a group or cluster of about 43 RyRs (depending on species) facing a group of about 10 to 25 LTCCs. This fundamental Ca^{2+} release unit of cardiac excitation-contraction (EC) coupling is called a couplon. This observation was made by (Hayashi *et al.*, 2009) using 3-D electron tomography which challenged the previous observation by (Franzini-Armstrong *et al.*, 1999) who proposed that there were 100 RyRs / cluster, studying the cardiac muscle with conventional electron microscopy. Notably Hayashi and colleges discovered that, about 1/3rd of the dyadic clefts are equal or smaller than the size that can host 15 RyRs tetramers.

The individual functional states of a couplon range from random spontaneous activation, triggered activation (by an L-type Ca^{2+} channel), refractoriness and recruitment

between neighboring couplons (crosstalk), which control the release of Ca^{2+} to the cytosolic space during CICR (Weiss *et al.*, 2011). It has been noted that adjacent couplons normally do not activate their neighboring couplons during individual Ca^{2+} releases. They are working in isolation by a process called local control, which was described by a computer model of cardiac EC-coupling and Ca^{2+} signaling implemented by Stern (Stern *et al.*, 1999).

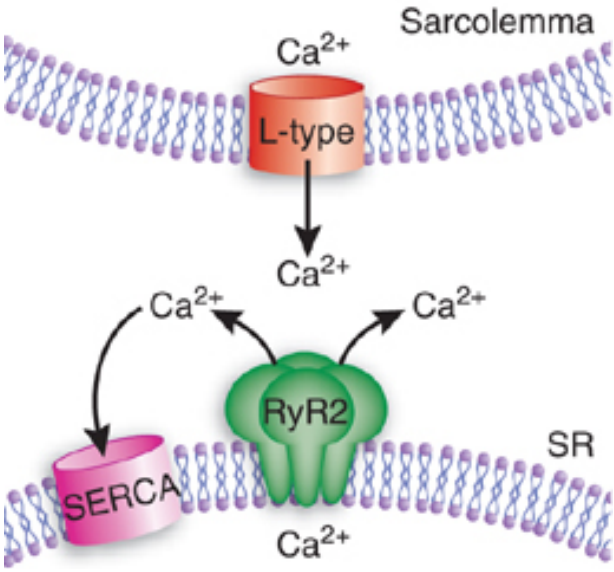


Fig 12: This scheme puts together the studies that have characterized the location and functional characterization of the LTCCs and the RyRs. It describes the regulatory domains during ECC and Ca^{2+} cycling, figure modified from (Molkentin, 2005). For more information please see (Cannell *et al.*, 1994; Soeller & Cannell, 1997).

During ECC, Ca^{2+} influx via LTCCs located at the T-tubular structure would increase dramatically the Ca^{2+} levels in the dyadic cleft of cardiac myocytes (to the order of ~100 μM concentrations). This high Ca^{2+} concentration then triggers Ca^{2+} release from intracellular Ca^{2+} stores, the SR, through the RyRs. Activation of a single couplon leads to a Ca^{2+} spark, a small elementary release signal. The dyadic cleft is a gap that has been measured to be around 12 nm in separation between the T-tubular and SR membranes.

4.4.2.L-type Ca^{2+} channel

The cardiac voltage sensitive L-type calcium channels (LTCCs) or $Ca_v1.2$, also known as (1,4)-dihydropyridine receptors (DHPRs), are responsible for influx of Ca^{2+} ions from the extracellular space to the cytosol in the form of an I_{Ca}. During ECC, these channels play a crucial role in activating the CICR process. Ca^{2+} channels display a fast activation and inward I_{Ca} transmission, but rather a slower inactivation, $\tau \sim 200$ ms (Lacinová & Hofmann, 2005), which is better appreciated in patch clamp experiments (see Fig 1D upper panel of the original manuscript). These channels are highly regulated during physiological activity. For example, modulation by the protein kinase CaMKII occurs upon high action potential frequencies, a process known as “facilitation”, which would increase I_{Ca}. (Bers, 2001). Other regulatory mechanisms, for example by PKA and NO, will be explained in the section “4.6. β-Adrenergic modulation of EC-coupling”.

Upon a depolarization of the cell membrane (usually from ~ –80 mV to > 0mV), which is physiologically triggered upon arrival of the AP, the LTCCs open due to the change in voltage across the cell membrane initiated by I_{Na}. Activation of LTCCS starts at potentials around –40 mV. Towards more positive values, more and more channels open and the I_{Ca} takes place. Its worth mentioning that at positive voltage values (> -50 mV) the LTCCs slowly inactivate after the rapid activation, as it can be seen in whole-cell voltage clamp experiments when Ca^{2+} is replaced by Ba^{2+} (which does not lead to the more rapid Ca^{2+}-induced inactivation of I_{Ca}, see below) (Branchaw *et al.*, 1997).

Other mechanisms for inactivation and more prominent than the voltage-dependent inactivation, is the Ca^{2+} itself, after it reaches the dyadic cleft, which is a ~20 nm gap between the SR and the LTCCs. Together with Ca^{2+} released from the SR via the RyRs, the high Ca^{2+} levels inactivate the LTCCs from the cytosolic side. Because of this mechanism, low resting cytosolic Ca^{2+} levels are also needed for LTCC reactivation.

Hormones such as adrenaline also modulate this channel by signaling via second messengers (e.g. cAMP) and by its phosphorylation through the activation of protein kinases, e.g. PKA (Kamp & Hell, 2000). Stimulation by phosphorylation yields more I_{Ca} at physiological depolarizing voltages in order to trigger a very robust I_{Ca}-dependent release from intracellular Ca^{2+} stores and for more pronounced filling of the SR with Ca^{2+}. This effect is observed specially during exercise, where β-adrenergic stimulation induces the heart to pump faster and stronger.

As stimulation during stress by the sympathetic nervous system increases the heart rhythm, faster recovery of these channels after inactivation would be expected, to become ready to the next depolarization. This type of behavior is in fact seen. In addition, faster Ca^{2+} re-uptake into the SR due to SERCA stimulation (Periasamy *et al.*, 2008), induces the sytosolic Ca^{2+} levels to decrease faster, hence contributing to a more rapid LTCC reactivation (Branchaw *et al.*, 1997; Bers, 2001).

In patch clamp experiments these channels exhibit rapid deactivation, which, unlike inactivation, is the transition from the open to the closed state. This gives rise to “tail currents” at re-polarizing voltage levels, substantial but very short Ca^{2+} currents (Zhou & Bers, 2000).

Finally this channel has also been shown to conduct Ba^{2+} and other divalent ions, and even Na^{+} (in the absence of Ca^{2+}). The channels are sensitive to blockage by dihydropyridines (such as nifedipine) or activation by Bay K 8644 (methyl 2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-1,4-dihydropyridine-3-carboxylate). Furthermore, some divalent cations such as Cd^{2+} and Ni^{2+} bind to the selectivity filter of the channel and block it from conducting (Sheets & Hanck, 1992).

4.4.3.The RyR macromolecular complex

Features like CICR, Ca^{2+}-induced inactivation, gating and Ca^{2+}-dependent sensitivity, are some of the many mechanisms that this multifaceted SR membrane Ca^{2+} channel displays. The RyR is composed by at least by 4 transmembrane domains M1 to M4 (Fig 6) through the SR membrane. Inside the SR membrane are the interaction sites of this channels with calsequestrin / junctin. Presently, its is thought that junctin and triadin assist calsequestrin in its Ca^{2+}–dependent regulation (Allen, 2009; Royer & Ríos, 2009; Györke & Györke, 1998).

The Ca^{2+} binding sites on the cytosolic part of the RyR are directly involved in activation of CICR, depending on the cytosolic Ca^{2+} levels. The RyRs start to activate at elevated $[Ca^{2+}]_i \sim 1$ μM and exhibit maximal P_o at $[Ca^{2+}]_i \sim 100$ μM. In lipid bilayer experiments reconstituted RyR are inhibited when the $[Ca^{2+}]_i$ reaches very high values ~ 1 mM, values which are unlikely to exist in-situ (Lanner *et al.*, 2010). The activation site presents a low Ca^{2+} affinity, which helps the channel to remain closed during low cytosolic Ca^{2+} levels during diastole. As previously mentioned, this channel also presents specific

phosphorylation sites for PKA and CaMKII which are serine 2808, 2030 and 2814 (Hain *et al.*, 1995; Marx *et al.*, 2000; Kockskämper & Pieske, 2006; Valdivia, 2012; Fischer *et al.*, 2013).

These sites are believed to regulate the RyR, for example during β-AR stimulation, by increasing their sensitivity to Ca^{2+}.

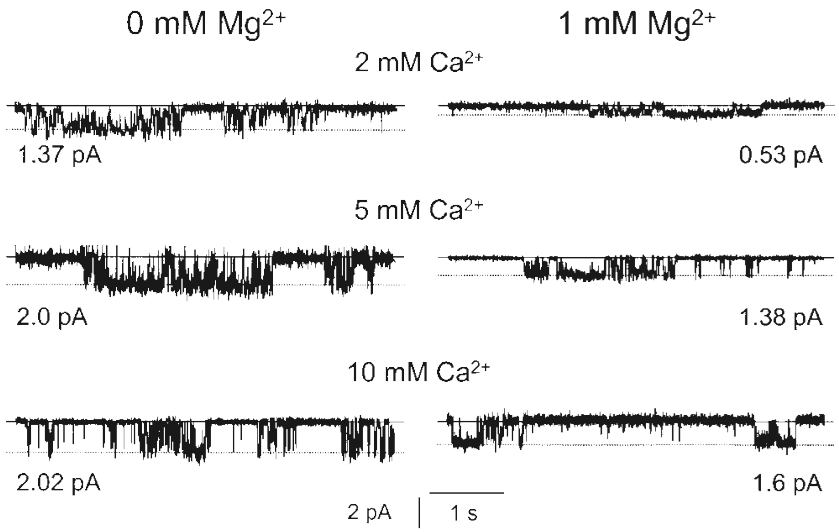


Fig 13: Effect of Mg^{2+} concentration on unitary RyRs Ca^{2+} current amplitude. Current amplitude was measured in single RyRs reconstituted in a lipid bilayer at 0 mV, in the absence and presence of 1 mM Mg^{2+} left and right respectively. Taken from (Mejía-Alvarez *et al.*, 1999).

The RyRs also present a mechanism opposing their opening when sensing Ca^{2+}, which is called refractoriness, related to the recovery from the still elusive mechanism terminating Ca^{2+} release. Refractoriness helps the SR to refill its Ca^{2+} content and prevents RyRs from opening during diastole. This phenomenon has been extensively studied and described to be dependent on the global SR Ca^{2+} depletion during a whole cell Ca^{2+} transient. Refractoriness seems to be much shorter locally (i.e. after Ca^{2+} sparks), presumably because of rapid Ca^{2+} redistribution within the SR network by diffusion. This was first described by (DelPrincipe *et al.*, 1999) when investigating local and global Ca^{2+} release dependent refractoriness of the RyRs, by comparing ultraviolet-laser flash and two-photon photolysis of caged Ca^{2+} compounds. Other small molecules as Mg^{2+}, ATP and CaM, modulate this receptor on the cytoplasmic side. Depending on their

concentration they exert stimulation (ATP) or inhibition (Mg^{2+} and CaM) of the RyRs function, also during ECC (Lanner *et al.*, 2010). CaM is an endogenously expressed 17-kDa Ca^{2+}–binding protein which regulates the RyRs P_o by direct interaction. CaM contains four Ca^{2+} binding pockets and binds to one site per RyR subunit (four per tetramer) (Lanner *et al.*, 2010). Mg^{2+} also plays an important role in modulating the RyRs open probability during CICR, as it was shown by (Gusev & Niggli, 2008), where removing free magnesium had a biphasic effect by inducing an avalanche of Ca^{2+} sparks which showed a recovery phase afterwards, presumably due to low SR Ca^{2+} content.

Taken together the RyR macromolecular complex displays a variety of features which can depend on each other, to regulate the characteristics of ECC. Other sites on the complex also play important roles in the regulation of the receptor during physiological regulation. The PP1 and PP2A protein phosphatases regulate the de-phosphorylation of the receptor during β-AR stimulation. More recently, the importance of a number of cysteine thiol sites for oxidation and S-nitrosation has been recognized (For reviews see: (Hess *et al.*, 2005; Espey *et al.*, 2006; Lim *et al.*, 2007)). However, the complexity of the regulation of this macromolecular complex is still largely unexplored and physiological role of many domains on the channel remain unclear. This is accentuated by the fact that several laboratories have obtained contradictory results on its regulation. A controversy has developed in the area of this thesis, specially regarding downstream consequences of β-adrenergic receptor stimulation (Marx *et al.*, 2000; Niggli *et al.*, 2012; Valdivia, 2012; Bers, 2012).

4.4.4. Cardiomyocyte contraction, SERCA, NCX, sarcolemmal Ca^{2+} pump and mitochondria

During ECC, coordinated Ca^{2+} signaling between systole and diastole is a key factor to ensure a proper cell signaling and initiate the cellular mechanical contraction (and the consequent cardiac muscle contraction). In the steady state, the amount of Ca^{2+} that enters the cell during I_{Ca} has to be removed to avoid intracellular Ca^{2+} overload. In addition, Ca^{2+} that has been released from the SR need to be pumped back into the store from beat to beat, to allow diastolic relaxation. Therefore, after I_{Ca} and Ca^{2+} release from the SR to the cytosol to produce contraction, SR re-uptake and elimination of the excess of Ca^{2+} is achieved by four already identified and quantified main mechanisms, as summarized below. Although the balance of the Ca^{2+} transport capacity between SERCA

and NCX is somewhat species dependent, the SERCA is usually the most powerful of the four mentioned Ca^{2+} transport mechanisms. For example, in rabbit ventricular cardiomyocytes, the SERCA moves ~ 70% of the total Ca^{2+} back into the SR, while the NCX extrudes 28 %. The mitochondria and the sarcolemmal Ca^{2+} ATPase participate with only 2 % (Bers, 2001).

In other species like rat, which has a Ca^{2+} signaling that is extremely based on the SR and has only small trans-sarcolemmal Ca^{2+} fluxes, the SERCA accounts for the majority of the Ca^{2+} movements during relaxation, ~92% followed by only a 7% of NCX participation and only a 1% of mitochontrial and the membrane Ca^{2+} ATPase participation. As the Ca^{2+} transport by the NCX depends on the Ca^{2+} and Na^{+} concentrations inside and outside of the cell and their electrochemical gradients, mathematical models are required to predict its role during the AP in terms of determining the AP shape during the depolarizing or re-polarizing phases, even tough I_{NCX} have been shown to be mainly inward during the majority of the cardiac cycle (corresponding to Ca^{2+} removal by the NCX) (Weber *et al.*, 2002).

In guinea pig cardiomyocytes the NCX removes about 30% of the cytosolic Ca^{2+} (Bers, 2001), hence contributing to diastolic or resting conditions to an extent similar to rabbit cells. As mentioned above, the NCX can also work in the reverse mode by carrying Na+ ions out of the cell. This can happen early during the AP, because there may be some accumulation of Na^{+} under the membrane during I_{Na} (Leblanc & Hume, 1990). This mechanism extrudes 3 Na^{+} ions by importing one Ca^{2+} ion to the cytosolic space.

4.5.Ca^{2+}-induced Ca^{2+} release

CICR is the process that every cardiomyocyte has to undergo to perform a contraction. Upon a depolarization of the cell membrane robust inward I_{Ca} through the L-type Ca^{2+} channels (LTCC) will open RyRs to release SR Ca^{2+}, which is the main source of Ca^{2+} for contraction. This process was first described when working with skinned skeletal muscle fibers by (Endo *et al.*, 1970), however, it was not until the work of (Fabiato, 1983) when it was thoroughly characterized in the heart.

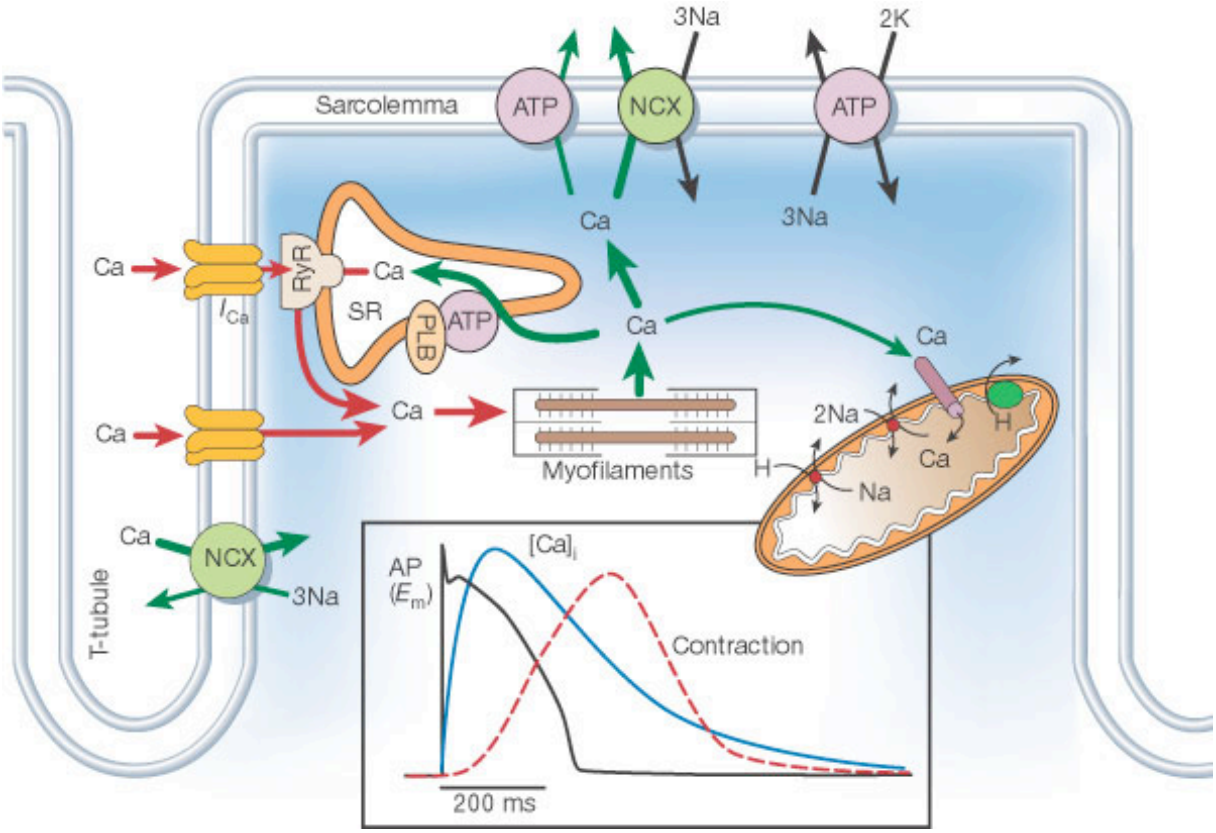


Fig 14: Scheme representing the Ca^{2+} fluxes during the ECC process. The four major contributors for Ca^{2+} regulation are presented: SERCA, NCX, Ca^{2+} ATPase and mitochondria. The small insert at the lower side shows the time course of the action potential, accompanied by the corresponding Ca^{2+} transient and contraction. Taken from (Bers, 2002).

4.5.1.Activation

To activate CICR, the LTCCs need to be available to open. Therefore, both a low resting membrane potential (~ –80 mV) and low cytosolic Ca^{2+} levels are required. Once the Na^{+} channels open upon arrival of the AP, the robust I_{Na} current depolarizes the sarcolemma, inducing opening of the voltage-dependent LTTCs to conduct Ca^{2+} entry as I_{Ca}. In Fig 15 the LTCC availability is shown at different voltages.

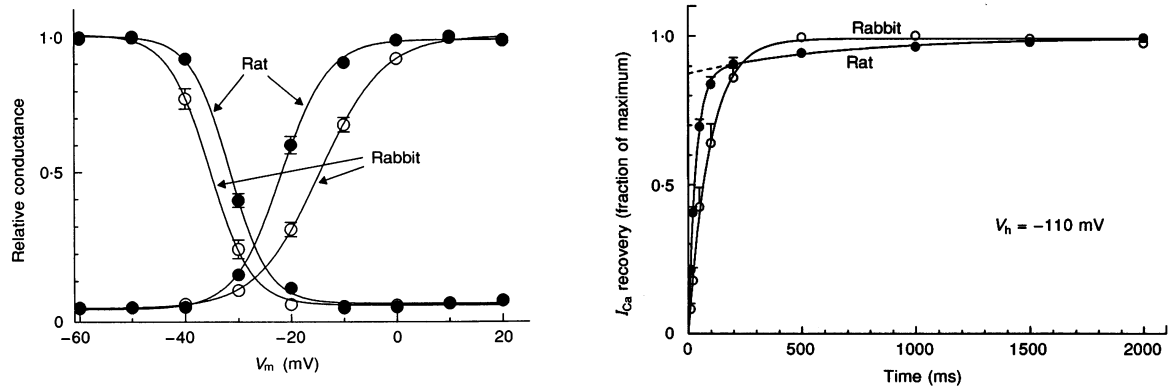


Fig 15: This figure shows the LTCC availability. The left panel shows the LTCC activation and availability to conduct I_{Ca}, measured by depolarizing from –90 mV to the indicated E_m for 2 seconds and then testing the remaining I_{Ca} at E_m = 0 mV. The right panel shows the I_{Ca} recovery from inactivation; when V_h was – 110 mV, it only toke about 200 ms to recover. In other figure (not shown here) the same recovery at V_h –50 mV was slower, hence showing that at more positive repolarizing voltages, the I_{Ca} takes longer to recover from inactivation. Taken from (Yuan *et al.*, 1996).

Once the Ca^{2+} reaches the dyadic cleft, within a couplon, the Ca^{2+} binds to the RyRs Ca^{2+} binding site (the activating A-site) (Zahradník *et al.*, 2005), this triggers a conformational change in the structure of RyR called domain unzipping (Oda *et al.*, 2013), which finally opens the channel pore. In the work of Fabiato 1985, a mechanism which could explain the CICR activation process was explained. In Fig 16, such a model is presented, where upon I_{Ca} activation, the lower “affinity binding site” senses the I_{Ca} therefore moving the RyRs from a closed to an open state.

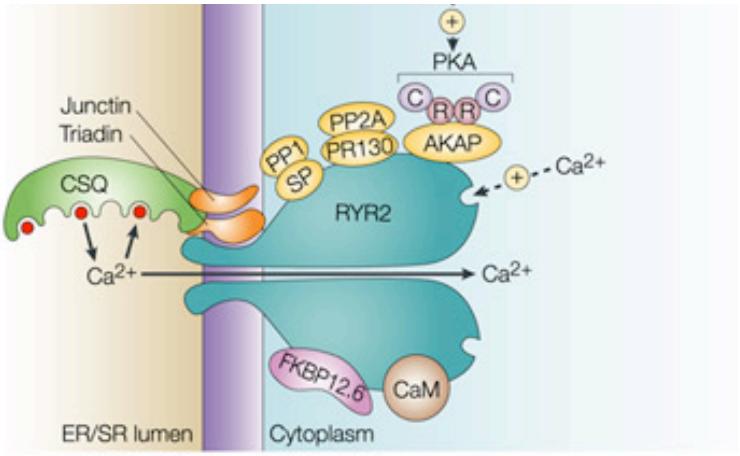


Fig 16: Scheme showing the activating Ca^{2+} binding sites of the RyRs, and the interactions with regulatory proteins. The left side corresponds to the SR which is the Ca^{2+} store. It contains calsequestrin, triadin and junctin which regulate and buffer the Ca^{2+} inside this organelle. On the sytosolic part we find other Ca^{2+} regulatory proteins such as the PKA regulatory site the FKBP 12.6 and the site for CaM; all working in synchrony to regulate Ca^{2+} release for contractile purposes. Modified from (Berridge *et al.*, 2003).

4.5.2.Termination

The mechanism(s) leading to Ca^{2+} release termination is not fully clear yet. Initially, Ca^{2+} which is released from the SR, was thought to bind to a higher affinity binding site, moving the RyRs to an inactivated state. After contraction and as $[Ca^{2+}]_i$ declines, Ca^{2+} dissociates from the lower affinity Ca^{2+} binding site, which allows to return the RyRs to the resting state. In a more recent view, the CICR process terminates mainly due to SR Ca^{2+} depletion and subsequent decline of the release flux, which contributes to deactivation of the RyRs by pernicious attrition (Gillespie & Fill, 2013). After release shutdown, CICR remains transiently refractory and the RyRs are less sensitive to be triggered by Ca^{2+}, as described in the work of (DelPrincipe *et al.*, 1999).

As previously mentioned both decline of sytosolic Ca^{2+} levels and SR Ca^{2+} re-uptake by the SERCA, after cardiomyocyte contraction, allows to refill the SR Ca^{2+} content, which then permits recovery of the CICR process and RyR to become availability to be triggered for another cycle of opening and subsequent Ca^{2+} release.

CICR termination may be somewhat more complex. Calsequestrin has been implicated to play a major role in RyR regulation by intra-SR Ca^{2+}. This protein has been extensively studied in this context as it is the major Ca^{2+} buffer inside the SR. Calsequestrin has been proposed to control the SR Ca^{2+} release, by extending the duration of Ca^{2+} fluxes (Terentyev *et al.*, 2003). New mechanism as the "luminal Ca^{2+}-dependent deactivation" proposed by (Terentyev *et al.*, 2002), suggests that calsequestrin exerts its effects by influencing the local luminal Ca^{2+} concentration-dependent gating of the RyRs via an allosteric interaction mediated by triadin and junction. Therefore, it seems that the regulation of the openings of RyRs by $[Ca^{2+}]_{SR}$ is responsible for the termination of CICR and for the following restitution of junctional SR release sites in cardiomyocytes, either directly or mediated via calsequestrin dependent RyR modulation. However more research is needed to understand the underlying mechanisms governing CICR termination.

4.5.3.Local control

This mechanism describes the independence of a group of RyRs and LTCC (couplons), to work without being affected by neighboring Ca^{2+} signals coming from other couplons/cluster. This model considers an array of RyRs, which are clustered in a dense, regular, two-dimensional lattice in the dyadic junction (Stern *et al.*, 1999). To examine the local control mechanism in situ, there is a very useful phenomenon that allows to assess couplon to couplon interactions. This is the elementary Ca^{2+} signal, called a Ca^{2+} spark.

This spatially localized cytosolic elevation of Ca^{2+} was visualized by (Cheng *et al.*, 1993), after the introduction of the confocal microscopy to Ca^{2+} signaling research. The existence of these Ca^{2+} signals also helped to understand that Ca^{2+} sparks underly the global Ca^{2+} transient during CICR (Wier *et al.*, 1994). The Ca^{2+} sparks can be triggered in several ways.

One of them, the Ca^{2+} "sparklet" (Wang *et al.*, 2001), is a inward I_{Ca} current mediated by a single LTCC that triggers SR Ca^{2+} release and a Ca^{2+} spark by activating one of the aforementioned clusters of RyRs.

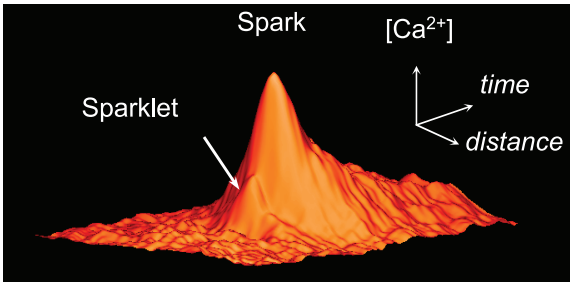


Fig 17: Detection of a "sparklet"-dependent triggering of a Ca^{2+} spark. A small influx of I_{Ca} through one LTCC triggers an opening of a RyRs cluster evoking a Ca^{2+} spark. Please note that not all sparklets trigger Ca^{2+} sparks. Figure taken from (Cheng & Lederer, 2008).

Another mechanism to achieve this local elevations in cytosolic Ca^{2+} is by a "spontaneous" opening of the RyRs in one of these couplons. Spontaneous sparks in resting cells are rare incidents because the open probability of the RyRs at 100 µM resting Ca^{2+} is very low (but not zero). Experiments with low concentrations of caffeine (acting as a RyR agonist) and without extracellular Ca^{2+} enhance this phenomenon by increasing the P_o of the RyRs and therefore the frequency of appearance of the Ca^{2+} sparks (Porta *et al.*, 2011). A third way to trigger Ca^{2+} sparks is known to occur under pathological conditions by openings of neighboring couplons which can induce Ca^{2+} release. By this possibility, the Ca^{2+} sparks and the associated local control theory can also explain the situation when Ca^{2+} waves are triggered and propagated by CICR. This happens especially when the SR is Ca^{2+} overloaded and can be seen as a failure of the local control mechanism (Weiss *et al.*, 2011).

And finally is has to be mentioned that Ca^{2+} signals smaller than a Ca^{2+} sparks seem to exist, termed a Ca^{2+} quark (Lipp & Niggli, 1998). These events were shown to appear from the opening of a smaller number of RyRs (possibly one), or from RyRs exhibiting a different gating mode. Recently, it was discovered that around 50 % of the continuous SR Ca^{2+} leak occurs via invisible Ca^{2+} quarks (Brochet *et al.*, 2011). Therefore, the local control results in the natural ability of the local Ca^{2+} fluxes to become manifest in an independent way, without significantly affecting neighboring release sites. This attribute can be exploited to investigate the sensitivity of the RyRs to control their opening under

physiological conditions or when affected by regulatory mechanisms and pharmacological interventions.

4.5.4.Ca^{2+} sparks as a read-out of the RyR's P_o

The frequency of Ca^{2+} sparks is very low during physiological conditions. They were discovered as spontaneous events in rat cardiomyocytes, but also found in other species, although at even lower frequencies, particularly in guinea pig cardiomyocytes. However, under proper control of the SR Ca^{2+} content, sparks can be an excellent tool, which serves as a readout to determine the open probability of the RyRs in situ. Once this channels open, Ca^{2+} can be visualized in the cytosol in the form of Ca^{2+} sparks and therefore assessment of RyR Ca^{2+} sensitivity or changes in RyR gating can be evaluated (for more information about RyR gating please see (Zahradník *et al.*, 2005)).

The RyR function is affected by different modulators such as protein kinases and other molecules like NO or ROS. This implies that post-translational modifications, such as phosphorylation, nitrosation or oxidation among others, can affect the way they work, especially regarding their opening and release Ca^{2+}.

Therefore, this approach to study the P_o of RyRs has been widely applied in real time experiments with isolated cardiomyocytes, either during acute or chronic interventions modifying the function of cardiac muscle. In this thesis, the frequency of the Ca^{2+} sparks and changes of their appearance were correlated with functional alterations exerted by various pharmacological tools. During such experiments, proper control of the SR Ca^{2+} content is essential and can be easily assed by a rapid application of caffeine, which induces RyRs to open and release almost all of its Ca^{2+} content. As mentioned above, the Ca^{2+} sparks can occur randomly in cardiomyocytes and originate from a cluster of RyRs.

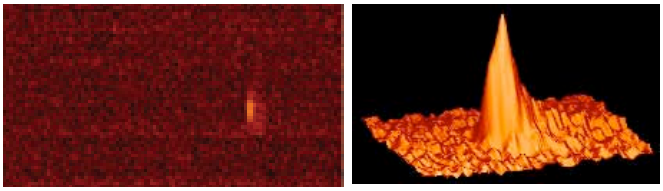


Fig 18: The Calcium Spark: **Left**: typical Ca^{2+} image of a cytosolic Ca^{2+} spark by laser scanning confocal microscopy using the Ca^{2+} indicator Fluo-3 (Ogrodnik & Niggli, 2010). **Right:** 3D intensity profile and spatial distribution of a Ca^{2+} spark (Lipp & Niggli, 1998).

After the initial localized accumulation of the released Ca^{2+} in the sytosolic space, it will dissipate away from the micro-domain of Ca^{2+} release by diffusion. In the model proposed in the research of (Porta *et al.*, 2011), applications of low-dose caffeine showed that not all RyR openings during diastole can evoke spontaneous Ca^{2+} sparks. They suggested that a spark may result only when a spontaneous single RyR opening lasts at least 6 ms.

Taken together, the Ca^{2+} spark is a useful tool to asses the propensity of the RyRs to open, upon a physiological change, such as the post-translational modifications caused by the aforementioned molecules.

4.5.5.Calcium waves (loss of local control)

In the original local control theory of Stern (called “cluster bomb model” (Stern, 1992; Stern *et al.*, 1999)) and in the one he presented later on, it is proposed that Ca^{2+} release within a couplon and more specifically within a cluster of RyRs, is an all or none process. However, accidental spontaneous Ca^{2+} sparks only very rarely trigger Ca^{2+} release from neighboring clusters in healthy cardiomyocytes. For example, a Ca^{2+} spark can trigger another Ca^{2+} spark from a different lateral cluster along the same Z-line, which could be as close as 0.76 μm (Parker *et al.*, 1996).

Under specific pathological conditions, the aforementioned local control mechanism can fail. This usually results in Ca^{2+} waves that travel along the cells in the cytosolic space,

as a reaction-diffusion wave and driven by CICR. The classical view of the wave traveling mechanism proposes that upon a higher sensitization of the RyRs from the cytosolic side (due to the arrival of the wave front) the RyRs open and trigger Ca^{2+} release (Fabiato, 1985; Jaffe, 1991). However, more recently several groups provided some arguments to include an additional feedback mechanism that helps to maintain the wave propagation. In a pioneering study of this type, it was proposed by (Keller *et al.*, 2007), that the RyR activation could even take place before the actual arrival of the Ca^{2+} wave, driven by a luminal RyR–dependent sensitization, mediated by a SR Ca^{2+} overloading via the SR Ca^{2+} pump (SERCA). The results of this investigation showed that not only CICR could govern the propagating mechanism of Ca^{2+} waves, and it linked SR Ca^{2+} load with potential mechanisms causing cardiac arrhythmias triggered by spontaneous SR Ca^{2+} releases.

4.6.β-Adrenergic modulation of EC-coupling

When the heart needs to provide high performance during physical exercise or in situations of mental stress, activation of the adrenergic system takes place. This mechanism provides norepinephrine (and epinephrine) to the cardiac muscle, which upon binding to the β-adrenergic receptors, triggers intracellular pathways that boosts cardiomyocytes activity and therefore the cardiac output.

4.6.1.The classical β-adrenergic pathway, fight or flight reaction

When a rapid reaction is needed, the sympathetic neurons in the cardiac atria and ventricles release norepinephrine. In cardiomyocytes, this modulates several cellular functions after binding to the β_1-adrenergic receptors. Once bound, these receptors trigger a conformational change that liberate the G_α subunit of the G_s protein, which diffuses towards activating the enzyme adenylate cyclase (AC) to produce cAMP.

In ventricular myocytes the main effect of cAMP is to activate PKA. Via phosphorylation of downstream Ca^{2+} regulatory proteins, this increases Ca^{2+} current influx through LTCC phosphorylation (larger I_{Ca}), which in turn enhances SR Ca^{2+} release by several mechanisms (e.g. by more Ca^{2+} loading of the SR and by providing a larger trigger CICR). In addition, acceleration of the SR Ca^{2+} re-uptake mediated by the SERCA occurs after phosphorylation of phospholamban (PLB). These mechanisms are discussed in more detail below.

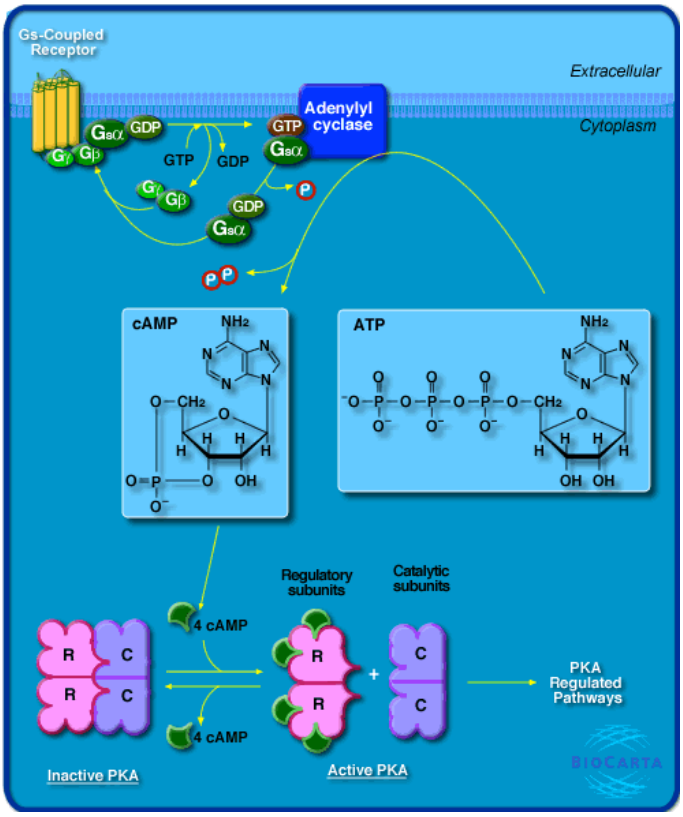


Fig 19: Description of the β-adrenergic pathway. The G_s coupled receptor at the top left side of the scheme, can represent the β-adrenergic receptor in the heart. Subsequent activation of the downstream regulatory proteins such as PKA, regulate inotropy in the cardiac muscle. Taken from: www.biocarta.com/pathfiles/h_gspathway.asp

β-Adrenergic receptor, isoforms

Three isoforms of β-AR are known and all are expressed in the heart: 1,2 and 3. With about a 90% of them being β_1, this receptor subtype dominates the adrenergic stimulation in cardiac muscle (Tilley, 2011). Almost all changes of Ca^{2+} signaling described later in this chapter are the result of the β_1 isoform activation. Regarding the β_3 isoform and partially in the case of β_2 they are rather involved in the negative inotropic effect, preventing activation of the protein kinase A, PKA, by inhibiting cAMP through activation of the G_i protein.

In the healthy heart the β_3-AR isoform may display a moderate negative inotropic effects (Dessy & Balligand, 2010; Gauthier *et al.*, 2011), but in heart failure, it could protect of adverse effects due to excessive catecholamine stimulation, as it couples with the G_i

protein subunit as explained above. Furthermore its cardioprotective effect is enhanced during heart failure where it has been shown that the β_1 isoform is down-regulated about 62% (Bristow *et al.*, 1986). In ventricular cardiomyocytes, β_1 receptor activation has been shown to be involved in the SR Ca^{2+} leak, especially observed in failing hearts (Petroff, 2002; Kushnir *et al.*, 2010) for reviews see (Lohse *et al.*, 2003).

G-protein coupling

This intermediate messengers of membrane ligand gated receptors, translate information about molecules outside the cell and activate intracellular signal transduction pathways. G-proteins are activated after binding to the receptor structure that has undergone a conformational change upon extracellular ligand binding.

The G-protein coupled receptor, has one main signal transducer, the G_α and its inhibitor $G_{\beta\gamma}$, although the last one in some cases can also act as a signal transducer. The G_α activity is regulated by hydrolyzing bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) to render inactivation.

There are many classes of G_α protein subunits, among them the most important in the regulation of signal transduction in the heart: $G_s\alpha$ (stimulator of positive inotropy), $G_i\alpha$ (inhibitor of positive inotropy) and $G_q\alpha$ which accounts for the activation of PLC-β isoforms which stimulate inositol lipid (i.e. calcium/PKC) signaling (for reviews see (Sprang, 1997)).

Adenylate cyclase and cAMP

As mentioned above, cAMP is the second messenger that is produced upon $G_s\alpha$-dependent activation of the enzyme AC. The mechanism of cAMP production occurs upon binding of the complex ($G_s\alpha$ + GTP) to AC, which then catalyzes the conversion of ATP into cAMP + 2 phosphates. Subsequently, cAMP binds to other downstream regulatory proteins, most importantly to PKA, which then becomes activated (Das *et al.*, 2007).

4.6.2.PKA and its main targets involved in EC-coupling

Once cAMP is bound to the regulatory subunit of PKA, the catalytic subunit detaches and is phosphorylated by ATP, to turn PKA on. Then PKA phosphorylates several downstream Ca^{2+} regulatory proteins that are involved in excitation-contraction coupling

(EC-coupling), such as the L-type calcium channels (LTCC) and the natural SERCA inhibitor phospholamban (PLB). All these modifications together favor the ability of the cardiomyocyte to handle another systole in a shorter time and pump blood much stronger to meet the circulatory demand during exercise (Layland *et al.*, 2005; Dong *et al.*, 2007).

L-type Ca^{2+} channels

The main function of the PKA-dependent phosphorylation of the LTCC during β-adrenergic stimulation, is to increasing the gating, the mean channel open time and the opening probability of functional Ca^{2+} channels (Kamp & Hell, 2000). A more clear appreciation of the LTCC phosphorylation by PKA can be achieved by using the drug Forskolin which bypasses the β-adrenergic receptor by acting directly on the AC. This maneuver can be of great use when trying to avoid other unspecific effects of the β-adrenergic stimulation with adrenaline.

Phospholamban (PLB)

PLB phosphorylation was discovered by Arnold Katz and coworkers in 1974 (Tada *et al.*, 1974). PKA phosphorylates phsopholamban (PLB) at the Ser-16, which leads to shorter intervals between contractions as it makes faster the Ca^{2+} re-uptake by the SERCA. In the absence of PLB phosphorylation, SERCA is inhibited by this protein, but inhibition is relieved upon phosphorylation by PKA. But other kinases have also been reported to phosphorylate PLB, at Thr-17 (Mattiazzi *et al.*, 2005). Therefore this protein plays a mayor role in the acceleration of relaxation, which is necessary to increase the cardiac rhythm.

Ryanodine receptors (RYR)

This has been one of the most controversial phosphorylation sites for PKA on a receptor or a channel. It has been shown that PKA--dependent phosphorylation of the RyRs at Ser-2808 dissociates the FKBP12.6 protein (now called calstabin-2) destabilizing the closed state of the channel (Marx *et al.*, 2000). Other studies have indicated that the basal phosphorylation level of this site by PKA is already 50% and no large changes are

observed after β-adrenergic stimulation (Valdivia, 2012). PKA-dependent phosphorylation of RyRs during β-adrenergic stimulation with isoproterenol has been proposed to increase the velocity of the release kinetics of the Ca^{2+} transient during EC-coupling, but not other parameters such as transient amplitude (Ginsburg & Bers, 2004). This main discrepancy between laboratories makes a role of PKA-dependent phosphorylation of the RyRs very unclear during EC-coupling in the presence of catecholaminergic stimulation.

Houser and Valdivia groups have provided recent evidence that preventing S2808 phosphorylation has a negligible impact on the β-adrenergic response of the heart (Benkusky *et al.*, 2007; Zhang *et al.*, 2012). Therefore it has been proposed that phosphorylation of PKA on two sites of the RyR (S2808 and S2030) could coordinate channel openings in response to I_{Ca} and phosphorylation of other sites (i.e. S2814) by other protein kinases as it is CaMKII, could rather open the RyRs at diastolic levels, to increase the observed Ca^{2+} leak in the form of Ca^{2+} sparks during β-adrenergic stimulation. What is known to date is that PKA does phosphorylate the RyRs at S2808 and S2830, but the functional changes during EC-coupling are yet to be seen (Ullrich *et al.*, 2012), specially as PKA-dependent phosphorylation of the LTCC increases I_{Ca} and the corresponding observed transient, making it very difficult to quantify the participation of the RyRs (for reviews see (Niggli *et al.*, 2012)). Therefore, the role of PKA-dependent phosphorylation of the RyRs during physiological conditions and how it could influence heart failure still remains controversial.

4.6.3. CaMKII and its main targets involved in EC-coupling

CaMKII is an ubiquitous multifaceted protein that responds to elevations and oscillations of Ca^{2+} transients (De Koninck & Schulman, 1998) and recently has also been shown to be activated by oxidation. (Erickson *et al.*, 2008). It has been shown that during β-adrenergic stimulation, CaMKII is activated and able to phosphorylate the RyRs at S2814 (Huke & Bers, 2008). While the case is clear for beating cardiomyocytes, the mechanism of activation of CaMKII in resting cells is still unresolved.

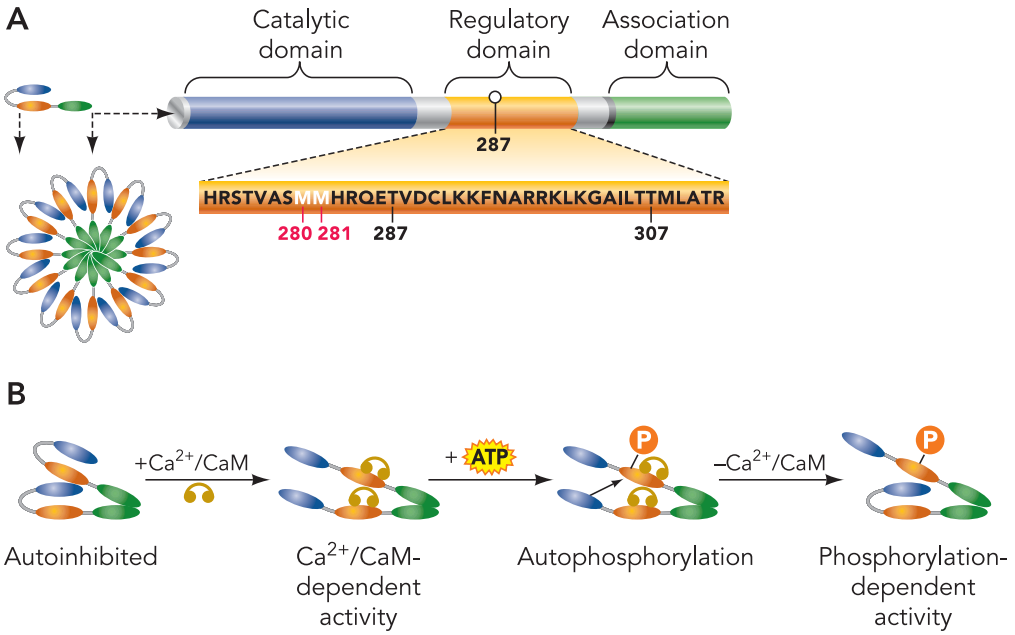


Fig 20: A. CaMKII structure. In the cartoon it is shown the NH_2-terminal catalytic domain that has the function to phosphorylate other proteins once the regulatory has opened after binding Ca^{2+}. The COOH associative domain participates in the assembly of CaMKII monomers into the holoenzyme. **B.** All together by making use of Ca^{2+} and ATP, this structure activates and regulates the protein which participates in the phosphorylation of other downstream Ca^{2+} regulatory proteins. Fig taken from (Couchonnal & Anderson, 2008).

Only pharmacological evidence by several groups has indicated that the observed diastolic SR Ca^{2+} leak is dependent on CaMKII activity (Curran *et al.*, 2007; Ogrodnik & Niggli, 2010).

Despite the fact that many targets of CaMKII have been identified, there are still are gaps in the description of the activating pathway of this protein during β-adrenergic stimulation.

Activation by Ca^{2+}

Ca^{2+} calmodulin-dependent protein kinase II (CaMKII) is activated by calmodulin with two bound calciums (Shifman *et al.*, 2006). CaMKII comprises an associative, a regulatory and a catalytic domain as shown in Fig 20. In the absence of Ca^{2+} the regulatory and a catalytic domain interact causing auto inhibition of the molecule.

Upon activation Ca^{2+}/CaM binds to the associative domain, the regulatory domain is exposed and auto-phosphorylation can occur within the dodecamer. This results in a CaMKII activity lasting longer than the actual Ca^{2+} transient (in the time domain of seconds). In tissues were Ca^{2+} oscillates, such as cardiac muscle, this results in the CaMKII activity also becoming dependent on the frequency of these oscillations, not only on their amplitude (De Koninck & Schulman, 1998). When active, the CaMKII uses its catalytic domain to phosphorylate several target proteins such as channels, transporters, enzymes and receptors. Some of these posttranslational modifications can directly affect EC-coupling of cardiomyocytes, as summarized below (Niggli *et al.*, 2012). CaMKII becomes inactive when Ca^{2+} levels are low. However, it has been shown by (Erickson *et al.*, 2008) that in the presence of oxidative stress CaMKII can maintain its activity for longer periods of time (see below for details).

L-type Ca^{2+} channels

In the absence of any adrenergic stimulation, rapid pacing of cardiomyocytes induces an increase of whole-cell Ca^{2+} current (I_{Ca}). This phenomenon is known as facilitation. It depends on enhanced CaMKII activity upon Ca^{2+} entry and cycling of in the cytosol. Once CaMKII its activated it phosphorylates the LTCC on the β_{2a} subunit at the Thr-498 and Ser-1512 and Ser-1570 on the α_{1c} subunit (Blaich *et al.*, 2010) to “facilitate” the whole-cell Ca^{2+} currents (I_{Ca}) during subsequent heart beats (Grueter *et al.*, 2006). As it was shown by (Dzhura *et al.*, 2000) CaMKII-dependent phosphorylation of the LTCC increases their P_o, this effect helps to overcome the Ca^{2+}/calmodulin-dependent inactivation of LTCC, which takes place at the α_{1c} subunit during the entry and SR release of Ca^{2+}.

As mentioned above, CaMKII has been shown to activate at fast pacing rates by auto-phosphorylation at Thr286/7, which enhances its affinity for calmodulin ~1000-fold and grants constitutive CaMKII activity until de-phosphorylated by a protein phosphatase (Grueter *et al.*, 2006). Therefore CaMKII-dependent phosphorylation of the LTCC appears to modulate EC-coupling in cardiomyocytes and this modulation would be enhanced when β-adrenergic stimulation takes place, even though PKA-dependent phosphorylation of the L-type Ca^{2+} channels seems to result in larger increments than those mediated by CaMKII (Kameyama *et al.*, 1985).

Phospholamban (PLB)

CaMKII-dependent phosphorylation of PLB at Thr-17 reduces the inhibition of the SERCA by PLB, which is responsible for the SR Ca^{2+} uptake. It has been observed that CaMKII-dependent phosphorylation of PLB during cardiomyocyte pacing only targets Thr-17 and not Ser-16 (which has been associated to PKA). However the activation of SERCA by CaMKII-dependent phosphorylation of PLB at Thr-17 is low (Zhang *et al.*, 2011), which makes PKA to dominate this function especially during β-adrenergic stimulation, when the intracellular Ca^{2+} levels are high. Valverde et al. 2005 (Valverde *et al.*, 2005) reported that PLB phosphorylation by CaMKII was not involved in frequency-dependent acceleration of relaxation (FDAR) associated to the relieve of SERCA inhibition; furthermore the CaMKII inhibitor KN-93 did not affect the observed FDAR.

Therefore, despite that the CaMKII-dependent phosphorylation of PLB appear to take place during both high pacing and β-adrenergic stimulation, it seems that the consequence of this modification is not determinant to increase SERCA activity, and negligible compared to PKA-dependent phosphorylation of PLB.

Ryanodine receptors

It has been shown in lipid-bilayer experiments with reconstituted channels that CaMKII dependent phosphorylation of the RyRs at Ser2814, increases their open probability (Wehrens et al., 2004). In intact myocytes, Li et al. (Li et al., 1997) demonstrated that endogenous CaMKII increases the amplitude of SR Ca^{2+} release for a given SR Ca^{2+} content and I_{Ca}. The was Ca^{2+} and CaMKII specific, because smaller conditioning Ca^{2+} transients failed to produce this phenomenon, as they were probably unable to activate CaMKII.

Accumulating evidence suggest that CaMKII participates in the modulation of the RyRs open probability and modulates gating, resulting in prolonged openings of RyRs and increased SR Ca^{2+} leak (Maier & Bers, 2007). However, the situation may be much more complex. One report (Rodriguez *et al.*, 2003), suggested that there may be at least four additional CaMKII phosphorylatable sites on the RyR. Their identity and functional characteristics are still unknown. In studies examining how Ca^{2+} sparks are affected by CaMKII activity, interesting observations were made with pharmacological interventions where the observed increase in spontaneous Ca^{2+} release events (Ca^{2+} sparks) where

drastically reduced when blocking CaMKII (using KN-93 or AIP). But also in transgenic animals which harbored either phosphorylation deficient (S2814A) or constitutively phosphorylated (S2814D) RyRs. In animal models of heart failure the hypothesis was studied that increased RyR phosphorylation by CaMKII is necessary and sufficient to induce lethal ventricular arrhythmias during stress, via increased spark frequency or triggered waves. This also resulted in reduced SR Ca^{2+} load and sudden death resulting from arrhythmias. Interestingly, the ablation of the serine S2814 CaMKII phosphorylation site on the RyRs (S2814A) protected the animals from sudden cardiac death (van Oort *et al.*, 2010). Other investigations have shown similar results when working with animal models of CaMKIIδ_C over-expression (Maier *et al.*, 2003), where it was found that Ca^{2+} handling was compromised by reduced SR Ca^{2+} load and by elevated SR Ca^{2+} release.

Taken together CaMKII appear to be responsible to sensitize the RyRs via phosphorylation of specific sites.

4.6.4.New concepts / pathways

cAMP dependent activation of Epac

Increasing evidence suggests that the exchange proteins activated by cAMP (Epac) participates in the activation of CaMKII. Pereira et al. (Pereira *et al.*, 2007) recently proposed new insight on this pathway. In their work, acute activation of Epac by 8-CPT led to CaMKII-dependent phosphorylation of the ryanodine receptor (RyR). 8-CPT is thought to be a cAMP analogue specifically activating Epac, but not PKA. Little evidence is known about the Epac-dependent activation of CaMKII. However it could probably involve products of PLC activity. PLC hydrolysis of PIP2 produce IP3 and diacylglycerol (DAG), which subsequent activate IP3 receptor-dependent Ca^{2+} release (a.k.a. Ca^{2+} puffs) which apparently sustains CaMKII activation. More evidence is needed to clarify this pathway/ activation mechanism and the role during physiology, for reviews see (Oestreich et al., 2007; Metrich et al., 2009).

ROS-dependent activation of CaMKII

In 2008 Erickson et al. (Erickson *et al.*, 2008) proposed a new mechanism for sustained activation of CaMKII via oxidation of methionine residues. As already mentioned, CaMKII is usually activated by Ca^{2+}/CaM, which after binding to CaMKII leads to auto-phosphorylation of the protein kinase, keeping it active for several seconds. However, this protein displays an alternative activation mechanism which makes activation to persist and even in the absence of Ca^{2+}/CaM. This occurs via oxidation of the methionines 281/282.

Upon initial Ca^{2+}/CaM-dependent CaMKII activation, exposure of the regulatory domain (which contains the methionine 281/282) allows these sites to be oxidized. After dissociation of the Ca^{2+}/CaM complex, the molecule is then unable to go back to the auto-inhibitory conformation, therefore yielding maintained activation (for several minutes).

Cardiac muscle generates ROS under many difference conditions, notably during a range of cardiac diseases, such as dystrophic cardiomyopathy (Jung *et al.*, 2008). Experimentally, ROS production has been detected during acute angiotensin II treatment of cardiomyocytes or during acute β-AR stimulation in beating cardiomyocytes (Bovo *et al.*, 2012).

Other protein kinases involved in EC-coupling regulation

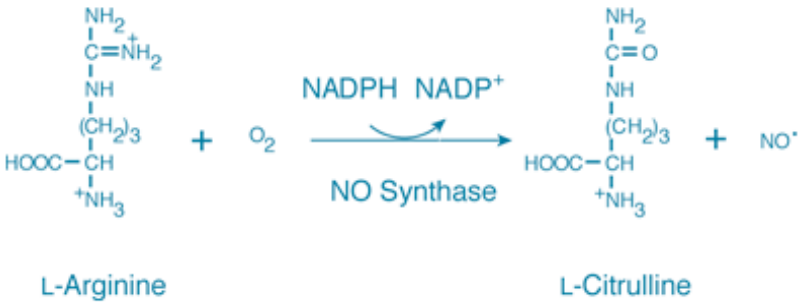
Other intracellular mechanism that coexist in physiological conditions such as AKT (a.k.a. protein kinase B), Epac, cGMP, PKG and PKC have been lately proposed to regulate EC-coupling. AKT for example has been shown to be activated during simulated ischemia and reperfusion. (Mockridge *et al.*, 2000) and it has also been shown to be activated during physical exercise, where apparently it increases I_{Ca}, which in turn enhances SR-Ca release(Bers, 2002) and lately AKT has been shown to be involved in the regulation of the signaling of inositol 1,4,5-trisphosphate receptors (IP_3R) as a target of their phosphorylation activity (Marchi *et al.*, 2008) for reviews see (Catalucci & Condorelli, 2006; Troncoso *et al.*, 2013).

Phosphorylation of the NCX could in principle accelerate the extrusion rate of Ca^{2+} during EC-coupling; for example by PKC phosphorylation. This has been a very discussed topic, the effect was initially observed in the giant axon but could not be mirrored in the cardiac tissue (Shigekawa & Iwamoto, 2001).

4.7.The role of NO and NOSs in EC-Coupling

4.7.1.cGMP independent: nitrosation

The identification of NO within the cardiovascular system is a fairly new, dating only since about 25 years (Espey *et al.*, 2006). For review see (Hare & Stamler, 2005). Investigations as those carried out by (Gonzalez *et al.*, 2010) and (Cutler *et al.*, 2012) have shown direct nitrosative effects of NO on the RyRs, which seem to increase the open probability of this channel.



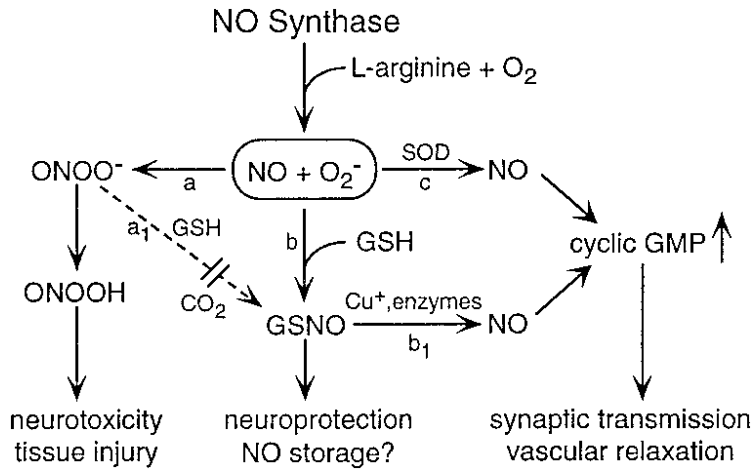


Fig 21: Description of the reaction of NO and its function on intracellular targets. Top Fig taken from the website of invitrogen.com and lower from (Mayer *et al.*, 1998).

NO is the second messenger for the cGMP which activates the phosphorylating kinase PKG; although this protein has been shown to phosphorylate the RyRs in-vitro only (Takasago *et al.*, 1991). Therefore, NO should not only be considered as a second messenger, but also as a compound directly nitrosating intracellular regulatory proteins.

4.7.2.cGMP-dependent NO signaling

Nitric oxide can display either positive or negative inotropic effects, depending on the experimental conditions. For example, it has been shown to modulate the β-adrenergic stimulation response, by decreasing intracellular levels of cAMP through a cGMP-dependent activation derived by iNOS (Joe *et al.*, 1998).

However this topic still remains controversial, partly because NO has also been proposed to modulate the positive inotropic response in cardiomyocytes. In a study by (Sarkar *et al.*, 2000) NO has been proposed to modulate the β-AR response, apparently also depending on the rate of NO delivery. In a report by (Petroff, 2002) it has been shown to modulate the response to mechanical stretch. This appeared to be specifically related to the eNOS isoform, which in concomitant activation through PI(3)K and Akt, augmented the amplitude of the Ca^{2+} transient but without altering the kinetics of the Ca^{2+} sparks.

Therefore NO plays a multifaceted role, which depending on the type of experimental conditions both regulate downstream Ca^{2+} regulatory proteins and modulate the positive or negative inotropic responses (for reviews see (Lim *et al.*, 2007)).

4.8.Known targets for NO signaling in EC-coupling

As mentioned above NO can be generated by different isoforms with distinct sub-cellular location. For example, eNOS derived NO has been shown to modulate the rate of activation of the LTCC (Suzuki *et al.*, 2010). Since eNOS is located in the caveolae of the cell membrane, its proximity to the L-type Ca^{2+} channels may account for this type of regulation.

As iNOS is not expressed in the healthy heart, there is one more isoform that can be important when properly stimulated, the nNOS. NO derived from this isoform apparently accounts mainly for the regulation of the RyRs, as it colocalizes with the RyRs on the SR membrane, as suggested by coimmunoprecipitation experiments (Barouch *et al.*, 2002).

4.8.1.L-type Ca^{2+} current

It has been shown that NO can directly inhibit the L-type Ca^{2+} channels which, would decrease positive inotropy (Hu *et al.*, 1997). This inhibition has been explained by NO acting on the sulfhydryl groups of the channel, which may be nitrosated or oxidized to render the channel's inhibitory behavior. But in contrast to what it was shown by (Suzuki *et al.*, 2010) the LTCC could actually be activated by eNOS derived NO, although this experiments were not done on cardiomyocytes which may explain these findings. In a study by (Sarkar *et al.*, 2000) the rate of NO donation was determinant to modulate the β-AR response, but it was not identified wether this was due to sensitization of the RyRs, change of the LTCC gating or other intermediate NO dependent mechanisms. Hence, this topic still remains controversial due to the aforementioned dual behavior of NO in cardiomyocytes and partly due to the location of the NOS isoforms (for reviews see (Ziolo, 2003)).

4.8.2.RyR

Under physiological conditions nNOS may primarily generate NO which in combination with GSH can form GSNO (Mayer *et al.*, 1998). The formation of this molecule either release NO again or transfer NO^+ to free thiols. Either way, the generation of this RNS may account for the observed cellular behaviors during certain pharmacological interventions through post-translational modification of cysteine residues.

As it was shown by (Sarkar *et al.*, 2000), NO can increase inotropy and independently of the type of RNS. In their work, they emphasized the role of a fast NO release by NO donors seemed to be a key factor for the observed effects. This may be explained as myoglobin could have acted as a fast NO scavenger (Flögel *et al.*, 2001). RyR nitrosation has also been reported to increase the SR Ca^{2+} leak. In the work of (Petroff, 2002) it was shown how during stretching of cardiomyocytes the NO derived from the eNOS isoform would in fact increase the frequency of Ca^{2+} sparks; which suggests that NO modulates the P_o of the RyRs. This effect was not seen in eNOS deficient cardiomyocytes.

nNOS is a Ca^{2+} activated enzyme, therefore it can be modulated during CICR, probably as a feedback mechanism for RyRs-dependent nitrosation. Further, (Xu *et al.*, 1998) found that the RyR2 has ~84 free cysteine residues per monomeric subunit; they also found that modification due to S-nitrosation of ~3 thiol sites per monomer, would increase 2 to 3 fold the Ca^{2+} transient. (Ziolo *et al.*, 2001) found that NO in combination with Iso (which would modify the levels of PKA dependent phosphorylation of the RyRs) can influence the functional effects of the RyRs-dependent S-nitrosation. Experimenting in high oxygen pressure conditions, as in the laboratory may promote the formation of ROS (Sun *et al.*, 2001). This molecules can change the levels of oxidation and behavior of RyR as the reaction between NO and O_2^- can, in some oxidizing conditions, generate peroxynitrite $ONOO^-$ (Sun *et al.*, 2008).

Finally has also been observed in failing cardiomyocytes that after myocardial infarction, the nNOS isoforms can actually migrate to the cell membrane, as it was shown by (Bendall *et al.*, 2004) that in a model of failing heart this isoform would co-localize with caveolin-3. The migration of the nNOS may have critical consequences as RyRs-dependent S-nitrosation might be lost, LTCC could get more inhibited and the xanthine oxidoreductase (which produces ROS) might be released from the nNOS-dependent

regulation(Xu *et al.*, 1998), hence creating more ROS which could alter the RyRs irreversibly.

Taken together, NO-dependent nitrosation of the RyRs might be important for the regulation of the channel and the effects that other reactive intermediates could exert on the channel.

4.8.3.Others

It has been reported that NO may act on the SERCA in a stimulatory fashion (Adachi *et al.*, 2004), which may increase the SR Ca^{2+} content; however we found in the attached manuscript that the increased frequency of Ca^{2+} sparks mediated via the action of NO on CaMKII was more relevant, ultimately leading to the opposite, SR Ca^{2+} depletion.

Other intracellular targets of NO might be intermediates of the family of the cysteine proteases such as caspases (Mitchell & Marletta, 2005), which have been shown to modulate apoptosis in cardiomyocytes (Communal *et al.*, 2002). Finally it has also been show that after Iso stimulation, nitrosation of the small family of proteins β-arresting, does modulate the trafficking of the beta receptors, hence accelerating internalization (Ozawa *et al.*, 2008), which might decrease the affect of adrenaline.

4.9.Proposed role in physiology and pathophysiology

4.9.1.NO/redox dysequilibrium

NO has been found to maintain an equilibrium that protects the RYRs from oxidation (Gonzalez *et al.*, 2010), also called a nitroso-redox balance (for reviews see (Hare, 2004)).

This was for instance shown in failing cardiomyocytes from Wistar- Kyoto rats which presented a dysequilibrium in the ROS/RNS balance, mainly by yielding low levels of S-nitrosation on the RyRs.

The RyRs present ~84 free thiols per homotetramer that can be either oxidized or nitrosated. The balance between these reactions seems to govern the behavior of the RYRs and their open probability. In physiological conditions (~10 mmHg PO_2 levels and ~5mM reduced glutathion GSH) (Sun *et al.*, 2008) showed that the RyRs present a 60% : 40% balance between free and occupied thiols, respectively. This information tells us that in physiological conditions ~60% of the free thiols can be either oxidized or nitrosated, which would determine the balance between this reactions and determine cell behavior.

Therefore it seems that NOS isoforms and their connection to different compartmentalized intracellular spaces, such as Caveolin 3 for eNOS (or nNOS during disease) and nNOS with XOR and the RyRs, need all an equilibrium to function. Otherwise, other species may take over and alter intracellular processes (For reviews see (Brieger *et al.*, 2012)).

4.9.2.Cardiac Arrhythmia

Direct RyRs S-nitrosation has been suggested to induce aberrant arrhythmias in the intact heart (Cutler *et al.*, 2012). This molecule can also target other Ca^{2+} regulatory proteins that upon nitrosation can alter their regulatory function, such as the L-type Ca^{2+} channels by their inhibition and Ca^{2+} re-uptake in the SR by stimulation of SERCA (Hu *et al.*, 1997).

A misbalance between these mechanisms then can produce arrhythmogenicity, an overloaded SR can trigger Ca^{2+} waves when in combination with an increased open probability of the RyRs (Venetucci *et al.*, 2007).

However the picture is not clear. NO has also been reported to prevent from cardiac arrhythmias (Burger & Feng, 2011), therefore it seems that it really depends on the type of experiments and whether if they include processes such as CICR or conditions when SERCA is stimulated as it happens in combination with β-AR stimulation (Ziolo *et al.*, 2001).

Moreover as proposed in this thesis NO can modulate CaMKII during β adrenergic stimulation; this conditions would favor the maintenance of a sustained SR Ca^{2+} content which would promote the appearance of Ca^{2+} waves.

4.9.3.DADs

Afterdepolarizations (ADs) are a class abnormal cardiac muscle depolarizations that are activated during the decline of the AP or after it has finished. Delayed afterdepolarizations (DADs) occur during phase 4 of the AP (see section “4.3. Action potential in Guinea-pig ventricular myocytes”). The potentially arrhythmogenic DADs are generated when Ca^{2+} leaks out of the SR, in the form of Ca^{2+} waves, inducing activation of the electrogenic NCX by extruding Ca^{2+} and introducing Na^{+} to the cell. The resulting inward current could produce arrhythmogenic delayed afterdepolarization (DADs) depending on the magnitude of the stimuli (Guo *et al.*, 2006; Shiferaw *et al.*, 2012).

In the heart, oxidative stress caused by ROS has been also shown to induce DADs. It was believed that ROS would affect the L-type Ca^{2+} channels but it was later also demonstrated that other targets of ROS may have become activated, especially by the CaMKII dependent LTCC facilitation mechanism (Xie et al., 2009; Sun & Pitt, 2011).

However, NO has been shown to inhibit the L-type Ca^{2+} channels (Sears *et al.*, 2003; Lim *et al.*, 2007), therefore we could hypothesize that the DADs we observed have to be triggered via SR-dependent Ca^{2+} release during diastole (Guo *et al.*, 2006). It has been shown that cardiomyocytes deficient in endothelial nitric oxide synthase ($eNOS^{-/-}$) exhibit long APs. They also had increased spontaneous DADs during β-adrenergic stimulation and an increased SR Ca^{2+} content (Wang *et al.*, 2012). This combination of features

clearly points towards other mechanisms, but not LTCC modulation, which could govern DADs in combination with β-adrenergic stimulation.

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5.ORIGINAL MANUSCRIPT AND PUBLISHED REVIEW

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NO-dependent CaMKII activation during β-adrenergic stimulation of cardiac muscle

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Aims During β-adrenergic receptor (β-AR) stimulation, phosphorylation of cardiomyocyte ryanodine receptors by protein kinases may contribute to an increased diastolic Ca^{2+} spark frequency. Regardless of prompt activation of protein kinase A during β-AR stimulation, this appears to rely more on activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), by a not yet identified signalling pathway. The goal of the present study was to identify and characterize the mechanisms which lead to CaMKII activation and elevated Ca^{2+} spark frequencies during β-AR stimulation in single cardiomyocytes in diastolic conditions.

Methods and results Confocal imaging revealed that β-AR stimulation increases endogenous NO production in cardiomyocytes, resulting in NO-dependent activation of CaMKII and a subsequent increase in diastolic Ca^{2+} spark frequency. These changes of spark frequency could be mimicked by exposure to the NO donor GSNO and were sensitive to the CaMKII inhibitors KN-93 and AIP. *In vitro*, CaMKII became nitrosated and its activity remained increased independent of Ca^{2+} in the presence of GSNO, as assessed with biochemical assays.

Conclusions β-AR stimulation of cardiomyocytes may activate CaMKII by a novel direct pathway involving NO, without requiring Ca^{2+} transients. This crosstalk between two established signalling pathways may contribute to arrhythmogenic diastolic Ca^{2+} release and Ca^{2+} waves during adrenergic stress, particularly in combination with cardiac diseases. In addition, NO-dependent activation of CaMKII is likely to have repercussions in many cellular signalling systems and cell types.

Keywords CaMKII • Ca sparks • Ca waves • NO-synthase

1. Introduction

In cardiac excitation–contraction coupling Ca^{2+}-induced Ca^{2+} release (CICR) is the mechanism that amplifies the Ca^{2+} signal initiated by entry of Ca^{2+} via voltage-dependent Ca^{2+} channels.[1] During each systole, CICR generates a robust Ca^{2+} transient by releasing Ca^{2+} from the sarcoplasmic reticulum (SR) via Ca^{2+} release channels (a.k.a. ryanodine receptors or RyRs). These are macromolecular complexes located in diadic clefts, microdomains of junctional SR, in close apposition to L-type Ca^{2+} channels.[2] Each group of RyRs and L-type Ca^{2+} channels forms a unit called couplon which can create elementary Ca^{2+} release events, Ca^{2+} sparks.[3] Faithful RyR functioning is crucial in the context of cardiac muscle Ca^{2+} release, its synchronization, and strength of contraction. Disturbances of these elementary mechanisms have been observed during various cardiac pathologies.[4,5]

Upon β-adrenergic receptor (β-AR) stimulation during emotional stress or physical exercise, key proteins of Ca^{2+} signalling, such as the L-type Ca^{2+} channels, the RyRs, and phospholamban are phosphorylated by protein kinases,[6,7] enhancing Ca^{2+} cycling. There is recent evidence indicating that elevated phosphorylation levels of the RyR mediated by protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) may increase their activity[8–11] (for reviews see refs[7,12]). During chronic β-AR stimulation, this could constitute a Ca^{2+} leak and deplete the SR of Ca^{2+}, which would reduce the amplitude of Ca^{2+} transients, eventually contributing to weak heartbeats.[13]

Experimentally, it has proven difficult to clearly assign a role for the different kinases in modulating RyR behaviour. The generation of various transgenic mouse lines, specifically targeting phosphorylation by PKA and CaMKII, has not clarified the situation and provided apparently contradictory results and interpretations. However, analysis of Ca^{2+} spark frequencies in transgenic mice expressing constitutively phosphorylated RyRs (S2808D, S2814D) suggested that PKA- and CaMKII-dependent phosporylation may increase resting Ca^{2+} spark frequencies.[5,9]

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Here, we examined Ca^{2+} sparks as signals closely reflecting RyR open probability in their native environment. While there is controversial literature on the relative importance of PKA- and/or CaMKII-dependent RyR phosphorylation,[8,14] only few studies specifically investigated changes of Ca^{2+} spark frequencies resulting from β-AR stimulation during diastole or in resting cardiomyocytes.[5,9,15,16] Based on these findings, it has been suggested that PKA-dependent phosphorylation of RyRs may not be significantly involved in the observed increase in the resting Ca^{2+} spark frequency. It has been reported that this entirely hinged on the SERCA stimulation resulting from phosphorylation of phospholamban[17] or occurred via a pathway that could involve CaMKII.[14,16,18–20] Since the CaMKII-dependent increase in spontaneous spark frequencies was observed in resting cardiomyocytes without detectable Ca^{2+} signals, which are typically required for significant Ca^{2+}-dependent activation of CaMKII,[21,22] it remained unresolved by which pathway(s) CaMKII would become activated under these conditions.

The current study represents an effort to reveal the mechanism involved in the activation of CaMKII in resting cardiomyocytes during β-AR stimulation and to identify an alternative pathway that could underlie the observed increase in Ca^{2+} spark frequency.

In the literature, several possible mechanisms have been mentioned. The 'exchange protein activated by cAMP' (Epac) may participate in the modulation of RyR open probability, either directly or via CaMKII.[18] Another alternative is activation of CaMKII by reactive oxygen species (ROS), which is known to occur independently of detectable Ca^{2+} signals.[23] Therefore, we carried out experiments investigating Ca^{2+} sparks and CaMKII activity to characterize the putative involvement of these and other cellular signalling pathways.

Together our findings reveal that upon β-AR stimulation, CaMKII becomes activated in a manner that does not require Ca^{2+} transients, initiated by formation of endogenous nitric oxide (NO). This represents an unexpected and newly discovered mode of CaMKII activation occurring in parallel to stimulation of PKA by cAMP. Preliminary findings have previously been presented in the abstract form.[24]

2. Methods

For additional information on methods, see Supplementary material online.

2.1 Isolation of Guinea-pig ventricular myocytes

For all electrophysiological and confocal Ca^{2+} and NO imaging experiments, we used freshly isolated Guinea-pig ventricular cardiomyocytes[16] following the animal handling procedures conforming with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996) and with the permission of the State Veterinary Administration and according to Swiss Federal Animal protection law (permit BE97/09). Animals were euthanized by stunning and cervical dislocation, followed by rapid removal and enzymatic dissociation of the cardiac tissue.

2.2 Experimental solutions

All drugs, inhibitors, and donors used during our experiments were freshly prepared daily from aliquots. Extracellular and intracellular (patch pipette) solutions were used from a ready-made stock.[16]

2.3 Electrophysiological recordings

I_{Ca} recordings were carried out in the whole-cell configuration of the patch clamp technique, resting membrane potential was set at −80 mV during Ca^{2+} imaging.[16]

2.4 SR Ca^{2+} content pre-conditioning

To ensure a constant SR Ca^{2+} content, we performed a SR Ca^{2+} loading protocol with a train of 20 membrane depolarizations from −80 to 0 mV (*Figure 1A*). Pharmacological interventions were applied, as indicated. Changes in SR Ca^{2+} content were compared with control conditions without the drug, after an identical loading protocol. In all cases, content was estimated by recording a Ca^{2+} transient triggered with caffeine.

2.5 Confocal Ca^{2+} and NO imaging

The Ca^{2+} spark frequency and SR Ca^{2+} content are shown after normalization and expressed as mean values ± SEM. For these recordings, we used fluo-3 as Ca^{2+} indicator. NO measurements were carried out using DAF-2DA. Confocal imaging was performed with either a FluoView-1000 (Olympus) or a MRC-1000 confocal laser-scanning microscope (Bio-Rad). Indicators were excited at 488 nm with a solid-state laser (Sapphire 488–10) and fluorescence was detected >515 nm.

2.6 *In vitro* $CaMKII_{\delta}$ activity and nitrosation assays

Ca^{2+}-independent, H_2O_2 and NO-dependent CaMKII activities were assessed by using an ELISA kit. Values are shown normalized to the maximal CaMKII activation levels reached in low Ca^{2+} (<10 nM Ca^{2+}; CaM/EGTA). Nitrosation of CaMKII was quantified using an antibody specifically detecting *S*-nitrosated cysteines.

2.7 Statistics

Paired or unpaired Student's *t*-tests were applied as appropriate to determine significance. In figures *P*-values of <0.05 or <0.01 are indicated by * or **, respectively. *N* refers to number of animals, and *n* to number of cells.

3. Results

3.1 Ca^{2+} spark frequency during β-AR stimulation is modulated by CaMKII but not PKA

We investigated cAMP-dependent pathways (e.g. PKA, Epac) to determine the involvement of PKA and/or CaMKII in the modulation of resting Ca^{2+} spark frequencies. Constant SR loading was achieved with Ca^{2+} pre-loading involving a train of L-type Ca^{2+} currents. SR Ca^{2+} content was estimated with caffeine before and after the experiment in each cardiomyocyte (*Figure 1A*).[16] As shown in *Figure 1B* and *C*, superfusion of resting cells with 1 μM isoproterenol (Iso) increased the frequency of Ca^{2+} sparks around 4-fold within 3 min, without significantly altering the $[Ca^{2+}]_{SR}$ in this time window (*Figure 1E*).[16] Next, cAMP was raised independently of the β-AR receptors by direct activation of adenylate cyclase with forskolin.[11] Surprisingly, and unlike Iso, 1 μM forskolin did not change the Ca^{2+} spark frequency significantly, even though the SR content increased to 125%, presumably resulting from SERCA stimulation without activation of sparks in parallel. Importantly, both drugs resulted in almost identical amplification of the L-type Ca^{2+} current (I_{Ca}), confirming that forskolin activated PKA to an extent corresponding to Iso application (*Figure 1D*). Inclusion of the specific PKA inhibitory peptide PKI in the patch solution did not prevent the increase in the Ca^{2+} spark frequency, but resulted in a drop of SR Ca^{2+} content, presumably resulting from the suppression of SERCA stimulation during Iso.

These results indicate that acute β-AR stimulation increases Ca^{2+} spark frequency, independently of cAMP and therefore makes an involvement of PKA and Epac very unlikely. Hence, we did not follow

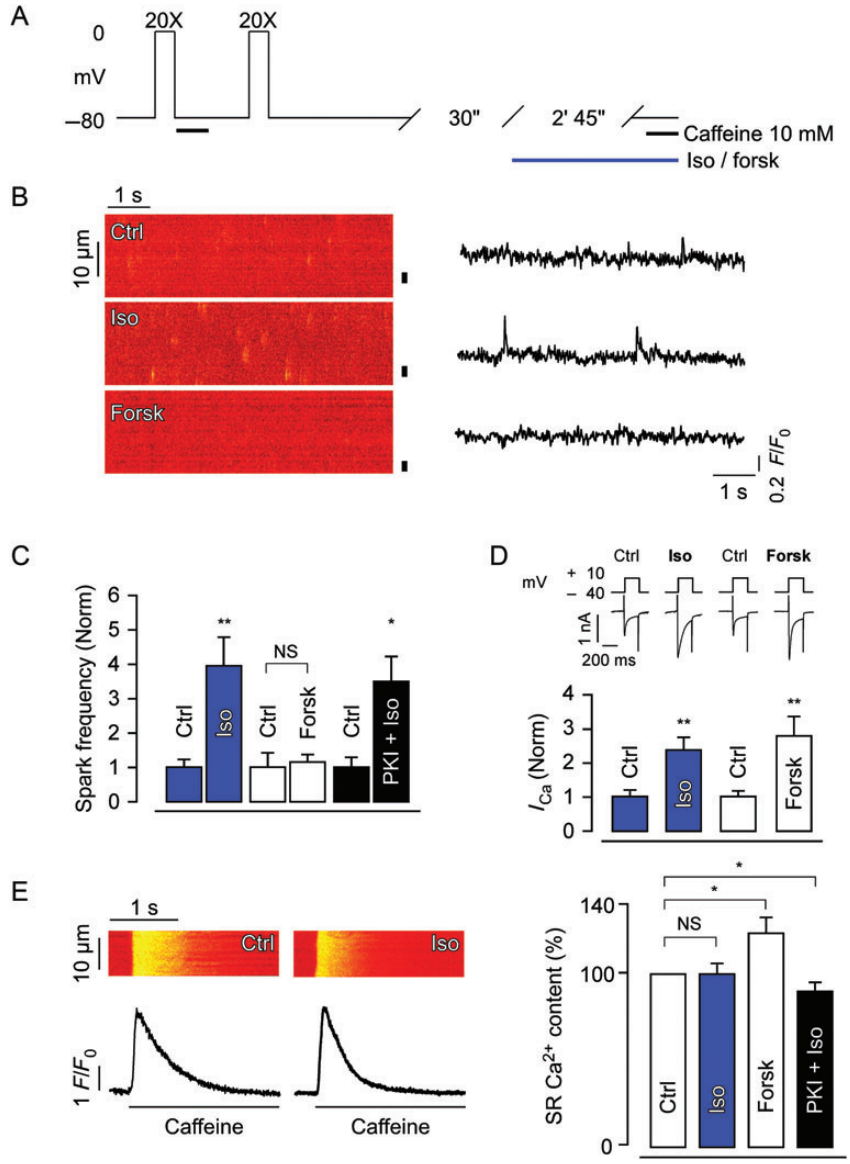

Figure 1 The increase in Ca^{2+} spark frequency by Iso is mediated by β-adrenergic receptors but not by cAMP. (*A*) The experimental protocol used trains of depolarizations to load the SR with Ca^{2+} and caffeine to estimate SR Ca^{2+} content (for details see Supplementary material online, information). (*B*) Confocal line-scan images showing Ca^{2+} sparks in control solution (Ctrl) and after ∼3 min of 1 μM Iso or 1 μM forskolin. (*C*) Normalized Ca^{2+} spark frequency in Ctrl, after ∼3 min of Iso ($n = 8$, $N = 8$) or forskolin ($n = 6$, $N = 2$) and after Iso in the presence of the PKA inhibitor PKI ($n = 7$, $N = 3$), respectively. In control, Ca^{2+} sparks are relatively indistinct and sparse in resting guinea pig cardiomyocytes[16] (around 1 s^{-1} 100 μm^{-1}). While Iso led to a ∼4-fold increase in Ca^{2+} spark frequency, this was not observed with forskolin and was not prevented by the PKA inhibitor PKI. (*D*) Ca^{2+} current in Ctrl and after ∼3 min Iso or forskolin. For this experiment, the cells were held at −40 mV to inactivate Na^{+} currents. Normalized Ca^{2+} current in Iso ($n = 5$, $N = 4$) or forskolin ($n = 6$, $N = 5$). Current stimulation by forskolin was similar to that by Iso, documenting comparable PKA activation. (*E*) Typical SR Ca^{2+} content assessment by 10 mM caffeine. In forskolin, SR content is increased to 125 ± 8.8% ($n = 15$, $N = 5$) because of SERCA stimulation without activation of sparks. In contrast, in PKI the SR Ca^{2+} content decreased, partly because PKA inhibition prevents SERCA stimulation.

up on either of these pathways. A potential alternative pathway could be CaMKII, despite the fact that in quiescent cells, there were no Ca^{2+} signals that could activate this kinase. To confirm involvement of CaMKII, as also suggested by our previous study,[16] we carried out experiments in the presence of either KN-93 or KN-92 or included 10 μM of the specific CaMKII inhibitor autocamtide-2-related inhibitory peptide (AIP) in the patch solution. KN-93 and AIP prevented the increase in Ca^{2+} spark frequency in Iso without changing the SR Ca^{2+} content (*Figure* 2*A*–*C*). KN-92, the inactive negative control for KN-93, did not suppress the increase in spark frequency, as expected. Thus, under these experimental conditions, we can use the Ca^{2+} spark frequency as biological indicator for CaMKII activity.

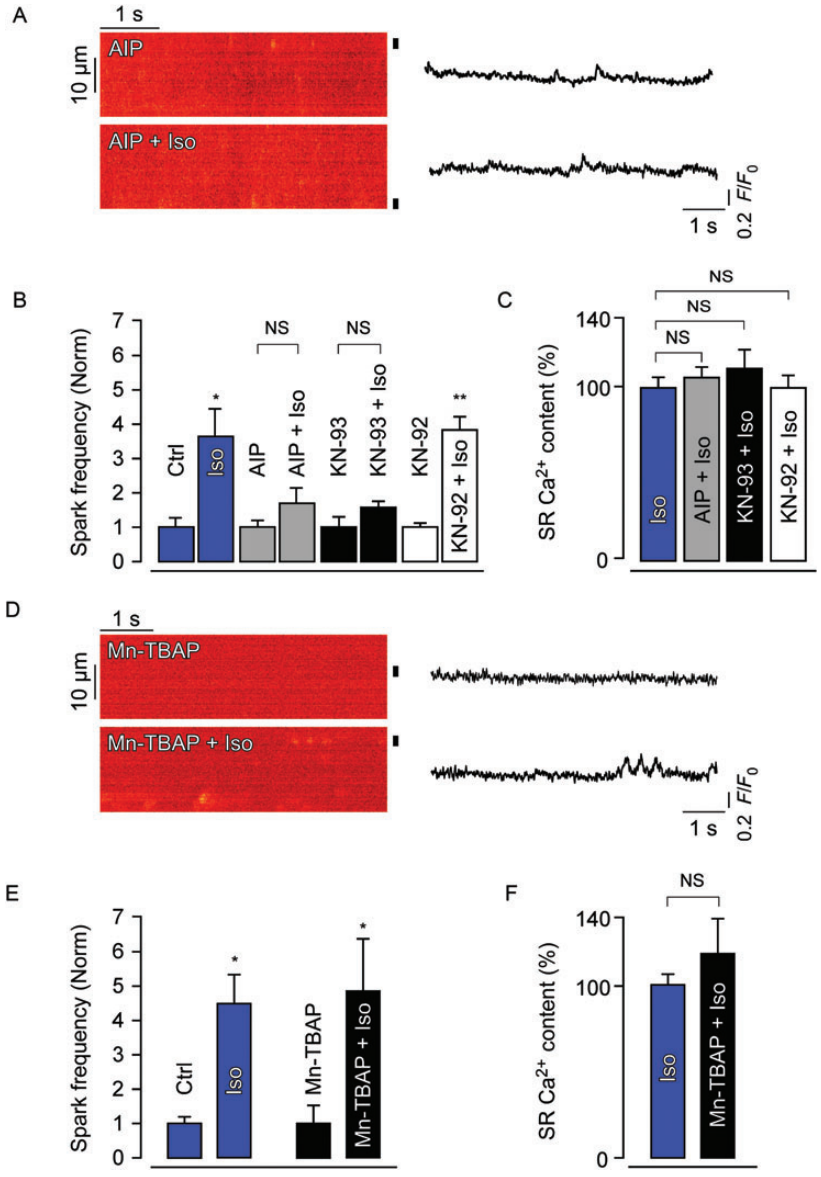

Figure 2 CaMKII is involved in spark frequency modulation, but not via ROS-dependent CaMKII activation. (*A*) Line-scan images in Ctrl and after 1 μM Iso, both in the presence of 10 μM of AIP in the patch pipette. (*B*) Ca^{2+} spark frequency in Ctrl and in Iso, in the absence ($n = 8, N = 8$) or presence of the CaMKII inhibitors AIP ($n = 9, N = 5$), KN-93 ($n = 5, N = 2$), and its inactive analogue KN-92 ($n = 5, N = 3$). (*C*) Unchanged SR Ca^{2+} content in the presence of AIP, KN-93, and KN-92. (*D*) Line-scan images recorded after 1 h pre-incubation with 100 μM ROS scavenger Mn-TBAP in Ctrl and after ~3 min of 1 μM Iso. (*E*) Ca^{2+} spark frequency during Iso without ($n = 8, N = 8$) and with Mn-TBAP ($n = 5, N = 5$). (*F*) Unchanged SR Ca^{2+} content in the presence of the ROS scavenger Mn-TBAP.

3.2 ROS scavengers fail to prevent the increase in Ca^{2+} spark frequency

We then examined whether Ca^{2+}-independent CaMKII activation by ROS[23] could underlie the higher spark frequency. For this, we used Mn-TBAP, a superoxide dismutase (SOD) mimetic, which has previously been shown in our experiments to reliably suppress Ca^{2+} sparks initiated by oxidative stress.[25] Interestingly, 100 μM Mn-TBAP failed to prevent the increase in Ca^{2+} spark frequency and did not significantly alter SR Ca^{2+} load (*Figure 2D–F*), indicating that CaMKII activation by β-AR stimulation is not ROS dependent.

3.3 NO modulates Ca^{2+} spark frequency upon β-AR stimulation

It has been suggested that after β-AR stimulation the increase in SR Ca^{2+} leak, determined with a dedicated leak protocol, is dependent on NO production and independent of PKA, because NO-synthase (NOS) inhibition prevented the leak observed in the presence of Iso.[11,26] To examine whether a similar mechanism could be involved in the higher frequency of Ca^{2+} spark observed here, we inhibited the synthesis of NO while stimulating the cardiomyocytes with Iso, analogous to experiments described in *Figure 1*. Pre-treatment with 500 μM of the NOS

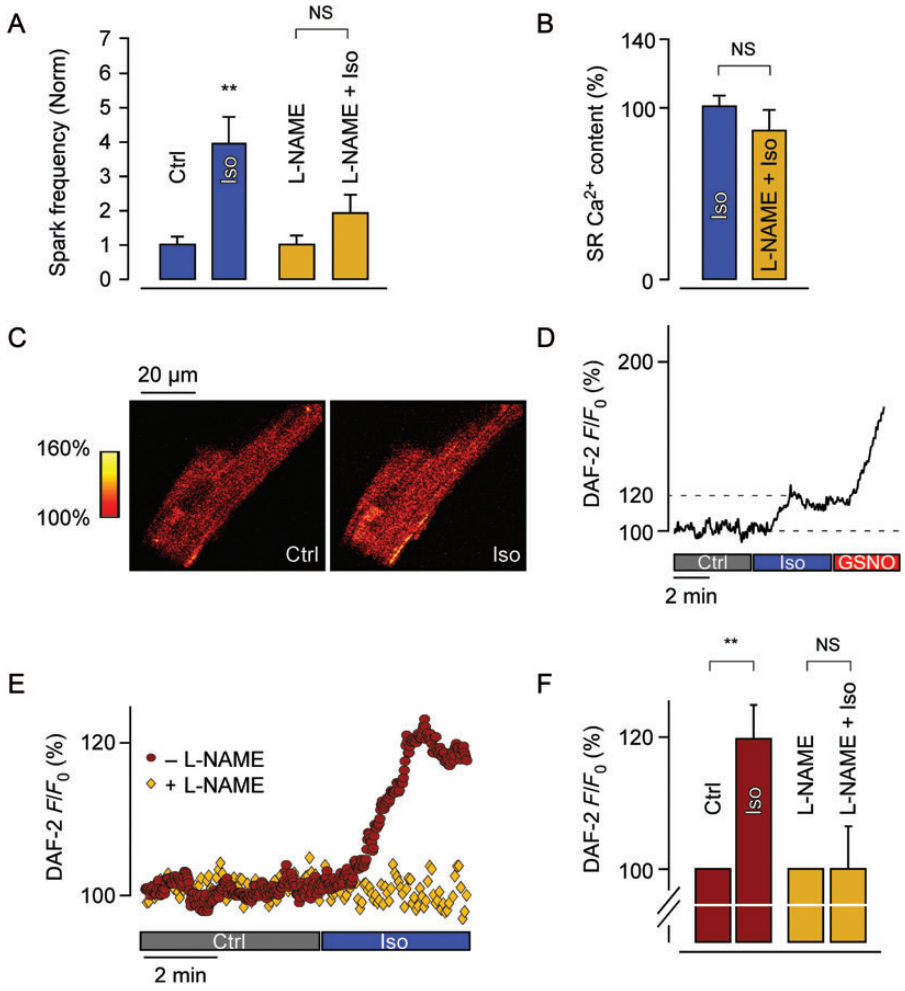

Figure 3 The increase in Ca^{2+} spark frequency is dependent on NO. (*A*) Comparison of the Ca^{2+} spark frequency stimulation by 1 μM Iso without ($n = 8$, $N = 8$) and with ($n = 7$, $N = 5$) 1 h pre-incubation with 500 μM L-NAME. (*B*) SR Ca^{2+} content remained unchanged in the presence of L-NAME. (*C*) Representative signal of DAF-2 fluorescence before (Ctrl) and after ~3 min of 1 μM Iso. (*D*) Representative time-course of NO production upon 1 μM Iso application. At the end, GSNO is applied as a positive control for NO detection. (*E*) Averaged time-course of DAF-2 fluorescence in Ctrl and during Iso recorded in the absence (red trace $n = 7$, $N = 6$) and presence of L-NAME (orange trace, $n = 6$, $N = 4$). (*F*) Normalized NO-induced DAF-2 fluorescence upon Iso (red bar) and after pre-incubation in 500 μM L-NAME (orange bar). L-NAME prevented the NO signal observed in control, confirming that the DAF-2 fluorescence resulted from NO production.

inhibitor L-NAME prevented a significant increase in Ca^{2+} spark frequency (*Figure 3A*), indicating that upon β-AR stimulation, the spark frequency is modulated by endogenous NO produced by NOS of the cardiomyocyte.

To confirm NO involvement, cells were loaded with diaminofluorescein (DAF-2) by exposure to the ester (DAF-2DA; 0.1 μM). After application of Iso, we recorded a substantial increase in DAF-2 fluorescence (19 ± 5%; *Figure 3C* and *E*). In some cells, the NO donor *S*-nitroso-L-glutathione (GSNO; 500 μM) was added at the end of the protocol, to confirm that DAF-2 resolves NO signals (*Figure 3D*). Please note that these recordings were corrected for dye bleaching and should not be taken quantitatively for NO concentrations. Because DAF-2 has been reported to detect other types of reactive species,[27] we repeated the same experiment in 500 μM L-NAME to suppress NO formation. This prevented the increase in DAF-2 fluorescence (*Figure 3E* and *F*, orange symbols and columns), confirming that the signal reflects NO production. Cellular ROS production induced by GSNO was excluded with the ROS sensitive fluorescent indicator CM-H_2DCF (see Supplementary material online, *Figure S1*). Together, these findings firmly establish a link between Iso-induced production of intracellular NO by the cardiomyocytes and the observed increase in Ca^{2+} spark frequencies mediated by CaMKII. This interpretation is in line with the inability of Mn-TBAP to prevent the increase in Ca^{2+} spark frequency (*Figure 2D* and *E*) and is consistent with the observation that Mn-TBAP does not significantly scavenge NO.[28]

3.4 The NO donor GSNO reproduces the increase in Ca^{2+} spark frequency induced by β-adrenergic stimulation

NO can affect the function of proteins, including the RyRs, via several known pathways.[29–31] This can occur directly as post-translational protein modifications, such as *S*-nitrosation (also referred to as *S*-nitrosylation or transnitrosylation).[12,32] Alternatively, NO can lead

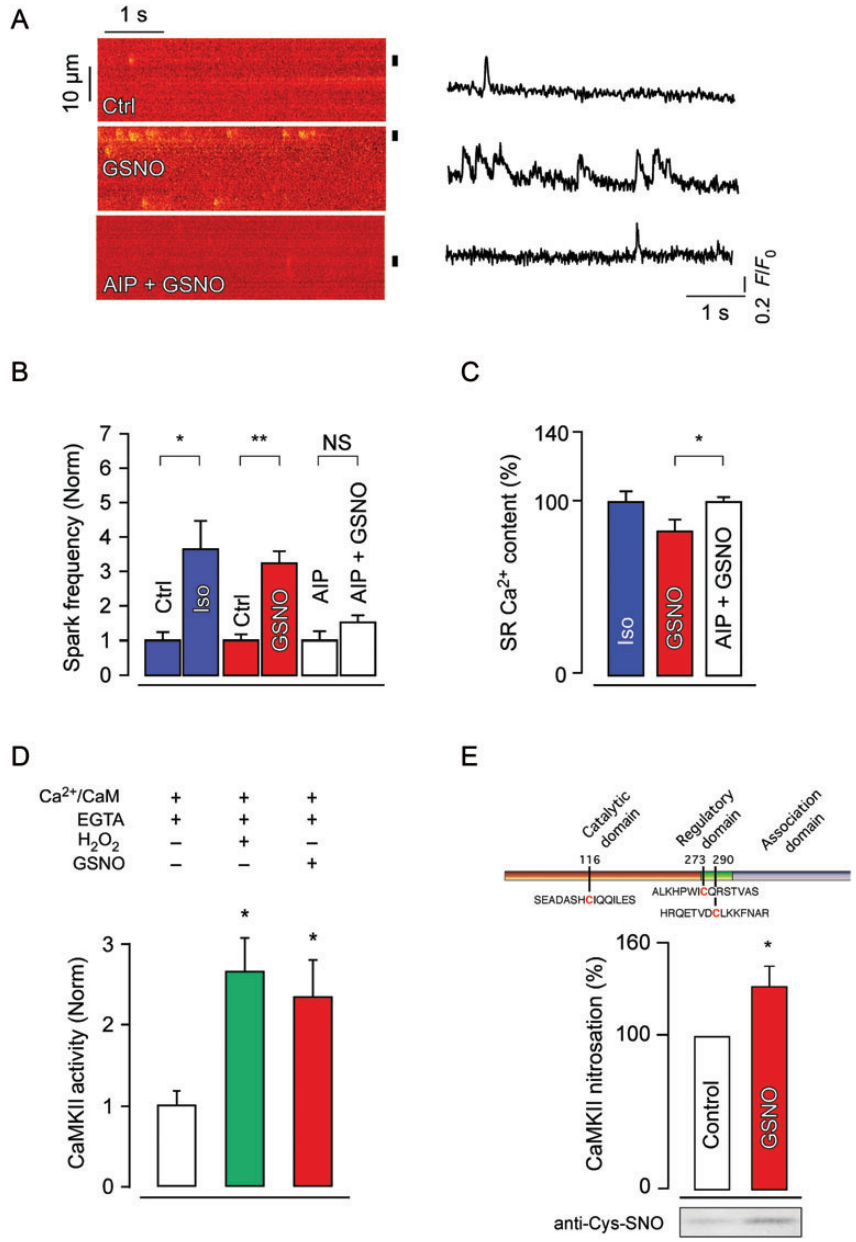

Figure 4 The NO donor GSNO increases Ca^{2+} spark frequency upon CaMKII activation via nitrosation. (*A*) Line-scan images of Ca^{2+} spark recordings in Ctrl (top), during 150 µM GSNO alone (middle), and GSNO in the presence of 10 µM AIP in the patch pipette (bottom). (*B*) Normalized spark frequencies after ~3 min of 1 µM Iso (blue bar $n = 6$, $N = 6$) and GSNO alone (red bar $n = 14$, $N = 11$) and with 10 µM AIP (white bar $n = 9$, $N = 4$). Inhibiting CaMKII with AIP prevented the higher frequency induced by GSNO. (*C*) After GSNO, the SR Ca^{2+} content was not maintained (83 ± 6% of control) due to a higher spark frequency without SERCA stimulation in parallel. (*D*) Quantitative *in vitro* CaMKII activation in response to 1 µM H_2O_2 (blue bar) or 500 µM GSNO (red bar) in comparison with control (white bar). CaMKII activities were normalized to full Ca^{2+}/CaM-dependent and control activities ($N = 5$). (*E*) Detection of nitrosated CaMKII *in vitro* by anti-Cys-SNO specific antibody reveals increased nitrosation in GSNO.

to the formation of cGMP and activation of protein kinase G (PKG), which has been shown to phosphorylate RyRs, but so far only *in vitro*.[33] The findings in *Figure 3* indicate that after Iso application, NO mediates the increase in Ca^{2+} spark frequency. To support this interpretation, we used a NO donor instead of Iso. One hundred fifty micromolar GSNO resulted in a 3.22 (±0.31)-fold increase in Ca^{2+} spark frequency, thereby quantitatively mimicking the changes observed in Iso (*Figure 4A* and *B*). Note that in these experiments, SR Ca^{2+} content did not maintain the control level, presumably because GSNO increased the Ca^{2+} spark frequency without concomitant SERCA stimulation (*Figure 4C*). To distinguish between a direct *S*-nitrosation of the RyRs and an indirect modification via CaMKII (suggested by the findings above), we tested whether AIP could prevent the GSNO-dependent occurrence of sparks, similar to what was observed in Iso. In the presence of AIP, GSNO did not significantly elevate spark frequency (*Figure 4B*). These findings confirm that the higher spark frequency in GSNO resulted largely from activation of CaMKII and not from a direct *S*-nitrosation of the RyRs or activation

of PKG. In summary, these data indicate that NO can activate CaMKII, in the absence of Ca^{2+} signals.

3.5 Quantification of NO-dependent CaMKII activation *in vitro*

To confirm that NO could activate CaMKII in the absence of elevated Ca^{2+}, we used an *in vitro* assay.[34] CaMKII activity was detected with an ELISA test and normalized to that observed in low Ca^{2+} (<10 nM; Ca^{2+}, CaM, and EGTA, *Figure 4D*). As already established,[23] addition of H_2O_2 activated CaMKII in low Ca^{2+}. The activity observed in 1 μM H_2O_2 was 2.65 (±0.47)-fold higher than in control. The NO donor GSNO resulted in comparable CaMKII stimulation of 2.31 (±0.39)-fold. CaMKII activity under these oxidative and nitrosative conditions represented ~16% of the maximal Ca^{2+}/CaM dependent activity (250 μM Ca^2 and 120 nM calmodulin). This confirms a direct activation of CaMKII by NO, as suggested by our findings in cardiomyocytes.

Each CaMKII monomer is predicted by GPS-SNO software[35] to have three potential sites for *S*-nitrosation (*Figure 4E*, upper panel). We used an antibody specifically recognizing *S*-nitrosated cysteines to quantify CaMKII nitrosation after pre-incubation of $CaMKII_\delta$ with GSNO (*Figure 4E*, lower panel). Indeed, a 32.1 ± 13% increase of CaMKII nitrosation was observed with this assay.

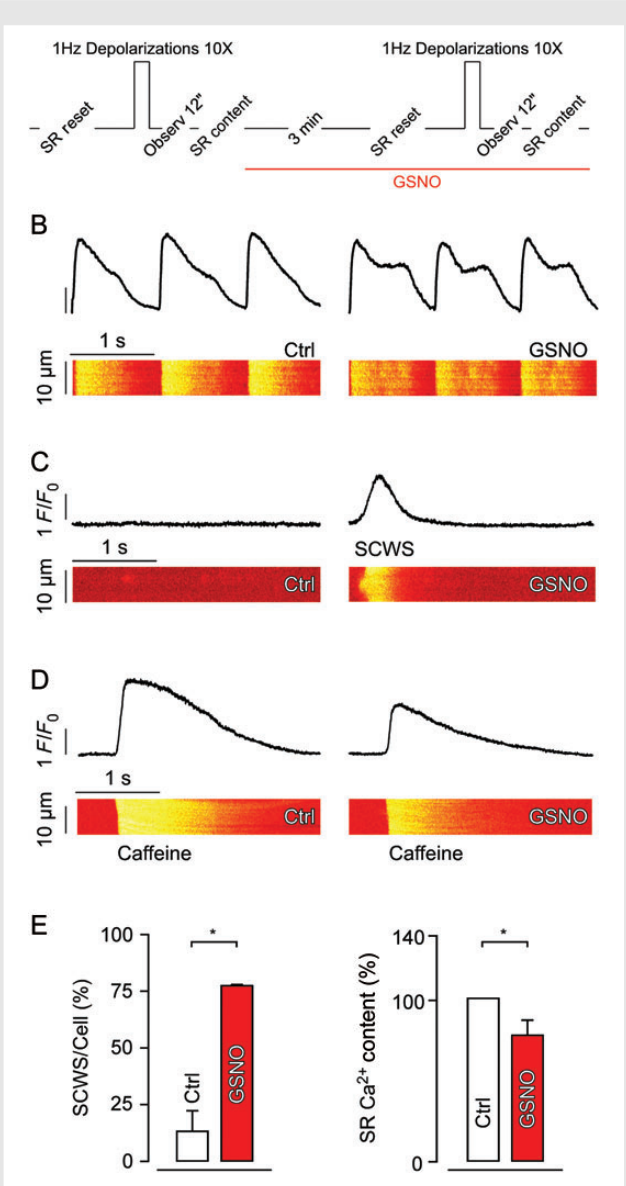

Figure 5 In beating cardiomyocytes, the NO donor GSNO increased diastolic Ca^{2+} spark frequency and induced arrhythmogenic diastolic Ca^{2+} waves. (*A*) Protocol used to record Ca^{2+} transients and Ca^{2+} waves from myocytes during and immediately after stimulation, with or without the presence of GSNO. (*B*) GSNO resulted in more diastolic Ca^{2+} sparks,(*C*) in a higher propensity for spontaneous Ca^{2+} waves (SCWS) and (*D*) in reduced SR Ca^{2+} content. (*E*) Statistical analysis of SCWS ($n = 9, N = 5$) and SR content ($n = 9, N = 5$).

3.6 GSNO leads to arrhythmogenic Ca^{2+} signals in beating cardiomyocytes

At the cellular level, spontaneous Ca^{2+} waves (SCWS) are considered to be indicators for arrhythmogenic conditions. Since the elevated Ca^{2+} spark frequencies shown above could result in SCWS, we tested the arrhythmogenic potential of the NO donor in field stimulated cardiomyocytes (*Figure 5*). In these experiments, NO presumably modified several relevant Ca^{2+} signalling proteins and membrane channels. However, the recordings revealed an increase in the diastolic Ca^{2+} spark frequency, similar to what was observed in resting cells. Furthermore, after a train of 10 depolarizations, 13.3% of the control myocytes exhibited SCWS, while in the presence of GSNO 77.8% showed waves. This elevated wave frequency was accompanied by a reduced SR content (to 77 ± 6.5% of control), confirming that the waves were resulting from altered function of the RyRs and not from SR Ca^{2+} overload.

Taken together, our results provide compelling evidence that the observed increase in Ca^{2+} spark frequency upon β-AR stimulation results from an activation of CaMKII, which is mediated by NO but is not dependent on Ca^{2+} transients (see *Figure 6* for a diagram of this pathway). This represents a new mechanism for CaMKII activation that may have far reaching implications.

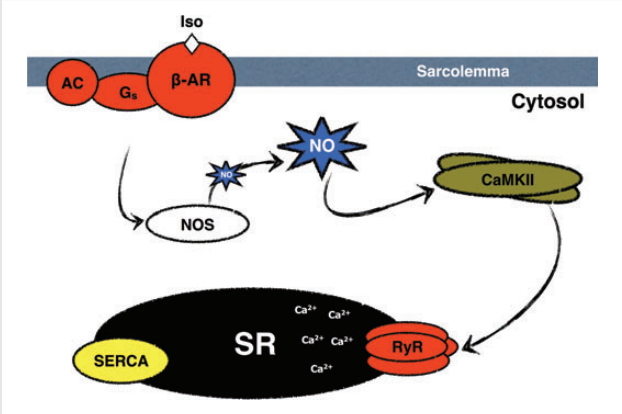

Figure 6 Diagram of the involved pathways during β-AR stimulation of resting cardiomyocytes. Our results show that during β-AR stimulation by Iso, endogenous production of NO derived from NOS activates CaMKII and subsequently modulates the RyRs open probability, as reflected by the higher Ca^{2+} spark frequency.

4. Discussion

RyRs have attracted considerable research interest, due to discoveries such as RyR mutations causing life-threatening arrhythmias.[36] Alterations of their behaviour are observed during several diseases and are often caused by post-translational modifications, most notable phosphorylation and oxidation/nitrosation (for review see ref.[12]). The participation of the RyRs in diseases such as catecholaminergic polymorphic ventricular tachycardias (CPVTs) and heart failure suggests that they may be potential drug targets.[37,38]

4.1 Modulation of RyR function

Changes of RyR function are expected to have a significant impact on cardiac Ca^{2+} signalling. Several laboratories have examined functional consequences of RyR phosphorylation on various levels of complexity, from single channels to isolated cells, partly using transgenic animal and disease models.[7,39,40] These studies have resulted in a considerable controversy and confusion regarding the functional role of the involved protein kinases PKA and CaMKII.[14,41] The reasons for this are not clear, but may arise from different disease models, protocols, and experimental designs. As suggested by the present study, they may partly arise from unexpected cross-talks between complex cellular signalling pathways.

4.2 Modes of CaMKII activation

When examining the importance of protein kinases for changes of diastolic Ca^{2+} spark frequencies after β-AR stimulation, we made a surprising observation. Even though there were no visible Ca^{2+} signals in resting cells that could lead to significant CaMKII activation, the increase in Ca^{2+} spark frequencies could be prevented by pharmacologically blocking CaMKII (but not PKA). This immediately raised the question how under these circumstances CaMKII could become activated? In the literature, several possibilities have been reported, including a pathway involving 'exchange factor directly activated by cAMP' (Epac),[15,42] or requiring oxidative modification of the CaMKII.[23] The observation that the application of forskolin did not elevate the propensity of diastolic Ca^{2+} sparks, unlike β-AR stimulation, is in line with our conclusion, based on the experiments with protein kinase inhibitors for CaMKII and PKA, that any PKA involvement is highly unlikely. Furthermore, the negative result with forskolin regarding spark frequencies makes activation of CaMKII via the Epac pathway improbable and is consistent with the finding that forskolin does not increase SR Ca^{2+} leak.[11] Therefore, we carried out experiments to test for the second possibility of Ca^{2+} spark activation, oxidative stress. Of note, redox modifications of the RyRs are well known to increase their openings,[43,44] although direct RyR oxidation would not be sensitive to the CaMKII inhibitor AIP, as observed in this study. Thus, we were left with the alternative that an oxidative modification of CaMKII could be responsible for its activation. However, we were unable to prevent the Ca^{2+} sparks with Mn-TBAP, a SOD mimetic which we found to reliably suppress ROS induced sparks in a model of oxidative stress.[25] This finding suggests that ROS generation after β-AR stimulation is not involved in CaMKII activation and is consistent with a recent report showing that ROS production does not increase during β-AR stimulation of resting cells.[45]

4.3 NO activates CaMKII in cardiomyocytes

An interesting observation has been reported in a study examining SR Ca^{2+} leaks in cardiomyocytes during β-AR stimulation using a dedicated leak protocol.[11] In this study, the SR Ca^{2+} leak has been quantified from the drop of the cytosolic Ca^{2+} concentration after blocking the RyRs with tetracaine. These authors showed that the function of CaMKII, but also NO synthases, were important determinants for the SR Ca^{2+} leak.[26] Surprisingly, our experiments along these lines revealed that inhibition of NOS also prevented the increase in spark frequency, suggesting an involvement of NO signalling. To confirm a key role of NO, we used a multi-pronged approach. We were able to detect endogenous NO production by the cardiomyocytes upon Iso application, while the NO donor GSNO mimicked the effects of Iso on the spark frequency. In contrast, the GSNO effect was almost completely inhibited by the specific CaMKII inhibitor AIP, suggesting that direct RyR nitrosation was not involved. Rather this appeared to be mainly mediated by CaMKII. Could it be that NO maintains CaMKII active at resting Ca^{2+} concentrations, similar to what has been reported for ROS?[23] For this to occur, an initial Ca^{2+}-dependent activation of CaMKII is required, which could be mediated by invisible Ca^{2+} signals, such as the Ca^{2+} quarks suggested to underlie a fraction of the SR Ca^{2+} leak.[46,47] To address the intriguing question of NO-dependent CaMKII activation directly, we applied a biochemical *in vitro* assay of CaMKII activity. The results obtained confirmed that NO can maintain CaMKII active to an extent similar to H_2O_2, without requiring elevated Ca^{2+} concentrations. Since this occurred *in vitro* and was initiated by significant *S*-nitrosation of the CaMKII protein, it seems very likely that NO can directly activate CaMKII. Interestingly, a strikingly different regulation by NO and ROS has been reported for the $CaMKII_{\alpha}$ isoform of this kinase in pituitary tumour GH3 cells. This isoform was inhibited after nitrosation, but became activated in reducing conditions.[48]

4.4 Relevance of NO-dependent CaMKII activation

Taken together, these findings provide compelling evidence for a stimulation of endogenous NO generation by cardiomyocytes upon β-AR stimulation. In the beating heart, this presumably additive mechanism may be even more pronounced, since further CaMKII activation will occur by enhanced Ca^{2+} transients. Several studies have previously reported positive or negative inotropic effects of NO, which may be partly related to modifications of CaMKII and RyR function, involved NOS isoforms, or crosstalk between activated pathways.[49,50] Related to this, it has been suggested that NO may have a biphasic effect on RyR open probability, depending on the extent of pre-existing β-AR stimulation.[51] Although the precise mechanism of this phenomenon remains unresolved, it may involve CaMKII and could be related to changes of the nitroso/redox balance, as several post-translational modifications will modulate RyR function in an exceedingly complex fashion.[52]

The pathway characterized here represents a newly discovered mechanism for CaMKII activation. This results in a surge of diastolic Ca^{2+} sparks and increased wave propensity. Since Ca^{2+} sparks are signals faithfully reporting the function of the RyRs, this likely occurs via CaMKII-dependent modulation of the RyRs. In addition, the direct activation of CaMKII by NO observed here and the pathways participating downstream of CaMKII are expected to have additional repercussions for cardiomyocyte Ca^{2+} signalling. For example, NO-mediated CaMKII activation contributes to the modulation of Ca^{2+} cycling upon β-AR stimulation, such as during physical exercise or emotional stress. During sustained β-AR stimulation, such as during heart failure, this could lead to SR Ca^{2+} depletion, weaker heartbeat, and

arrhythmias.[40,53] The additional CaMKII activation prompted by NO may be particularly detrimental if it occurs in conditions with already hypersensitive RyRs, for example in the presence of CPVT mutations or oxidative RyR modifications.

Finally, the identification of this pathway adds to the experimental complexity of studies with cardiac muscle because it represents a possibility for crosstalk between PKA and CaMKII activation, downstream of β-AR stimulation. The existence of such a cross-talk may explain some of the controversial experimental results and interpretations regarding the regulation of the RyRs by PKA or CaMKII, respectively.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflicts of interest: none declared.

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Review

Posttranslational modifications of cardiac ryanodine receptors: Ca^{2+} signaling and EC-coupling☆

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ABSTRACT

In cardiac muscle, a number of posttranslational protein modifications can alter the function of the Ca^{2+} release channel of the sarcoplasmic reticulum (SR), also known as the ryanodine receptor (RyR). During every heartbeat RyRs are activated by the Ca^{2+}-induced Ca^{2+} release mechanism and contribute a large fraction of the Ca^{2+} required for contraction. Some of the posttranslational modifications of the RyR are known to affect its gating and Ca^{2+} sensitivity. Presently, research in a number of laboratories is focused on RyR phosphorylation, both by PKA and CaMKII, or on RyR modifications caused by reactive oxygen and nitrogen species (ROS/RNS). Both classes of posttranslational modifications are thought to play important roles in the physiological regulation of channel activity, but are also known to provoke abnormal alterations during various diseases. Only recently it was realized that several types of posttranslational modifications are tightly connected and form synergistic (or antagonistic) feed-back loops resulting in additive and potentially detrimental downstream effects. This review summarizes recent findings on such posttranslational modifications, attempts to bridge molecular with cellular findings, and opens a perspective for future work trying to understand the ramifications of crosstalk in these multiple signaling pathways. Clarifying these complex interactions will be important in the development of novel therapeutic approaches, since this may form the foundation for the implementation of multi-pronged treatment regimes in the future. This article is part of a Special Issue entitled: Cardiomyocyte Biology: Cardiac Pathways of Differentiation, Metabolism and Contraction.

1. Introduction

In cardiac muscle, ryanodine receptors (RyRs) serve as Ca^{2+} release channels of the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR). Thereby, they provide a large fraction of the Ca^{2+} required to initiate muscle contraction from beat to beat. They are normally activated by a small amount of Ca^{2+} entering into cardiac muscle cells from the extracellular space, *via* voltage-dependent Ca^{2+} channels. This Ca^{2+}-induced Ca^{2+} release (CICR) from the SR is the mechanism which amplifies the Ca^{2+} signal and governs excitation–contraction (EC) coupling by activation of RyRs (for review see [1]).

Research on the RyR, both on its structure and function, has been carried out over the last decades using multiple experimental approaches and techniques to overcome the difficulty of examining a channel that is located intracellularly and therefore not easily accessible. This includes assays using isolated SR vesicles (*e.g.* [2,3]), single RyR channels reconstituted into lipid bilayers (*e.g.* [4–9]), permeabilized cardiomyocytes [10,11], but also various biochemical techniques (*e.g.* see [12,13]). Cellular ultrastructural and co-localization information has been obtained with immunocytochemistry and electron tomography [14,15] and structure on the molecular level has been assessed with cryo-electron microscopy [16,17].

Abbreviations: cADPR, cyclic ADP ribose; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CICR, Ca^{2+}-induced Ca^{2+} release; CPVT, catecholaminergic polymorphic ventricular tachycardia; EC, excitation–contraction; *mdx*, mouse model of muscular dystrophy; ROS, reactive oxygen species; RNS, reactive nitrogen species; NOX, nicotinamide adenine dinucleotide phosphate-oxidase; NOS, nitric oxide synthase; PKA, protein kinase A; PLB, phospholamban; RyR, ryanodine receptor; SERCA, sarco-(endo)plasmic reticulum Ca^{2+} pump; SR, sarcoplasmic reticulum; XO, xanthine oxidase

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Many of these studies have confirmed the potential of RyRs to undergo several of the numerous known posttranslational modifications and a number of reports have provided evidence for functional consequences resulting from some of these modifications. These data were frequently obtained in artificial experimental systems and under conditions far away from the natural environment of the RyRs. Therefore, it often remained unclear whether and how these observations on or near the molecular level would translate into intact and living cardiomyocytes and into the entire organ or organism [18].

Some time ago it became practical to closely examine RyR function *in-situ* and within its native environment, which means inside living cells. This has become possible because of groundbreaking developments of technologies to faithfully image subcellular and microdomain Ca^{2+} signals with appropriate spatial and temporal resolution. These developments were significantly driven by the chemical synthesis of bright and kinetically fast fluorescent Ca^{2+} indicators [19,20] and the simultaneous advancements of laser-scanning confocal microscopy combined with digital image acquisition and processing [21].

Since several excellent reviews cover many aspects of RyR posttranslational modifications on the biochemical and molecular level [22–30], here we will concentrate mainly, but not exclusively, on recent findings that have been obtained by examining RyR activity and cardiac Ca^{2+} signaling on the cellular level, where the channels can be examined under conditions not far from their native environment. In particular, we will focus on the consequences of a combined impact of several posttranslational modifications and their mutual interactions during physiological regulation of RyRs and during the development of cardiac diseases affecting RyR function.

2. The ryanodine receptor

2.1. The RyR macromolecular complex

In mammals three RyR isoforms are known: the skeletal muscle form RyR1, the cardiac RyR2 and the more broadly expressed brain form RyR3. The cardiac RyR2 is a large macromolecular complex consisting of a homo-tetramer with 4 subunits comprising a molecular mass of 565 kDa each, totaling 2.2 MDa (for review see [31]). This complex is regulated and modulated in numerous ways by ions (*e.g.* Ca^{2+}, Mg^{2+}, H^{+}), by small molecules (*e.g.* ATP, cADPR) and by proteins (*e.g.* sorcin, calstabin2, junctin, triadin). Important for this review, the macromolecular complex is also connected to protein kinase A (PKA), phosphatases (*e.g.* phosphatase 1 and 2A) and phosphodiesterase (PDE4D) which are tethered to the channel and held near their target sites by means of anchoring proteins [32,33]. This allows for a tight and spatially confined homeostatic regulation of the balance between PKA-dependent RyR phosphorylation and phosphatase dependent dephosphorylation. Ca^{2+}/calmodulin dependent kinase II (CaMKII) was also found to be associated with the RyRs, but the nature and target specificity of this connection are less clear [34]. On the RyR itself, a number of phosphorylation sites have been identified (see Section 3). Furthermore, the RyR complex comprises several free cysteines that can be subject to reversible oxidative modification (see Section 4).

2.2. The Ca^{2+} signaling microdomain in the vicinity of the RyRs

In cardiac muscle, a large fraction of the RyRs are organized in dyads, where the SR membrane contains a cluster of 30–250 RyRs [35] and comes in close contact (gap of ~15 nm) with the T-tubular membrane, which harbors the voltage-dependent L-type Ca^{2+} channels. Opening of one or more L-type channels can activate CICR *via* several RyRs within a cluster. The tiny SR Ca^{2+} release generated by these few opening channels gives rise to a Ca^{2+} spark, an elementary Ca^{2+} signaling event, which can be detected and analyzed using confocal imaging of Ca^{2+} sensitive fluorescence indicators (for reviews see [36,37]). During each heart beat, a large number of Ca^{2+} sparks are activated simultaneously, summing up to form the cardiac Ca^{2+} transient for the activation of contraction. Ca^{2+} sparks and even smaller Ca^{2+} release events, Ca^{2+} quarks, can also occur spontaneously, for example during diastole [38,39]. Spontaneous Ca^{2+} sparks and Ca^{2+} quarks are considered to occur accidentally and partly underlie the SR Ca^{2+} leak. Accidental spontaneous Ca^{2+} sparks do not normally trigger larger Ca^{2+} signals, such as Ca^{2+} waves, and are therefore not arrhythmogenic. Eventless or "quarky" SR Ca^{2+} release through single (or very few) RyRs was recently proposed to contribute substantially to the leak [38–42]. However, under conditions of SR Ca^{2+} overload and in circumstances which sensitize the RyRs, single Ca^{2+} sparks can initiate Ca^{2+} waves traveling along the myocytes in a saltatory fashion from sarcomere to sarcomere [43–46]. These Ca^{2+} waves have a substantial arrhythmogenic potential, since they are able to initiate Ca^{2+} activated currents, such as the Na^{+}–Ca^{2+} exchange current (I_{NCX}), which in turn may depolarize the cardiomyocyte to generate a delayed afterpotential (DAD) and even trigger premature action potentials.

2.3. Ca^{2+} dependent activation and inactivation of the RyRs

The open probability of RyRs depends steeply on the cytosolic Ca^{2+} concentration, whereby Ca^{2+} is thought to bind to the RyR activation site [47]. The increase of the RyR open probability subsequent to openings of L-type Ca^{2+} channels and entry of Ca^{2+} into the dyadic cleft is the main mechanism for activation of CICR during physiological activity. The Ca^{2+} concentration prevailing in the dyadic cleft can only be estimated with computer models at present [48], thus we use "Ca^{2+} sensitivity" of RyRs as a descriptive term. In any case, the Ca^{2+} sensitivity of the RyRs *in-situ* is low enough to ensure independent activation of adjacent Ca^{2+} spark sites, to allow for the regulation of cardiac Ca^{2+} signals by virtue of local control and recruitment of Ca^{2+} sparks. The Ca^{2+} sensitivity of the channels for this type of activation is known to depend on a number of modulators as mentioned above, but also on several regulatory or disease-associated posttranslational protein modifications (see Sections 3 and 4). Inactivation of the RyRs and termination of the Ca^{2+} sparks *in-situ* are less well understood and are the focus of significant ongoing research efforts. One proposed mechanism is based on the regulation of the RyRs by the Ca^{2+} concentration inside the SR. Thereby, lowering the SR Ca^{2+} concentration during a spark would make the RyRs insensitive for Ca^{2+} on the cytosolic side of the channels, which causes their deactivation. Based on observations in SR vesicles, RyRs in lipid bilayers and cells overexpressing calsequestrin, deactivation has been suggested to occur *via* a retrograde signal mediated by allosteric interactions between calsequestrin (acting as the Ca^{2+} sensor) and junctin and/or triadin and the RyR [2,8,49,50]. This mode of spark termination could be stabilized by a reinforcing mechanism that has been proposed recently based on model predictions. The local SR depletion and subsequent decay in Ca^{2+} release flux from the SR during a Ca^{2+} spark may contribute to the self-termination, because of the resulting decline of the dyadic Ca^{2+} concentration [51]. Other proposed mechanisms for spark termination include Ca^{2+} dependent inactivation of the RyRs [52], but up to 100 μM cytosolic Ca^{2+} no RyR inactivation was observed in permeabilized cardiomyocytes [53]. Another mechanistically attractive possibility is stochastic attrition, where the probabilistic simultaneous closure of all RyRs in one cluster would interrupt their mutual activation by CICR within the dyadic cleft [54]. When all channels close, the very high dyadic Ca^{2+} concentration drops to low cytosolic levels within a few milliseconds [48]. However, the probability of all channels to be closed simultaneously is quite low given the estimated number of RyRs in a cluster [35], unless their gating is partly coupled [55].

The Ca^{2+} release termination mechanism mediated by lowering of the SR luminal Ca^{2+} concentration and deactivation of the RyRs could also be important under conditions of SR Ca^{2+} overload, the opposite of the depletion during a Ca^{2+} spark and CICR. By sensitizing the RyRs for Ca^{2+} on the cytosolic side, elevations of intra SR Ca^{2+}

could initiate or facilitate store-overload induced Ca^{2+} release (SOICR) and arrhythmogenic Ca^{2+} waves [46,56].

3. RyR phosphorylation

As mentioned above, the mechanism and functional consequences of RyR phosphorylation have attracted much recent attention. Fig. 1 shows a summary of the involved pathways. Interest in this issue was inspired by a report suggesting that PKA dependent "hyperphosphorylation" of RyRs could occur during heart failure (HF) thereby aggravating this condition. Hyperphosphorylation was proposed to promote the Ca^{2+} sensitivity of RyRs resulting in elevated open probability. This in turn would cause a substantial diastolic SR Ca^{2+} leak, which could contribute to low SR Ca^{2+} content, smaller Ca^{2+} transients and hence weak heart beat [12]. Using mainly biochemical and molecular biology approaches, serine 2808 and 2030 on the RyR have been identified as possible phosphorylation sites for protein kinase A (PKA), and serine 2814 for CaMKII. However, the specificity of these sites for the mentioned kinases remains a disputed issue [12,57–59] and additional sites are likely to exist [60]. Moreover, a fierce controversy revolves around the functional consequence and pathophysiological relevance of the phosphorylation at these sites [61,62]. This debate may result from differences in experimental approaches, methods and tools, but also from variations of the particular animal and disease models.

3.1. Phosphorylation by CaMKII

The picture which emerges from the literature seems to be more clear for the consequences of CaMKII activation which leads to phosphorylation of serine 2814 on the RyR and possibly other sites [60,62], among many collateral targets. CaMKII activity seems to produce quite consistent functional changes of the RyRs that are reconcilable with the general prediction over a wide range of experimental settings and approaches, extending from single channel experiments to cellular Ca^{2+} signaling and a variety of transgenic animals. In single channel experiments the open probability of the RyRs was generally found to be increased upon phosphorylation by CaMKII [63] (but see [64,65]). In isolated cardiomyocytes activation of CaMKII was associated with an increase of the Ca^{2+} spark frequency [66]. Transgenic mice overexpressing the cardiac isoform of CaMKII showed a marked hypertrophy, altered expression and phosphorylation levels of several proteins involved in Ca^{2+} signaling. Despite lower SR Ca^{2+} content, the cells also showed elevated Ca^{2+} spark frequencies, leading to pronounced SR Ca^{2+} leak and a susceptibility for arrhythmias [67,68]. Ablation of CaMKII resulted in a protection of the animals from cardiac hypertrophy, possibly mediated by the unavailability of CaMKII signaling in the pathways of excitation–transcription coupling [69,70]. To obtain further insight into the functional role of serine 2814 on the RyR several mouse models were engineered to specifically scrutinize this site. In one animal serine 2814 was replaced by an alanine, which removes its capability to become phosphorylated by CaMKII (S2814A mouse). Hearts of these animals and cardiomyocytes isolated from them showed blunted force–frequency relationships [71] and the mice were protected from arrhythmias induced by tachypacing after being subjected to transverse aortic constriction (TAC) to induce hypertrophy and failure [72]. Conversely, the S2814D RyR, where serine is replaced by aspartic acid, mimics constitutive CaMKII dependent RyR phosphorylation and increases the open probability of the channels in bilayer experiments. Cardiomyocytes isolated from S2814D mice

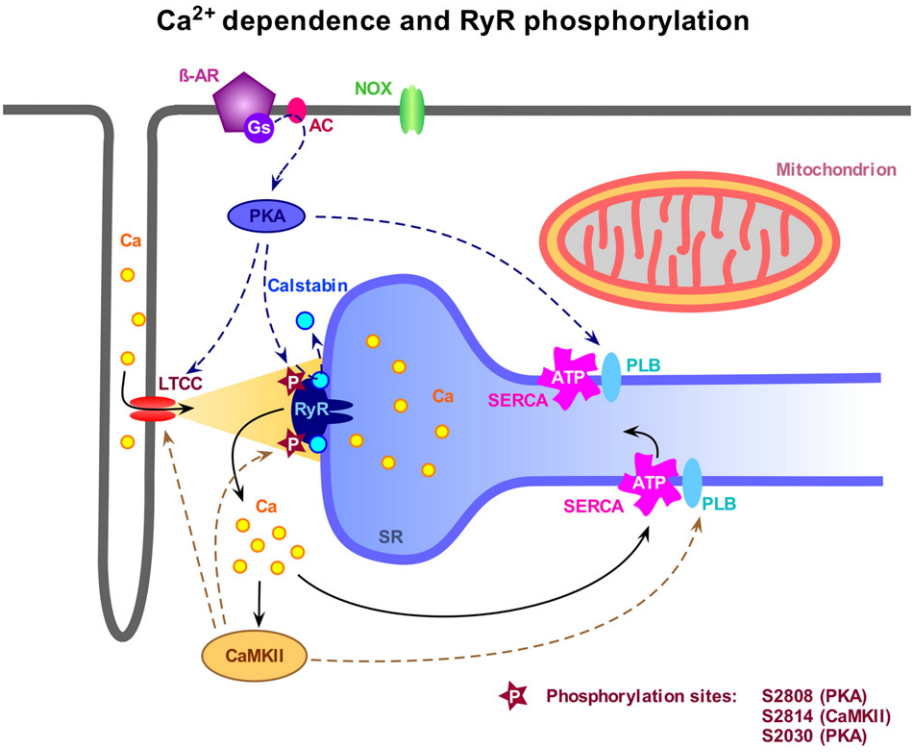

Fig. 1. Modulation of the ryanodine receptor (RyR) by Ca^{2+} and phosphorylation. Ca^{2+} influx *via* the L-type Ca channel (LTCC) activates the RyR and triggers Ca^{2+} release from the sarcoplasmic reticulum (SR), a process referred to as Ca^{2+} induced Ca^{2+} release or CICR, leading to myocyte contraction. The levels of free cytosolic Ca^{2+} are tightly regulated by the SR Ca^{2+} ATPase (SERCA) and the sarcolemmal Na^{+}–Ca^{2+} exchanger (not indicated). After CICR and contraction, the Ca^{2+} store is refilled by pumping Ca^{2+} back into the SR thereby re-establishing diastolic Ca^{2+} levels. The sensitivity of RyR toward activating Ca^{2+} is modulated by phosphorylation. Stimulation of the $ß_1$-adrenoreceptor (ß-AR) leads to Gs-protein-mediated activation of adenylyl cyclase (AC) and further cAMP-dependent activation of PKA. PKA can directly phosphorylate RyR at several phosphorylation sites, presumably at S2808, possibly inducing dissociation of calstabin 2, and at S2030, but also modulates the LTCC and SERCA function, the latter by phosphorylation of phospholamban (PLB). Increased cytosolic Ca^{2+} levels activate CaMKII, which directly phosphorylates RyR at S2814. Similar to PKA, CaMKII also phosphorylates PLB and the LTCC leading to global changes in myocyte Ca^{2+} homeostasis.

showed elevated Ca^{2+} spark frequencies that could not be further increased by CaMKII activation [72] and the mice developed a propensity for arrhythmias and sudden cardiac death when stressed with catecholaminergic challenges or tachypacing subsequent to TAC.

Taken together, these and numerous other studies draw a picture whereby in the short-term CaMKII dependent phosphorylation substantially modifies RyR function, cardiac Ca^{2+} signaling and EC-coupling. Overall, these signaling systems seem to become boosted, more active and Ca^{2+} sensitive but less well controlled, from the molecular to the cellular and organ level. Thus, CaMKII has been considered as a treatment target for multiple short term and long-term cardiac conditions that are associated with disturbances of Ca^{2+} signaling and CaMKII activation [73–76].

3.2. Phosphorylation by PKA

PKA dependent phosphorylation and "hyperphosphorylation" of the RyRs at serine 2808 during heart failure (and in a transgenic mouse model overexpressing the catalytic domain of PKA in the heart) have been proposed to dissociate the stabilizing protein calstabin 2 (a.k.a. FKPB-12.6) from the RyR macromolecular complex, a sequence of events that is suggested to be followed by major functional changes of the channels resulting in diastolic Ca^{2+} leak, SR Ca^{2+} depletion and weak heart beat [12,77]. Obviously, this mechanism could be very important both for the physiological regulation of the channels during stress as well as for their pathophysiological malfunctioning. Therefore, it has attracted substantial research efforts from several laboratories. While in general phosphorylation of the S2808 site has been confirmed by various laboratories, specificity for PKA of this site, the conditions under which phosphorylation would occur and whether or not this leads to calstabin 2 dissociation have remained equivocal [57]. An additional PKA site has been identified at serine 2030 [58,78]. On the single channel level, functional changes after PKA-dependent phosphorylation have been described some time ago [79,80]. On the cellular level, the consequences of PKA-dependent RyR phosphorylation have been more difficult to pinpoint, partly because of the complex adjustments of multiple signaling networks downstream the activation of PKA in intact or permeabilized cells. Changes of Ca^{2+} spark parameters indeed were observed upon application of cAMP in permeabilized mouse cardiomyocytes, but were entirely attributable to the concomitant SERCA stimulation resulting from PLB phosphorylation, as they were not present in cells isolated from PLB ablated mice, where SERCA is already maximally stimulated [11]. Two-photon photolysis of caged Ca^{2+} to artificially trigger Ca^{2+} sparks suggested changes of RyR gating after β-adrenergic stimulation, since in resting Guinea pig myocytes larger Ca^{2+} release events were observed despite a decline of SR content [81]. However, when analyzing the frequency of spontaneous Ca^{2+} sparks at rest, this was later found to most likely depend on CaMKII activation [82].

Because of these difficulties to dissect the consequences of β-adrenergic stimulation on RyR function, transgenic animals have been engineered specifically targeting the serine 2808 site. Several animal models have been created where this serine is replaced by alanine, resulting in S2808A channels which can no longer be phosphorylated at this site [59,83]. Another model is the S2808D mice, which have a modification which corresponds to constitutively phosphorylated RyRs. Unfortunately, the generation of these animals has not fulfilled the expectation to clarify the open issues, as the results published in several reports have again been controversial. Initial studies with the S2808A mice showed that the modification was very subtle, did not disturb normal cardiac function and the animals had no overt phenotype. However, after myocardial infarction (MI) these mice were protected from developing heart failure and from arrhythmias induced by phosphodiesterase inhibition [59]. Reconstituted S2808A channels did not show elevated open probability after MI, in contrast to those from WT mice (an observation which is puzzling by itself, because the CaMKII phosphorylation site on these RyRs should still be functional [72]). This difference on the molecular level was proposed to be the underlying mechanism preventing SR Ca^{2+} leak, weak heartbeat and the susceptibility to arrhythmias in S2808A mice.

In a different laboratory, a further S2808A mouse was engineered and these animals were subjected to a pressure overload heart failure model after TAC [83]. In this study, no obvious cardioprotection was conferred to the animals by ablating the 2808 phosphorylation site. Furthermore, no substantial differences between WT and S2808A RyRs were present in the open probability and gating kinetics of reconstituted channels. This study then examined Ca^{2+} signaling and EC-coupling on the cellular level, including an analysis of Ca^{2+} sparks and waves. Again, no significant differences were found between the two groups of animals. These observations led the authors to conclude that the serine 2808 site only has a limited role in the pathogenesis of heart failure.

At present it remains unsettled why these apparently similar studies led to essentially opposite conclusions. One has to consider that the used disease models and the particular pathomechanisms activated in each of them (*e.g.* pressure overload after TAC versus ischemia/inflammatory disease without pressure overload but potentially more oxidative stress [84]) could result in quite different outcomes, as has been observed in another study investigating CaMKII dependent RyR phosphorylation [85], or that the RyRs of the two engineered animals do not operate in a perfectly identical way [86]. Alternatively, some of the resulting functional modifications may be rather subtle, and can be compensated by auto-regulatory features of the cardiac EC-coupling machinery [87] and are therefore difficult to detect.

Starting from the latter possibility, a detailed study was carried out to examine SR Ca^{2+} release kinetics, their spatial synchronization, and the improvement of this parameter by β-adrenergic stimulation when the communication between L-type Ca^{2+} channels and RyRs was challenged [88]. The reasoning for this approach was the notion that these events occur at the very interface between the L-type Ca^{2+} channels and the RyRs and might therefore reveal even subtle changes. When this communication was tested by using very small Ca^{2+} currents as triggers, substantial spatial desynchronization was observed. This was resynchronized upon β-adrenergic stimulation in the WT [89] but not in the S2808A cells. Furthermore, unlike WT cells, Ca^{2+} wave propagation was not accelerated upon β-adrenergic stimulation in S2808A cells. Together with the long delays observed in the release synchronization, this suggested the possibility of an intra-SR mechanism [46,56], whereby SR Ca^{2+} loading *via* SERCA would lead to sensitization of the RyR from the luminal side, thereby pushing the channel over the trigger threshold. The possibility of an intra-SR mechanism was then confirmed in reconstituted single RyR channels. At high SR Ca^{2+} concentrations, and only under this condition, WT channels indeed responded with a significantly larger increase in open probability upon PKA dependent phosphorylation than S2808A channels. Regarding the ongoing controversy, the main conclusion from these studies is that the effects of serine 2808 phosphorylation are present but delicate and may be difficult to detect when SR Ca^{2+} content is not controlled experimentally (*e.g. in vivo*, when the auto-regulatory adjustments of SR Ca^{2+} content mentioned above may compensate for small changes of RyR open probability).

Taken together it appears that the mechanisms and consequences of PKA dependent RyR phosphorylation are less clear and potentially more subtle than those mediated by CaMKII. Whether and how these delicate changes translate into the *in vivo* situation is difficult to extrapolate and will require more research. In support for this expectation, a recent cross-breeding experiment between dystrophic *mdx* and S2808A mice indicated that the RyR mutation confers significant protection for cardiac disease manifestations and progression of the dystrophic cardiomyopathy in these animals (see below) [90].

4. Redox modification of RyR

4.1. RyR oxidation by ROS

Changes of the cellular redox state give rise to another category of posttranslational RyR modifications, which do not only have a modulatory function but also play an important role in the development of various cardiac diseases. The term "intracellular redox potential" broadly describes the balance between reduced and oxidized proteins within cells, which in turn is determined by the level of generation and buffering of cellular reactive oxygen and reactive nitrogen species (ROS/RNS). There are multiple sources of ROS/RNS within the cell (see Fig. 2). They include but are not limited to NADPH oxidase (NOX), xanthine oxidase (XO), mitochondria and nitric oxide synthase (NOS). On the other end there are various cellular antioxidant defense components such as catalase, superoxide dismutase, thio- and glutaredoxins, glutathione peroxidase, glutathione, vitamins A, C and E, *etc.* Under physiological conditions, the extent of ROS/RNS accumulation is finely controlled by these scavenging and reducing mechanisms, and at low concentrations ROS/RNS serve as important intracellular messengers. An imbalance between generation of ROS/RNS and the efficiency of cellular defense systems can lead to a transient or persistent oxidative/nitrosative stress resulting in redox modifications of various cellular proteins, including those involved in Ca^{2+} homeostasis. The RyR is an important example, since it is known to be very susceptible to redox modifications. Each cardiac RyR tetramer contains a total of 364 cysteines [91]. In the presence of a physiological concentration of one of the major cellular "redox buffers" glutathione (5 mM) about 84 of these cysteines are free. The sulfhydryl groups of these cysteines are subject to reversible cross-linking, S-nitrosation (often referred to as S-nitrosylation) and S-glutathionylation. Numerous studies of RyRs incorporated in lipid bilayers convincingly showed that reversible redox modifications significantly affect the activity of RyR channels. Oxidative conditions generally increase the RyR open probability, while reducing agents do the opposite (*e.g.* [91–94]). Therefore, the functional consequence of a moderate cellular oxidative/nitrosative stress could be immediate enhancement of Ca^{2+} release from the SR in response to a given physiological trigger. This possibility has been supported by experiments with isolated SR vesicles (*e.g.* [91,95]). The increased Ca^{2+} sensitivity of RyRs and subsequently larger Ca^{2+} transients could have a positive inotropic effect on the cardiac function [96]. However, severe oxidative stress can cause irreversible and sustained activation of RyRs [91], increased Ca^{2+} leak from the SR, decreased SR Ca^{2+} load and finally a decline of beat-to-beat cellular Ca^{2+} transients with contractile dysfunction. Such conditions are usually associated with or even caused by the development of various cardiac abnormalities. Therefore, the role of RyR redox modifications in cardiac pathophysiology is currently under intensive investigation in multiple laboratories around the world.

When the experimental gear was shifted from molecules and vesicles towards studies of cells, organs and organisms, it became obvious that the findings obtained from isolated RyR channels cannot be translated to more complex biological systems without a critical reevaluation. Besides the presence of various cellular sources for ROS, redox modification targets multiple intracellular sites including major proteins involved in EC coupling and all of them need to be considered in order to identify the link between each modification and the resulting changes of RyR function [97]. To discriminate between correlative, adaptive and causal posttranslational RyR modifications is often a daunting task.

Cardiac muscle has a substantial NOX activity (for reviews see [98,99]). It has been reported that NOX2 is the predominant isoform expressed in T-tubular and SR membranes of mature cardiomyocytes. Therefore, it is strategically positioned to modulate the activity of the RyRs. NOX is an enzyme that utilizes NADPH to produce superoxide anion. NOX2 was found to be overexpressed and/or its activity

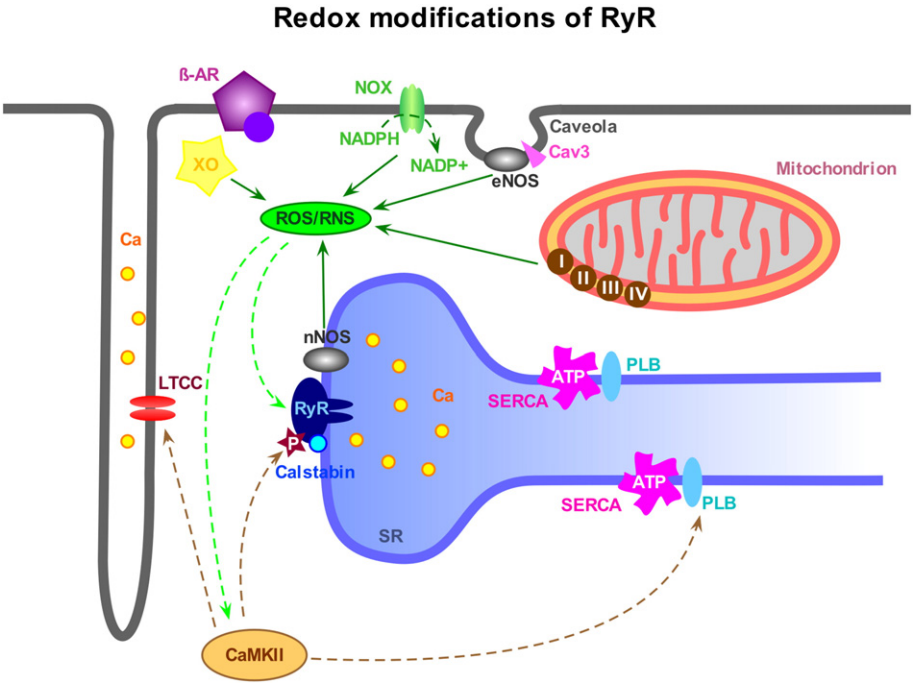

Fig. 2. Redox-modifications of RyRs. Changes in the redox potential of the myocyte have been shown to have a serious influence on protein function, especially at the level of the RyR. The main sources for the production of reactive oxygen species (ROS) in cardiomyocytes are the sarcolemmal NADPH oxidase (NOX), the xanthine oxidase (XO) and the mitochondrial electron transport chain (complex I through IV). ROS can glutathionylate free cysteine residues on the RyR and also act in an indirect way *via* CaMKII activation and subsequent RyR phosphorylation. Nitric oxide synthases (NOS) are mainly responsible for the production of nitric oxide (NO) and reactive nitrogen species (RNS). In cardiomyocytes, sarcolemmal endothelial NOS (eNOS), which co-localizes with caveolin-3 (Cav3) in caveolae, and RyR-associated neuronal nNOS are primarily responsible for the production of NO, causing S-nitrosation at free thiol groups of the RyR and many other proteins. Most likely, these mechanisms work synergistically and induce parallel modifications of RyR function.

increased in dystrophic hearts [100,101], in hearts of patients with a history of atrial fibrillation [102], and in hearts subjected to tachycardiac preconditioning [103]. Although the exact mechanisms of NOX activation under these pathological conditions remain unclear, it was shown that ROS produced by NOX stimulates SR Ca^{2+} release *via* at least two pathways: 1) direct oxidation or S-glutathionylation of RyRs or 2) indirectly through CaMKII activation [104] followed by phosphorylation of the RyRs. Reducing or ROS scavenging compounds could generally mitigate or prevent the consequences of oxidative stress in these experimental models. Another widely recognized source of ROS production in cardiac myocytes is mitochondria [105,106]. Mitochondria always generate a small amount of ROS through leakage in the electron transport chain during respiration. Under some pathophysiological conditions, such as ischemia/reperfusion, ROS produced by mitochondria become the main contributors to cellular oxidative stress. In this situation mitochondrial Ca^{2+} overload and subsequently ROS overproduction may trigger mitochondrial permeability transition, which in turn boosts ROS production *via* ROS-induced ROS release mechanisms [107,108]. There are also several reports indicating upregulation of XO activity in experimental models of heart failure [109]. Furthermore, contractile function and myocardial efficiency in HF could be improved by treating the animals with xanthine oxidase inhibitor allopurinol [110,111]. Overall, regardless of the source of their generation, ROS and subsequent oxidative modifications of RyRs have been directly held accountable for augmented stretch-induced Ca^{2+} responses and hypersensitive EC-coupling in dystrophic cardiomyocytes [101,112–115] as well as in impaired Ca^{2+} signaling in failing [116,117] and diabetic hearts [118,119].

4.2. RyR modifications by RNS

The two major isoforms of NO synthase (NOS) in cardiac myocytes are eNOS and nNOS. They have a specific sub-cellular localization and are possibly aimed at different targets in their microdomains, due to the short range of NO diffusion. The eNOS isoform is localized in the plasma membrane in caveolae through interaction with caveolin-3. In healthy cardiac muscle nNOS is mainly located in the SR membrane, linked to the RyRs. In failing or diseased hearts nNOS may partly redistribute to the sarcolemma. Normally, the iNOS isoform is not present in significant amounts, but this may be different during the development of cardiac diseases. NO produced by these enzymes can bind to free thiol groups on various proteins, including RyR, causing S-nitrosation and conformational changes. Alternatively, NO can act *via* the cGMP dependent pathway and activated PKG, a protein kinase which is thought to phosphorylate the RyR at the S2814 CaMKII site, at least *in vitro* [60]. However, whether this occurs *in vivo* is presently unclear.

An important role for direct RyR nitrosation in cardiac EC-coupling and Ca^{2+} signaling was suspected already some time ago (for review see [96]), when it was found that the stretch-induced enhancement of cardiac Ca^{2+} signals and elevation of Ca^{2+} spark frequency were blunted in the presence of L-NAME, an unspecific inhibitor of all NOS isoforms [120]. The effect of stretch could be mimicked by adding the NO donor SNAP, which nearly doubled the Ca^{2+} spark frequency. Additional studies reported that NO could have diverse actions, depending on the preexisting extent of β-adrenergic stimulation. NO donors increased the Ca^{2+} spark frequency in a cGMP independent way at low (10 nM) concentrations of ISO, presumably by RyR nitrosation (but other mechanisms were not excluded) [121]. At 1 μM ISO a decrease of the spark frequency was observed, however this was accompanied (or caused) by a reduction of the SR Ca^{2+} content. In $nNOS^{-/-}$ mice, but not in $eNOS^{-/-}$ mice, hyponitrosation of the RyRs was observed, indicating that the structural proximity between nNOS and RyR may be functionally relevant. Interestingly, these RyRs exhibited more extensive oxidative modifications, thought to lead to elevated SR Ca^{2+} leak [122]. Thus, constitutive RyR S-nitrosation in WT animals may confer some protection of the channels against more severe oxidative modifications. This may be important in various diseases, where changes of the nitrosation have been implied in their pathology, but also in conferring some cardioprotection [123]. However, in another study with myocytes from $nNOS^{-/-}$ mice, Ca^{2+} spark frequencies and the SR Ca^{2+} leak at a given Ca^{2+} load were found to be reduced, and both could be normalized (*i.e.* increased) by exposure to an NO donor [124]. In line with these findings, RyRs were hypernitrosated in cardiomyocytes with upregulated nNOS activity and this was paralleled by increased SR Ca^{2+} leak and elevated fractional Ca^{2+} release [125]. Taken together, and considering the caveats when interpreting experimental data obtained from transgenic animals, these findings indicate that, depending on the conditions (*e.g.* on the extent of oxidative stress), nNOS signaling can also increase RyR activity in cardiac muscle, either directly or indirectly.

In one disease related study the extent of RyR nitrosation was quantified in mice with dystrophic cardiomyopathy and found to be increased around 4–5 fold [126], while PKA dependent RyR phosphorylation was not significantly elevated. This was accompanied by a doubling of the frequency of spontaneous Ca^{2+} sparks and a propensity for arrhythmias. The extent of RyR oxidation and CaMKII-dependent RyR phosphorylation was not assessed directly, but the protective effect of N-acetyl cysteine (NAC) suggests an important role of oxidative stress in dystrophic cardiomyopathy, as reported earlier [100,101].

The general concept which emerges from these partly controversial studies, although rather diffuse, suggests that the reciprocal interactions between RyR modifications resulting from ROS and RNS and their functional outcome are very complex and not yet fully understood. While some observations suggest quite synergistic actions, in other experimental settings more competitive effects between ROS and RNS modifications become apparent. Interactions between ROS and RNS are possible in various ways, for example through their tightly connected chemistries (*e.g.* superoxide and NO can combine to form peroxynitrite [127]) or by competing for the same thiols on the RyR. A further complication in the interpretation of the experimental data may arise from the finding that RyR2 is nitrosated *via* S-nitrosoglutathione (GSNO) and not by NO directly [94]. Further, most experiments were carried out at ambient oxygen pressure (~150 mm Hg), but in the tissue there is much less oxygen (~10 mm Hg). The degree of oxidation and the function of the cardiac RyR are modified by ambient O_2 [94]. In any case, it seems that the precise balance between ROS and RNS is important, and that a NO/ROS disequilibrium can lead to abnormal RyR channel behavior [128].

5. Cross-talk between redox modifications and phosphorylation in disease

Recently, a number of studies have been carried out in a variety of cardiac disease models, focusing on modifications of RyR function and the conceivably underlying posttranslational modifications. A common finding in many of these studies was a sequential (*i.e.* during disease development) or simultaneous presence of several posttranslational RyR modifications. While such a pattern could result from parallel but unrelated changes of the involved pathways, it seems more plausible that these modifications are not independent from each other. There are numerous possibilities for significant cross-talk and synergisms among these signaling pathways such as ROS/RNS, phosphorylation and Ca^{2+} signals, from the origin (receptor or source of the signal) down to the target, the RyR itself (for reviews see [129,130]). In one scenario, boosting the Ca^{2+} transient by phosphorylating various Ca^{2+} signaling proteins may elevate mitochondrial Ca^{2+} content, followed by an increased mitochondrial metabolism and ROS production [105]. Mitochondrial ROS can further augment the Ca^{2+} signals by oxidizing multiple Ca^{2+} signaling proteins, as described above, but

also by activating CaMKII *via* redox modification. This occurs in addition to the stimulation by the larger Ca^{2+} transients themselves and will lead to extra protein phosphorylation [104], thereby establishing multiple and coupled positive feed-back loops, which further amplify these signals [131]. Moreover, receptors and enzymes involved in the generation, modulation and termination (*e.g.* phosphatases, phosphodiesterases, ROS scavengers, SNO reductases) of these associated signals are often regulated *via* other functionally interconnected pathways. For example, β-adrenergic responsiveness is regulated by NO, creating a link between NO, phosphorylation and Ca^{2+} signals [132]. In turn, the activity of NOSes is Ca^{2+} sensitive [133]. The eNOS isoform (but not nNOS) is stimulated by ROS [134]. Most likely, many more direct and indirect possibilities for cross-talk between these pathways exist within cells.

Interactions between RyR oxidation and phosphorylation have been studied in dystrophic cardiomyopathy, a disease that combines a high degree of oxidative stress and excessive Ca^{2+} signals after mechanical stress, resulting from the lack of the protein dystrophin [101]. In one example, a cross-breeding approach has been applied to test for rescue from this disease by eliminating not the main pathomechanism, but another step in the vicious cycle [90]. Dystrophic *mdx* mice were crossed with RyR-S2808A mice, which carry RyRs that cannot be phosphorylated at this site. Ablation of this phosphorylation site protected these animals, even though not the main pathomechanism was targeted, but rather one of the other steps in the positive feed-back loop. Unlike *mdx* mice, these animals did not develop cardiac hypertrophy with fibrosis and showed improved cardiac function. Further, they were protected from isoproterenol-induced arrhythmias and SR Ca^{2+} leak. These findings suggest that PKA dependent RyR phosphorylation contributes to the abnormal Ca^{2+} homeostasis in dystrophic cardiomyopathy. Interestingly, and in apparent contrast to these findings, another study was not able to detect significant PKA dependent RyR phosphorylation in *mdx* mice [126]. However, more recent studies suggest that the disease phenotype and the pattern of RyR posttranslational modifications change in the course of dystrophic cardiomyopathy, and other diseases as well. Unlike oxidative stress and CaMKII activation, RyR phosphorylation by PKA seems to become important only at later stages of the disease, and is associated with elevated SR Ca^{2+} leak and reduced Ca^{2+} content [115].

6. Possible clinical relevance

A multitude of cardiac diseases are accompanied by acute or chronic hyperadrenergic states and/or oxidative cellular stress which can initiate vicious cycles and pathomechanisms involving RyR posttranslational modifications similar to those described above. Since phosphorylation, as well as oxidation and elevated Ca^{2+} concentration will increase the RyR open probability, the CICR mechanism may become very sensitive and unstable. To what extent these multiple changes and the concomitant posttranslational RyR modifications exert additive effects and whether they progress rapidly or slowly during the development of a given disease is not yet established and remains to be investigated. However, a number of recent experimental studies on the cellular level are in line with this possibility. Examples for diseases where posttranslational RyR modifications have been reported are congestive heart failure [116], dystrophic cardiomyopathy [101], diabetes [119], ischemia/reperfusion [135] and atrial fibrillation [136]. However, the clinical relevance of the presented experimental findings in general and the impact of the identified pathomechanisms and suspected cross-talk pathways in particular can ultimately only be confirmed in clinical trials. In such future pilot studies multipronged therapeutic strategies would need to be compared with established treatments targeting only one mechanism. Nevertheless, some of the animal studies carried out with various disease models are already fairly developed and can provide a solid foundation on which to base future clinical trials, possibly with studies in human cardiomyocytes and in larger animals as intermediate steps. Based on the available data a few disease entities have been identified in which posttranslational RyR modifications seem to make a substantial contribution to disease progression. One category of examples is a variety of disease models leading to heart failure (*e.g.* myocardial infarction, transverse aortic constriction (TAC), artificial tachypacing, dystrophic cardiomyopathy). In these entities both, oxidative RyR modifications and CaMKII (and possibly PKA) dependent RyR phosphorylation have been found to be present concurrently, but in various proportions [85,115,116,126,137,138]. The concomitant destabilization of the RyRs may also favor or underlie the occurrence of various forms of arrhythmias, initiated by diastolic Ca^{2+} release leading to delayed afterdepolarizations and extrasystoles. Not unexpectedly, the arrhythmogenicity of phosphorylated RyRs also appears to be instrumental in some forms of atrial fibrillation [139–142].

Another layer of complexity is added to the intricate mutual interactions of all the posttranslational modifications discussed above in patients carrying a mutation of the RyR2 [143,144]. These mutated channels often exhibit destabilized gating behavior, possibly arising from altered interaction (*i.e.* zipping) of RyR channel domains [145,146]. Many of these patients are prone to stress-induced arrhythmias, manifesting themselves as catecholaminergic polymorphic ventricular tachycardias (CPVTs), potentially leading to sudden cardiac death. Cell lines and transgenic animals have been engineered expressing RyRs harboring mutations that were identified in families with CPVT patients [147–150]. These animals replicate the human disease phenotype and serve as disease models to investigate pathomechanisms arising from RyR mutations and to develop therapeutic approaches. In these disease models, arrhythmias could be provoked by tachypacing and/or by β-adrenergic stimulation. In cellular experiments, these cardiomyocytes exhibited elevated Ca^{2+} spark frequencies, a propensity for diastolic Ca^{2+} waves with delayed after-depolarizations and reduced wave thresholds. These are all signs for disturbed channel gating with a predisposition towards abnormal Ca^{2+} sensitivity (cytosolic or SR luminal) of mutated channels. In summary, the phenotypes resulting from RyR mutations share many features with the functional consequences of the posttranslational modifications discussed above. While it is well established that physical or emotional stress can prompt CPVTs in patients harboring cardiac RyR mutations, it is so far unknown how stress exactly triggers these arrhythmias. Are they provoked by additional RyR sensitization originating from PKA or CaMKII dependent RyR phosphorylation? Or are they the result of the concomitant stimulation of the SERCA after PLB phosphorylation, leading to elevated SR Ca^{2+} loading? Or is it the combination of these two possibilities which is particularly detrimental?

7. Conclusion and outlook

While we start to understand the consequences of various posttranslational RyR modifications on the molecular level, we also develop the awareness for the extraordinary complexity of this issue on the cellular and organ level. This partly results from the multiple crosstalks and interactions of the various signaling pathways and their intertwined positive and negative feed-back loops. A large amount of research will thus be required to address the question whether all the regulatory and/or pathophysiologically important mechanisms changing RyR function behave in additive, competitive or mutually exclusive ways. To answer these and many similar questions relevant for other cardiac conditions, it needs to be understood in more detail, on the molecular level, how these modifications interact to bring about functional change. These findings then need to be integrated into the more complex situation of intact cells, organs and organisms, to determine their physiological and clinical relevance. Based on the presently available literature, only partly discussed above, one is inclined to predict that such interactions exist and are very important. Understanding these interactions will

lay the foundation for the development of mechanism based therapies, potentially targeting several synergistically acting mechanisms simultaneously.

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6.UNPUBLISHED DATA

6.1.The β_1-adrenergic receptor modulates the Ca^{2+} sparks

We decided to confirm that the effects that we observed with isoproterenol were also reproducible as well with a specific β_1 adrenergic receptor agonist. As dobutamine is also a β_2 adrenergic receptor agonist, we decided to inhibit the β_2 isoform. In the non published Fig 22 we show the this effect is maintained, therefore confirming the increase in Ca^{2+} spark frequency, which is observed in Iso, is indeed dependent on the β_1 isoform.

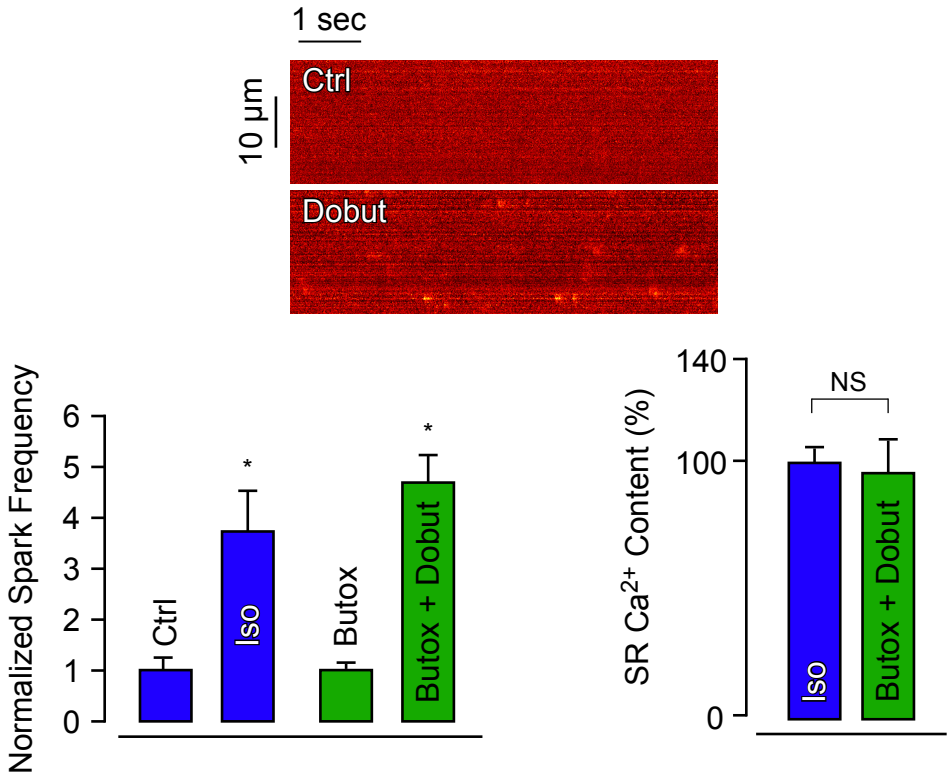

Fig 22: Dobutamine in the presence of Butoxamine (a β_2 adrenergic receptor antagonist), increases the frequency of Ca^{2+} sparks to the same extent as Iso alone. This confirms that Iso indeed activates the β_1 adrenergic receptor to modulate the Ca^{2+} sparks.

6.2.The ROS scavenger TIRON

To evaluate the effect that the gas form of NO had in the regulation of the frequency of Ca^{2+} sparks, we pre-incubated and ran our experiments in the presence of 10mM TIRON, a ROS scavenger. As well as it was observed with Mn-TBAP, TIRON did not manage to prevent the increase in Ca^{2+} spark frequency in the presence of Iso.

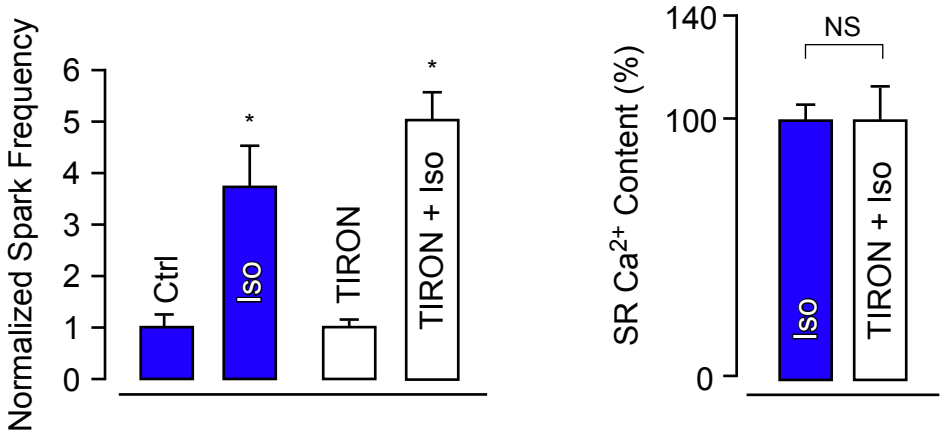

Fig 23: Pre-incubation with TIRON (a ROS scavenger) fails preventing the increase in Ca^{2+} spark frequency produced by Iso. This confirms together with the Mn-TBAP data of the published article (Fig 2E), that the increase in Ca^{2+} spark frequency in the presence of Iso, is not modulated by ROS but instead by NO alone as confirmed along the research presented in this thesis.

6.3.The eNOS inhibitor L-NIO and the nNOS inhibitor AAAN

To distinguish the source of NO we made 2 experiments using specific inhibitors for eNOS and nNOS. In the first set of experiments we used L-NIO, a reported specific eNOS inhibitor; unfortunately pre-incubation or acute application of L-NIO, resulted in an increased occurrence of spontaneous Ca^{2+} waves (SCWS). The fact that we had SCWS from the beginning of the experiments made the assessment of Ca^{2+} sparks impossible.

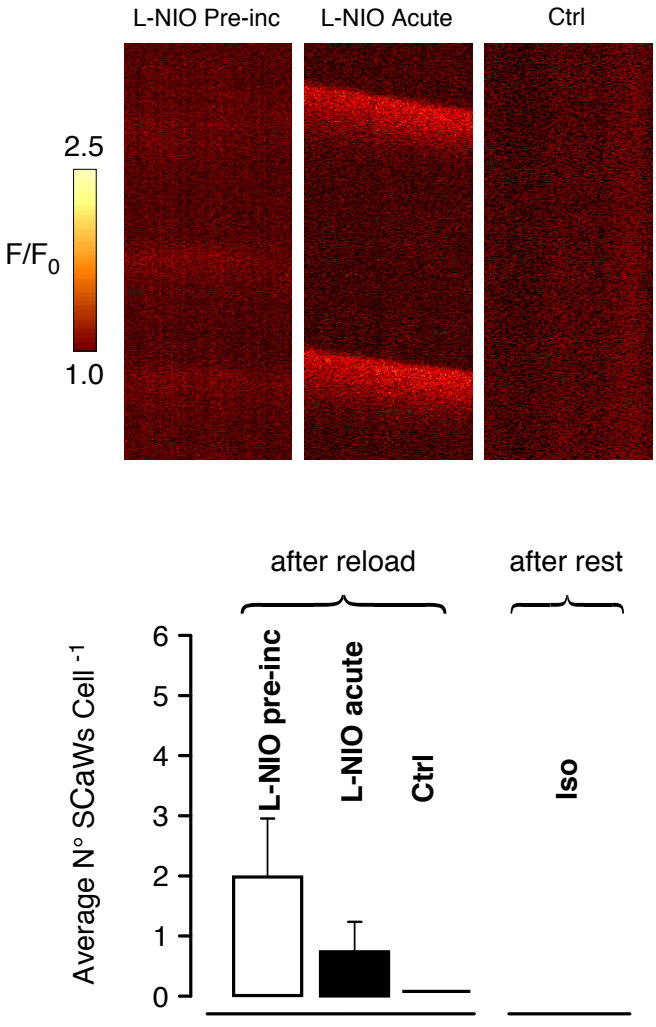

Fig 24: 5 µM L-NIO pre-incubation, triggered spontaneous Ca^{2+} waves (SCaWs) and during acute application of the inhibitor itself. To asses the effect of the drug on (SCaWs), the cells were controlled on the same experimental day **without** L-NIO and then exposed to Iso alone (showing increase in Ca^{2+} spark frequency as expected), interestingly these cells did not present (SCaWs) under identical experimental conditions, suggesting side effects of the drug L-NIO once is applied.

We decided to go for a different maneuver and asses whether if a more specific nNOS inhibitor prevented the change in the frequency of Ca^{2+} sparks when applying Iso. AAAN is potent and ~2600 times more specific for nNOS than for eNOS.

Therefore we decided to use this compound and asses if it could prevent the increase in the frequency of Ca^{2+} sparks observed in Iso. In the non published Fig 25 we could observe that this compound prevented the increase in the frequency of Ca^{2+} sparks.

Taken together this suggests that nNOS is the primary isoform that regulates the β_1 adrenergic receptor stimulation. However we cannot discard the eNOS isoform as it has also been reported to modulate the adrenergic response in the heart (for reviews see (Massion *et al.*, 2003)).

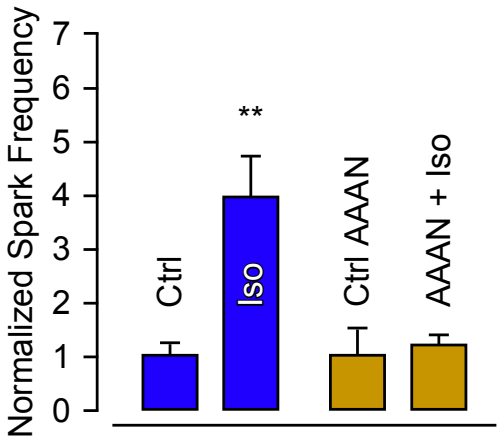

Fig 25: The specific nNOS inhibitor AAAN did prevent the increase in the frequency of Ca^{2+} sparks upon Iso application.

6.4.Detection of ROS production during β-AR stimulation

It has been recently suggested that ROS are generated during β adrenergic stimulation with Iso (Bovo *et al.*, 2012). Therefore we decided to assess if our model also presented production of this type of molecules which could directly affect the frequency of Ca^{2+} sparks (Shkryl *et al.*, 2009).

We pre-incubated the cells with the ROS indicator CM-H_2-DCF. After a base line we stimulated the cells with Iso, during the same time that we used for the detection of Ca^{2+} sparks, ~3 minutes. In the presence of Iso, there was no detectable increase in fluorescence; despite that at the end of the protocol the indicator responded positively to H_2O_2, as an indicator of ROS detection.

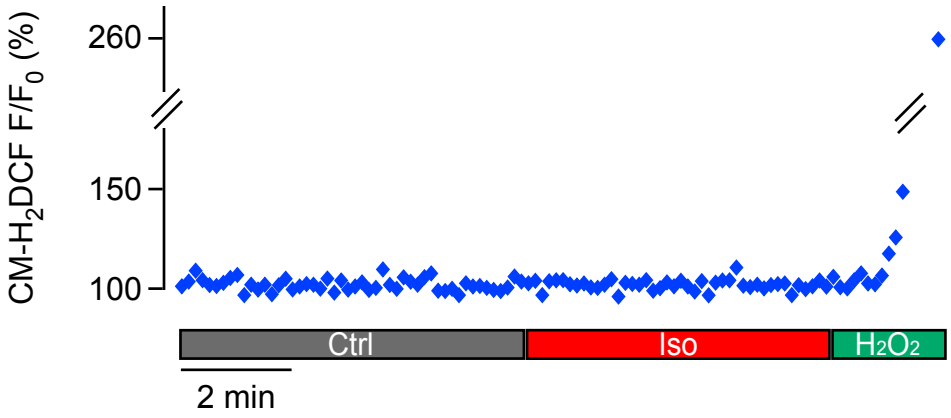

Fig 26: Average of 2 cells pre-incubated in 10 μM of the ROS indicator CM-H_2-DCF. ~6 minutes are allowed for baseline followed by ~5 min in Iso, at the end of the protocol H_2O_2 is added to confirm a positive response of the indicator towards detecting ROS.

Taken together we conclude that in our pathway there is no detectable increase in ROS production / formation during β adrenergic stimulation, which is also consistent to the research of (Bovo *et al.*, 2012) as they demonstrated that ROS are created during β

adrenergic stimulation of beating cardiomyocytes and not during resting conditions as in our case.

Finally to confirm in a more specific way that we did not have oxidation at a molecular level we tried to detect oxidation of CaMKII when in the presence of GSNO. For that we used anti-oxidized CaMKII antibody which was already used in the research of (Erickson *et al.*, 2008).

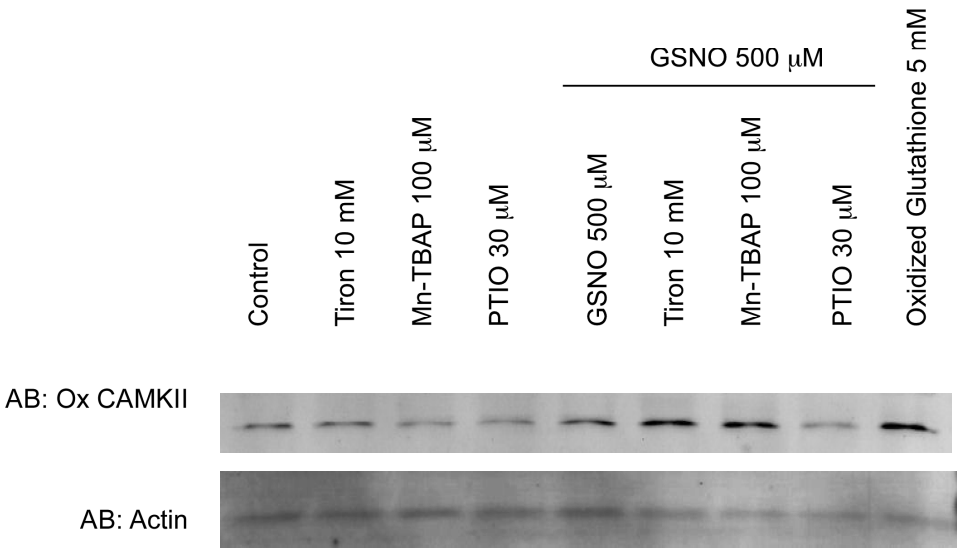

Fig 27: Proteins from cardiac iPS cells extract were exposed to 500 microM of GSNO to determine whether the NO donor could itself oxidize CaMKII. The samples were also treated with 10 mM of TIRON, a ROS scavenger, 100 μM of Mn-TBAP, a peroxinitrate scavenger, and 30 μM of PTIO, a nitric oxide scavenger. 40 μg of total protein was loaded in each well. To detect oxidizing CaMKII a specific primary antibody (Millipore) was used. Only PTIO prevented the signal increased induced by GSNO, by interfering with nitrosation. However, ROS scavengers do not reduce the signal induced by GSNO, indicating that CaMKII oxidation is not involved. As positive control for oxidation, 5 mM of oxidized glutathione was used. To control for gel loading we used an antibody against actin.

However, the anti-oxidized CaMKII antibody does not detect the purified CaMKIIδ we have used in the functional assay. This antibody also does not detect another commercially available CaMKII, produced in SF9 cells.

As an alternative, we used tissue homogenate for western blotting (see Fig 27 above). Here, the anti-oxidized antibody unfortunately detected both, the oxidized and the nitrosated CaMKII (for an example compare lanes with GSNO alone and lane with GSSG (oxidized glutathione). We then repeated these blots in the presence of a NO scavenger (PTIO) and ROS scavengers (Tiron, Mn-TBAP). Indeed, after GSNO treatment, the ROS scavengers did not prevent the CaMKII modification which made it detectable by the anti ox-CaMKII antibody. However, the NO scavenger PTIO prevented this modification. Taken together this means that 1) GSNO does not oxidize the CaMKII, but rather leads to nitrosation (as suggested by several findings in this thesis); 2) the commercially available anti ox-CaMKII antibody cannot distinguish between oxidized and nitrosated CaMKII.

6.5.References

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7.DISCUSSION

7.1. Discovery of a new pathway for CaMKII activation

In the framework of CaMKII involvement in the modulation of EC-coupling by the adrenergic system, Ca^{2+} has always been the prominent messenger that this protein-kinase needs to become activated. Solid evidence has suggested that after β adrenergic stimulation of cardiomyocytes CaMKII becomes active and phosphorylates, among others, the RyRs, the LTCCs and PLB, the SERCA inhibitor. More recently a revolutionary concept has been proposed by (Erickson *et al.*, 2008). This paper reported that oxidation of CaMKII can also enhance its activity without requiring Ca^{2+} signals.

In the included manuscript, yet another new and alternative pathway for CaMKII activation is presented. This is the main finding of my thesis. After a complex series of multifaceted experiments we found out and describe how during β adrenergic stimulation, endogenous NO formation by the cardiomyocyte could govern the activation CaMKII by protein nitrosation, also in the absence of detectable Ca^{2+} signals. This newly discovered pathway for CaMKII activation may be of great significance in many different biological systems.

7.2. Possible role in physiology and pathophysiology of cardiac Ca^{2+} signaling

CaMKII has been involved in many of the aberrant behaviors that failing cardiomyocytes present, such as lack of synchronization between contractions, elevated SR Ca^{2+} leak, and arrhythmias (Maier *et al.*, 2003; Cutler *et al.*, 2012). However, CaMKII is also used by cardiomyocytes as a mechanism to increase inotropy, as observed during I_{Ca} facilitation of LTCC. There the channels enter another gating mode, characterized by long-lasting and recurrent openings. Further, CaMKII causes a modest SERCA stimulation through phosphorylation of PLB at Thr-17 (Bers, 2001; Zhang *et al.*, 2011). As far as RyRs are concerned the picture is much more complex. CaMKII does phosphorylate the RyRs during β adrenergic stimulation, but its role during CICR and EC-coupling still remains up to debate (Bers, 2012). PKA-dependent phosphorylation of the LTCC is also activated during β adrenergic stimulation, this results in a larger I_{Ca} and corresponding elevated Ca^{2+} release, hence overpowering the changes that CaMKII may have exerted on the RyRs. Great efforts have been made by several research laboratories to understand the role RyRs-dependent CaMKII phosphorylation plays in cardiac muscle. In 2010 (Kushnir *et al.*, 2010) found that CaMKII phosphorylation of the RyRs is involved in mediating the positive force frequency relationship (FFR) in the heart, which was first described by (Bowditch, 1871).

However, CaMKII-dependent phosphorylation of the RyRs has been predominantly shown to be involved in other types of pathologies, such as atrial fibrillation in FKBP12.6 knockout mice (Li *et al.*, 2012), regulating the mitochondria stress response in the cardiomyocytes (Joiner *et al.*, 2012) and by participating in pathways which involve cardiac diseases and cardiomyocyte apoptosis leading to heart failure (Zhu *et al.*, 2003) (for reviews see (Anderson, 2011)). Taken together CaMKII is definitively a mechanism by which the cardiomyocytes regulate their function, but in some situations it might mediate the progression of heart failure, it can deliver undesirable effects and be involved in pathophysiological states, possibly due to the levels of phosphorylation on the RyRs (or other proteins) (Rodriguez *et al.*, 2003).

7.2.1.Contribution to inotropy

As described in the section 4.6. “β-Adrenergic modulation of EC-coupling” during β adrenergic stimulation of the cardiac muscle, both CaMKII and PKA become activated to increase performance. As mentioned in that section both PKA and CaMKII have specific intracellular targets which become phosphorylated to increase inotropy, such as PLB to accelerate SR Ca^{2+} re-uptake, LTCC-dependent increase of I_{Ca} to increase the magnitude of CICR and the phosphorylation of the RyRs to increase their sensitivity to open (Marx *et al.*, 2000; Wehrens *et al.*, 2004). The classical β-adrenergic pathway has been shown to mainly rely on PKA-dependent phosphorylation where I_{Ca} and SERCA become stimulated. However, in this thesis NO has been detected to increase intracellularly in isolated cardiomyocytes and during β-adrenergic stimulation for the first time. In agreement with other laboratories we propose in this research that during β-adrenergic stimulation the frequency of Ca^{2+} sparks increases, but most likely through a NO-dependent CaMKII activated mechanism, as the data suggests.

Increasing evidence also indicates that CaMKII is involved in the modulation of the sensitivity of the RyRs, especially during heart failure. In a study by (Li *et al.*, 1997) it was shown that CaMKII inhibition decreases SR Ca^{2+} transient amplitude, which would reduce force of contraction. This makes the entire picture somehow complex, as it has also been shown that activation of CaMKII could decrease the SR Ca^{2+} content, hence depleting the SR and the concomitant response to inotropy. (Guo *et al.*, 2006) made a substantial effort to elucidate this discrepancy. They showed in saponin-permeabilized wild type (WT) and phospholamban knockout (PLB-KO) ventricular cardiomyocytes, that the increased open probability was dependent on CaMKII phosphorylation of the RyRs and not due to PKA. This was complemented by the research of (Ginsburg & Bers, 2004) which by comparing similar I_{Ca} and SR Ca^{2+} content conditions, concluded that the time to peak of the Ca^{2+} transient was accelerated ~50% in the presence of Iso and that this effect was only PKA dependent.

Hence CaMKII seems to be pro-arrhythmogenic and to contribute to increased negative inotropy, it can increase the open probability of the RyRs which could deplete intracellular Ca^{2+} stores and lower cardiac contractility due to a low SR Ca^{2+} content (Curran *et al.*, 2007), as observed in heart failure (but see (Venetucci *et al.*, 2013) for an alternative opinion). For reviews see (Bers *et al.*, 2003; Kockskämper & Pieske, 2006))

7.2.2.Contribution to SR Ca^{2+} leak

During β-adrenergic stimulation of cardiomyocytes it has been a major challenge to characterize the components of the observed increase in SR Ca^{2+} leak (Curran *et al.*, 2007). In conditions of heart failure (where β-adrenergic stimulation has been treated with β-receptor blockers), CaMKII has been shown to phosphorylate the RyRs and to modulate such a Ca^{2+} leak (Ai *et al.*, 2005).

The additional pathway discovered during my thesis work adds to the understanding of this aberrant effect during β-adrenergic stimulation. As stated in the included manuscript, an increase in the frequency of Ca^{2+} sparks may significantly deplete the SR content if it is not compensated by SERCA stimulation. This can be clearly seen in Fig 4C and 5E, where the spark frequency was selectively increased (i.e. no SERCA stimulation) due to the presence of the NO donor, GSNO. This experimental conditions has similarities to conditions of congested heart failure, where SERCA expression has been show to be down-regulated in many studies (e.g. (Currie & Smith, 1999)). Therefore a progressive SR Ca^{2+} depletion may lead to smaller Ca^{2+} transients and impaired cardiac contractility. In addition, this situation may even induce arrhythmogenic Ca^{2+} waves due to the increased SR Ca^{2+} leak and abnormally high RyR Ca^{2+} sensitivity.

In the research of (Curran *et al.*, 2007) it was analyzed how during β-adrenergic stimulation with Iso, CaMKII but not PKA becomes activated and mediates the SR Ca^{2+} leak. This study was carried out following a specific protocol to assess SR Ca^{2+} leak, which was designed by (Shannon *et al.*, 2002). Furthermore (Santiago *et al.*, 2010) (Brochet *et al.*, 2011) showed that the sparks might not only account for the observed SR Ca^{2+} leak but that an “invisible leak” may also participate. As already discussed above in the section 4.5.3. “Local Control” a proposed mechanism that could explain the so called “invisible Ca^{2+} leak” are the Ca^{2+} quarks (Lipp & Niggli, 1996). These submicroscopic events may also account for the initial activation of CaMKII in resting cells before it undergoes nitrosation.

Therefore, CaMKII can significantly contribute to generation of SR Ca^{2+} leak specially during β-adrenergic stimulation. This newly discovered mechanism could increase the propensity of arrhythmogenic Ca^{2+} releases during diastole, where Ca^{2+} levels are low.

7.2.3.Arrhythmogenicity

When the SR is Ca^{2+} overloaded, the propensity for Ca^{2+} waves is higher. This type of Ca^{2+} waves has been termed “store overload induced Ca^{2+} release (SOICR) (MacLennan & Chen, 2009), as opposed to the Ca^{2+} release triggered by Ca^{2+} current. Other mechanisms that can catalyze the formation of Ca^{2+} waves are for example the changes of the threshold at which RyRs open to release Ca^{2+} (Jiang *et al.*, 2004). Recently it was shown that the propensity of developing ventricular arrhythmias in a mouse model of catecholaminergic polymorphic ventricular tachycardia (CPVT) increased during activation of CaMKII upon Iso application (Liu *et al.*, 2011). This would increase the phosphorylation at the CaMKII site on the RyRs and deplete the SR Ca^{2+} content.

In our model we also seek to understand how Iso increases the frequency of Ca^{2+} release events that could also evoke Ca^{2+} waves in beating myocytes. NO-mediated CaMKII activation during β-adrenergic stimulation would sensitize the RyRs and increase their P_o as observed indirectly by the increase in Ca^{2+} spark frequency. This goes in line with the observations from other laboratories, which have reported that NO is involved in increased inotropy and pro-arrhythmogenic behavior (Sarkar *et al.*, 2000; Cutler *et al.*, 2012). Thus, even in the more physiological condition of a stimulated cardiomyocyte we showed how this effect could be mimicked. As observed in Fig 5b (right) of the included manuscript diastolic sparks and spontaneous Ca^{2+} waves appear after GSNO application in field stimulated cells.

As mentioned above, an elevated SR Ca^{2+} content can also increase the frequency of Ca^{2+} sparks and elicit cytosolic Ca^{2+} waves via SOICR. However we show in this thesis that forskolin, a direct AC activator which activated PKA but did not increase Ca^{2+} spark frequency, increases the SR Ca^{2+} content significantly but not the occurrence of spontaneous Ca^{2+} waves. Taken together we can infer from this observation that in diastole PKA does not play a role in modulating the Ca^{2+} sparks frequency. Activating CaMKII seems to be sufficient to induce an arrhythmogenic behavior in resting cardiomyocytes. This becomes even more evident during EC-coupling, even though in stimulated cells the Ca^{2+} signal is much more complex than Ca^{2+} sparks in resting cells. Thus, as NO most likely will affect many mechanisms other than CICR and the RyRs, the observations made in beating cells cannot be exclusively attributed to the changes of RyR gating.

7.3.Implications for the interpretation of experimental data

7.3.1.Cross talk may have led to CaMKII activation when it was not expected / considered

Initially, we focused our investigation towards understanding the causes of the increased Ca^{2+} spark frequency, that was previously described by (Ogrodnik & Niggli, 2010), a former member of our lab that observed this effect when β stimulating isolated guinea pigs cardiomyocytes at rest and under whole-cell patch-clamp conditions. As it has been repeatedly shown in the literature, PKA is the principal modulator underlying positive inotropy in the heart. However, Ogrodnik found that the increase in Ca^{2+} spark frequency was not mediated by PKA, as suggested by protein kinase inhibitors for PKA and CaMKII. Despite potential limitations of this pharmacological approach, it rather appeared that CaMKII was involved in the increase in Ca^{2+} spark frequency. As already mentioned CaMKII is a molecule that is activated by CaM/Ca^{2+} which immediately raised the question: how is it possible that the RyRs open probability is modulated via CaMKII activation since these experiments were carried out with resting cardiomyocytes without detectable Ca^{2+} signals?

Since the increase in Ca^{2+} spark frequency required activation of the β-AR, we hypothesized that downstream along the pathway that at the end activates PKA, there had to be a crosstalk between the signaling pathways activation PKA and CaMKII.

As observed in Fig 1B,C and D of the “Original Manuscript” in chapter 5., forskolin-dependent activation of AC did not result in sparks, suggesting that the crosstalk finally activating CaMKII had to origin before the activation of AC. While we do not know this part of the pathway, CaMKII had to be activated before the AC. Thus, most likely from the receptor itself, or on the level of the G-protein.

So far we did not attempt to identify the exact point of crosstalk between the two signals activating PKA and CaMKII. Instead we determined the mechanism for CaMKII activation in the absence of Ca^{2+} and oxidation which resulted in the concept of CaMKII activation by nitric oxide.

Therefore after this thesis, a new door opens to investigate the details of the crosstalk mechanism that besides activating PKA, also somehow stimulates the NOS, directly or indirectly. It also opens a new field of study that can have significant repercussions in trying to understand the mechanism that modulate heart failure during adrenergic stimulation, which also considers consequences resulting from NO-dependent CaMKII activation.

7.4.Limitations of the study

As an approach to explain what is happening in each single cardiomyocytes of the heart, we tried to mimic the condition that could maintain the cells alive. This includes control of the pH, electrochemical potentials, source of energy, membrane voltages, antioxidants, among other, (for a better description please see the section 8.2. “Solutions”).

As an effort to explain physiological processes that are performed by single cardiomyocytes in the heart, we tried to mimic the conditions that could maintain the cells alive while at the same time allowing biophysical and pharmacological experiments (For a more detailed description please see the section 8. “Methodology”). No matter how hard we try, there will be differences between isolated cells and cells in the intact heart. As with all experimental studies, we cannot examine the cardiomyocytes without modifying and disturbing them, which is certainly changing their behavior. To study the cardiomyocytes we dialyze the cell interior with artificial solutions, and we provide pharmacological drugs to mimic what the body can supply naturally. So even if the conditions seem to be quite similar, they are not. They are only an approximation to explain what we want to know. All this has to be considered when interpreting and applying results obtained from this experimental system.

Cardiomyocytes behave differently in the heart compared to isolated conditions as they communicate through gap junctions, through which they share vital elements to maintain their homeostasis and deliver the desired output. Therefore lets have a closer look at the potential differences when studying an isolated system compared to a system in more physiological conditions.

7.4.1.Isolated and resting cells: difference to beating cells in the intact heart

Gap junctions are responsible of transmitting the AP through cardiomyocytes, but also for the exchange of ions and small molecules (Desplantez *et al.*, 2007). In isolated cardiomyocytes, the gap junctions close, this does not allow for redistribution of intracellular ions between them and therefore individual cells are by their own; this means

that cardiomyocytes can elicit autonomous activity and spontaneous contractions that might not be present in the issue, since adjacent cells would regulate between them. Once isolated, cardiomyocytes have limited supply of nutrients and extracellular agents that regulate the homeostasis of the cell. For example, cardiomyocytes are affected by physiological growth factors, external hormones such as acetylcholine and insulin. Another potential limitation is the fact that all experiments were carried out at room temperature, 22-23 °C. This are the usual conditions for the vast majority of the cellular cardiac research.

Therefore this thesis aimed to approach and extrapolate the observations that simplified system of single cardiomyocytes may provide. This simplifications are necessary, because well controlled cellular electrophysiological and EC-coupling studies are so far only possible in freshly isolated myocytes (for a review please see (Mitcheson *et al.*, 1998)).

7.4.2. Voltage-clamp conditions

Since CaMKII is a molecule that is modulated by alternating cytosolic Ca^{2+} levels, it was needed to control the membrane potential to keep it constant or apply well defined voltage protocols during the experiments designed to study this protein. Therefore by applying the patch clamp technique in the whole cell configuration (Neher *et al.*, 1978), the cell is dialyzed by a pipette solution which replaces the intracellular medium by an artificial solution. This new medium also allows to visualize the cytosolic Ca^{2+} concentration with fluorescent indicators. Using these indicators we can quantify both the increase in Ca^{2+} spark frequency and the SR Ca^{2+} content.

It has been reported that during the patch process some ~70 μM of the mobile intracellular Ca^{2+} buffers are dialyzed out of the cells. To compensate for this loss but to avoid over-buffering Ca^{2+} we included an equivalent amount of the fluorescent Ca^{2+} indicator fluo-3 in our solutions, DM-Nitrophen is an EDTA based light-sensitive Ca^{2+} chelator compound that is already mostly saturated with Ca^{2+} and therefore does not add to the cellular Ca^{2+} buffering (Kaplan & Ellis-Davies, 1988).

The trains of pre-pulse depolarizations (see Fig 1 of the “original manuscript”) aim to load the SR with a consistent amount of Ca^{2+}. One of the differences compared to physiological conditions is that there are no K^+ currents during these depolarizations

(blocked by Cs^{+}, Ba^{2+} and TEA-Cl). While the shape of the “action potential” is not as shown in Fig 10, it is a squared pulse from -80 to 0 mV during 200 ms which intends to mimic the actual physiological AP. While this it is not the exact mirror image of a physiological AP, it could change up to some extent the rate of Ca^{2+} fluxes, although not enough to produce undesired effects and sufficient to deliver the intended SR Ca^{2+} loading.

Once the cell has been patched and a giga-seal is formed, it could present some resistance to open the membrane upon gentle suction. Sometimes the patch pipette opening is less than optimal and a brief pressure pulse helps opening the patch which rapidly allows intracellular dialysis with the new medium. Sometimes it is seen how this process increases the size of the cell slightly which could induce some structural changes or modify protein interacting with the cell membrane (e.g. stretch activated channels).

Overall the patch-clamp technique is the best technique that we have available today. Under control of the proper conditions it is a great tool to observe changes in membrane currents due to pharmacological interventions and also to study the kinetics of Ca^{2+} fluxes under exact control of the membrane potential.

7.4.3.High ambient oxygen pressure

Working in the laboratory in conditions of ambient oxygen pressure might induce formation of reactive oxygen species (Sun *et al.*, 2001). Ambient oxygen pressure is about 150 mmHg, but in the tissue is much lower ~ 10 mm Hg. Ambient O_2 can change the level of oxidation and the function of the cardiac or skeletal RyR, this might result in functional changes of the RyRs, also via chemical interactions with other compounds (Sun *et al.*, 2008).

One example is the formation of peroxynitrite $ONOO^{-}$, this compound is formed after superoxide O_2^{-} interacts with nitric oxide NO. As our experiments were carried out in high oxygen pressure, we decided to control for this effect.

As observed in non published figures Fig 26, Fig 27 and in the “supplementary Figure” of the “original manuscript”, the formation of peroxynitrite either does not occur or is undetectable. This means that in our experiments the actual NO effect is maintained and that the probable $ONOO^{-}$ formation is negligible. This could be due to a fast action of the

superoxide dismutase which could decrease the levels of O_2^- into O_2 and H_2O_2, hence preventing the formation of this oxidant (Sentman *et al.*, 2006). This notion is supported by the absence of an inhibitory effect of Mn-TBAP, a peroxynitrite decomposition catalyst (Fig 2E of the “original manuscript”);

7.4.4.Species differences

Expression of different regulatory proteins may account for the differences observed in Ca^{2+} kinetics in the regulation of EC-coupling. For instance, NCX is more expressed in guinea pig cardiomyocytes than in mice, and they have more Ca^{2+} current. Mice show much more SR Ca^{2+} release and SERCA dependent Ca^{2+} re-uptake during relaxation. Thus, the Ca^{2+} signaling of guinea-pigs is more based on Ca^{2+} entry and removal, while in mice it is more based on SR Ca^{2+} release and re-uptake. This may be a reason why mice can elevate their heart rate up to ~ 600 beats per minute, which corresponds to 10 Hz. If we compare this high frequency to other species like human or even Guinea pig, this will never be possible to be achieved physiologically, as the energetic balance does not allow it (Loiselle & Gibbs, 1979) and because it could be harmful for the heart by inducing lethal arrhythmias between systole and diastole (Loiselle & Gibbs, 1979).

This adaptation to heart rhythms may be extrapolated to understand differences in the shape and frequency of Ca^{2+} sparks that are observed between for example mice, Guinea pigs, rats and human.

7.5.References

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8.METHODOLOGY

8.1.Isolation of ventricular myocytes

We used guinea-pig ventricular cardiomyocytes isolated following an established protocol of (Mitra & Morad, 1985b). To isolate the cardiomyocytes, hearts were first perfused at 37◦C for 6 min with a Ca^{2+} free solution composed in mM: 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.33 NaH2PO4, 5 HEPES, 11 glucose, pH 7.4, adjusted with NaOH. During the perfusion the cardiac muscle was digested by treatment with a cocktail of collagenase type II (0.5 mg/ml, Worthington, Switzerland) and protease type XIV (0.1 mg/ml, Sigma, Switzerland) during ~6min. After digestion, the two ventricles were cut into small pieces, which allowed the release of the cardiomyocytes in a solution containing 200 µM Ca^{2+} which was finally raised to 500 µM Ca^{2+} within ~30 min before experiments were carried out.

8.2.Solutions

During patch clamp experiments the cells were superfused with an extracellular solution containing (in mM): 10 glucose, 140 NaCl, 1.8 $CaCl_2$, 1 CsCl, 5 KCl, 0.5 $BaCl_2$, 10 HEPES, pH 7.4 adjusted with NaOH. A fast switching gravity-driven superfusion device allowed for rapid switching between experimental solutions and local superfusion of signal cardiomyocytes. The following was added to some of the solutions: 1 µM forskolin for adenylate cyclase (AC) stimulation (Alomone Labs), 1 µM isoproterenol (Iso) for β-AR stimulation , 10 mM caffeine for emptying of the SR, 500 µM L-NAME (N_ω-Nitro-L-arginine methyl ester hydrochloride) for NOS inhibition, 100 µM Mn-TBAP (Mn(III)tetrakis (4-benzoic acid) porphyrin) SOD mimetic / peroxynitrite decomposition catalyst (Merck),, 500 µM GSNO: S-Nitroso-L-glutathione (Cayman Chemical) for exogenous application of NO donor and DAF-2 DA (4,5-Diaminofluorescein diacetate) as an NO indicator (Cayman Chemicals). Drugs or inhibitors were prepared for every experiment from a stock or a fresh aliquot. Unless noted otherwise, chemicals were purchased from Sigma. Experiments were performed at (22°C)

8.3.The voltage-clamp technique

Pipettes with ~1.5 µm tip diameter and ~2.5 MΩ series resistance were pulled from borosilicate glass tubes (BF150-86-7.5, Sutter Instrument Company, Novato, CA, USA) by a horizontal puller (DMZ, Zeitz Instrumente, Augsburg, Germany). The cardiomyocytes were examined in the whole-cell configuration of the patch-clamp technique using an Axopatch 200 amplifier (Axon Instruments, Union City, CA, USA). Upon reaching whole-cell configuration cells were allowed at least 7 min for dialysis with a pipette solution to ensure equilibration with the intracellular solution composed, of (in mM): 0.5 $CaCl_2$,120 cesium glutamate, 20 HEPES, 5 K_2-ATP, 2 reduced glutathione (GSH), 20 TEA-Cl, 0.1 K_5-fluo-3 (Biotium), 2 Na_4-DM-nitrophen (Calbiochem) pH 7.2 adjusted with CsOH. In one specific case it was in addition included in the patch pipette, 10 µM AIP (Autocamtide 2-related inhibitory peptide) for CaMKII inhibition. Membrane currents were recorded using custom written software developed under Labview (National Instruments, Ennetbaden, Switzerland) and data were analyzed using IgorPro software (WaveMetrics, Lake Oswego, OR, USA). For field stimulation the myocytes were excited with a voltage 20% above threshold. The cells were loaded with fluo-3 by exposure to 5 μM of the ester form (fluo-3-AM) for 20 minutes, followed by at least 20 minutes for complete de-esterification.

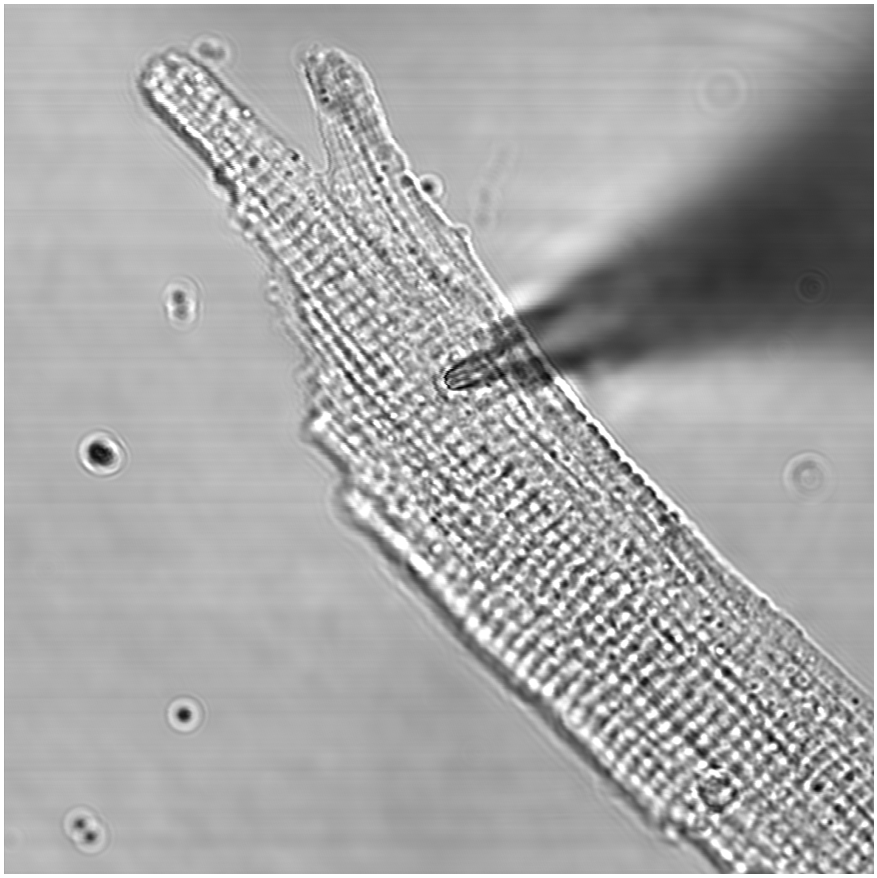

Fig 28: Typical image of a patched cardiomyocyte in the whole cell configuration. The top right pointy blurred shape is the patch pipette. Image self acquired.

To load the SR with Ca^{2+} to a well defined and steady state, we delivered an initial train of 20 depolarizations (from -80 to 0 mV each of them of 200 ms duration at a frequency of 0.5 Hz) followed by an assessment of SR Ca^{2+} content by acute application of 10 mM caffeine. After a second and identical train of depolarizations, three confocal line-scan images were acquired to assess Ca^{2+} spark frequency in control conditions. Subsequently the superfusion solution was switched to a perfusate containing 1 µM Isoproterenol (or a combination of compounds as indicated in the description of each experiment). Ca^{2+} spark frequency was again recorded by 3 line scans, within 3 min of application. At the end of the protocol caffeine was applied once again to asses SR Ca^{2+} content.

8.4.Confocal imaging of Ca^{2+}, NO and ROS

For confocal Ca^{2+} imaging of Ca^{2+} sparks and caffeine-induced Ca^{2+} transients, cells were loaded with the Ca^{2+} sensitive fluorescent indicator fluo-3 by dialysis through the recording pipette. For recording of Ca^{2+} transients during field stimulation, cells were incubated with 5 μM fluo-3 AM. Ca^{2+}, NO and ROS indicators (fluo-3, DAF-2, CM-H_2-DCF, respectively, see below) were excited at λ = 488 nm with a solid-state laser (Sapphire 488-10, Coherent, Santa Clara, CA, USA). The laser power used to excite fluo-3 was attenuated to ~150 µWatts. Fluorescence was detected at λ > 515 nm on an Olympus FluoView 1000 (Olympus, Volketswil, Switzerland) or on a MRC-1000 confocal laser scanning microscope (Bio-Rad, Glattbrugg, Switzerland). Line-scan images and Ca^{2+} sparks were analyzed using Image SXM with custom-written macros and ImageJ software, with the SparkMaster plugin (Picht *et al.*, 2007). Line-scan images are shown as self-ratio images, obtained by line-wise normalization for resting fluorescence. The amplitude of the Ca^{2+} transient evoked by caffeine application was used to compare SR Ca^{2+} content before and after any drug intervention in resting cardiomyocytes. Line-scan images are shown after normalization to its own resting fluorescence line by line.

NO and ROS were detected after pre-incubation with 0.1 µM DAF-2DA or CM-H_2-DCFDA for 30 min. After washout, 30 min was allowed for de-esterification. Inside the cell, the diacetate groups are hydrolyzed by cytosolic esterases, thus releasing DAF-2 or CM-H_2-DCF. In the presence of oxygen, binding of NO converts the non-fluorescent DAF-2 to its fluorescent triazolofluorescein derivative DAF-2T, which can be detected with confocal imaging. NO (and ROS) imaging was performed by using very low laser power (~20 µWatts) to minimize bleaching. In the absence of Iso, the NO related DAF-2T signal, exhibited a mono-exponential decay due to bleaching. We used Igor Pro software (WaveMetrics, Lake Oswego, OR, USA) to correct for bleaching by fitting a mono-exponential function to the decay (prior to the addition of Iso). The raw DAF-2T signal was subsequently normalized to the extrapolated exponential decay. In experiments where NOSs were inhibited by 1 hour pre-incubation with 500 µM L-NAME, a similar DAF-2 pre-incubation protocol and analysis were performed.

8.4.1.In-vitro CaMKIIδ Activity Assay

Recombinant full length human CAMKIIδ (Swiss-Prot Q13557-8) was supplied by Abcam (Cambridge, UK). CaMKIIδ (10 ng/sample) was pre-incubated in a kinase reaction buffer, of the CaMKII activity assay kit distributed by CycLex, with 250 μM $CaCl_2$ and 120 nM calmodulin (CaM) on ice during 1 min. Then, the protein was treated by addition of either 1 μM H_2O_2, 500 μM GSNO or vehicle (as a control condition) at 22 °C for 10 min. 10 mM EGTA was then added to the 3 different conditions and incubated for 10 min to buffer the Ca^{2+} ions. CaMKII activation was measured using the non-radioisotopic CaMKII assay kit (CycLex, Japan), following the manufacturer's instructions. Samples were loaded into microplate wells precoated with the polypeptide Syntide 2, which can be phosphorylated by CaMKII, upon 30 min pre-incubation at 30°C. The amount of phosphorylated substrate was detected by a specific antibody conjugated with peroxidase. The absorbance was measured ratiometrically at wavelengths of 450 and 550 nm. The maximal and the minimal Ca^{2+}-CaM–dependent CaMKII activity, were determined in 250 μM Ca^{2+} plus 120 nM CaM and in very low Ca^{2+} (<10 nM) plus 120 nM CaM after EGTA application, respectively. The Ca^{2+} concentration after EGTA application was assessed with the Nanodrop NP 3000 (Thermo Scientific, Allschwil, Switzerland). Activity values are represented as mean ± SEM normalized with respect to the maximal and minimal CaMKII activity.

8.4.2.Cys-SNO inmunobloting

To measure the nitrosation of $CaMKII_\delta$ we used a specific antibody against S-nitroso-cyteine (Cys-SNO). Purified CaMKIIδ was pretreated with 500 μM GSNO or vehicle (as in the previous CaMKII activity assay). CaMKII samples (80 ng of protein) were mixed with Laemmli buffer and loaded without reducing agents. CaMKII samples were separated by SDS-PAGE and transferred onto PVDF membrane. Membrane was incubated with a rabbit Cys-SNO antibody (1:500)(Sigma-Aldrich) overnight at 4°C. This was followed by incubation with anti-rabbit secondary antibody Dylight 800 conjugated (1:10.000) (Rockland) during 1 hour at room temperature. The bands were detected with an Odyssey scanner (Li-Cor Biosciences, Homburg, Germany) and the band intensities were quantified using GelAnalyzer 2010a software. Nitrosation values are represented as mean ± S.E.M. normalized with respect to control (in absence of NO donor).

8.5.Statistics

Statistical analysis was executed with IgorPro and Numbers software. All results are normalized to Ctrl conditions; sparks were quantified in (sparks/100μm/sec) and normalized to control to be presented as a change in Ca^{2+} spark frequency. SR Ca^{2+} content was determined as a percentage of control where both are expressed as normalized mean values ± standard error of the mean (SEM). Values were compared for significance using Student's t-test. Statistical significance ($*$) and ($**$) was assumed for P values less than 0.05 and 0.01 respectively.

8.6.References

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Declaration of Originality

Last name, first name: Gutierrez Daniel

Matriculation number: 06-819-601

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

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I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 3 October 2013

Signature