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THE MODULATION OF SERCA PUMP ACTIVITY AS A TOOL FOR THE MANAGEMENT OF HEART FAILURE.

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Relate, they are going to tell me. So we'll understand and file the case. They're wrong. Only what we don't understand can have an end. There will be no end.

Peter Høeg - Smilla's Sense of Snow

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Chapter 1: Introduction

Heart Failure: a General Overview

Heart failure is a highly widespread pathology, which accounts for at least 5% of acute hospital admissions and for about 2% of national health care expenditure in developed countries[1;2]. It is estimated that more than 15 million of people in Europe have heart failure, with a prevalence which rises sharply from 2-3% in the younger groups, mainly composed by men, to 10-20% in the elderly, equally distributed between sexes[1]. Although many definitions of heart failure have been made in the last decades[3], in the recent years the pathology has been generally defined as a syndrome in which the patients share the following features: symptoms typical of heart failure, like dyspnoea (shortness of breath) and fatigue; fluid retention such as pulmonary congestion; functional and/or structural abnormalities of the heart at rest[1;4;5]. Due to the complexity of the diagnosis (early stages of heart failure are often asymptomatic) and to the diagnosis of major co-morbidities such as diabetes, it is likely that the incidence of heart failure in the population is currently underestimated[1].

Aetiology

Many different causes, some of them not completely characterized yet, can lead to heart failure, either alone or in combination. The development of heart failure begins with an INDEX EVENT[6]: an injury or a condition of prolonged stress of the myocardium that is able to induce the activation of neurohormones and cytokines in response to it[7;8]. Coronary heart disease and systemic hypertension are the major

causes of heart failure[1;9], but virtually any form of heart disease can lead to the syndrome, from gene mutation to myocardial infarction[6]. The index event is usually followed by an asymptomatic period, during which structural and functional remodelling of the heart occurs, leading to left ventricular dysfunction[10;11] and lately to overt heart failure[12].

The main risk factors are asymptomatic left ventricular dysfunction and myocardial hypertrophy, which may underlie asymptomatic heart failure as well, myocardial infarction and hypertension[9]. Obesity, diabetes and smoking are other important risk factors[1].

Progression and Prognosis

There is now a general consensus about describing the progression of heart failure with the neurohumoral paradigm: the index event is associated to a diminished cardiac performance, which triggers compensatory mechanisms mediated by many neurohumoral pathways in order to increase the cardiac output (the volume of blood being pumped by the heart) and restore optimal blood perfusion throughout the body[8;13]. However, the chronic nature of these compensatory mechanisms results in cardiac remodelling and hypertrophy[14], alteration of contractile proteins levels[15;16], cardiomyocytes death[17] and fibrosis[18], and thus represents a major component of heart failure pathogenesis[6;8;13].

As a consequence of the maladaptive response arising from chronic neurohumoral stimulation the ventricular chambers become stiff and complete relaxation is impaired. The pressure developed by the heart increases to maintain an optimal cardiac input, at the expense of

increased pulmonary capillary pressure, which leads to pulmonary oedema[19]. The increased blood pressure results also in renal dysfunction[20] and in electrolyte imbalance (hyperkalaemia[1]). The stiffness of the heart limits coronary blood flow, and a state of energy starvation ensues[21]. As heart failure progresses, the contractile deficit of the heart increases and compensatory mechanisms are enhanced, leading to a general worsening of the pathology towards end-stage heart failure, where dyspnoea and fatigue are present even at rest. Many classifications of the severity of heart failure have been made according to the symptoms or the structural abnormalities observed. Table 1 reports the two classifications most commonly employed to describe heart failure stages[4;5].

Despite the continuous improvements in the therapies and in the non-pharmacologic management of heart failure, the syndrome retains a poor prognosis: 50% of patients dead at 4 years from diagnosis and 40% of patients admitted to hospital with heart failure are dead or readmitted within one year[1;22]. With the exception of lung cancer, heart failure is considered more malignant than common types of cancer[23].

Since the impaired contractility is the feature of the syndrome that triggers the maladaptive responses and determines its worsening, the cellular mechanisms responsible for contraction and relaxation, the alterations of such mechanisms induced by heart failure and the pharmacologic tools employed to restore optimal contractility will be detailed in the following sections.

Table 1 ***Most common classifications of heart failure stages***

American College of Cardiology/ American Heart Association (ACC/AHA) stages of heart failure (based on functional abnormalities)		New York Heart Association (NYHA) functional classification (based on symptoms and physical activity)	
Stage A	At high risk for heart failure but without structural heart disease or symptoms of heart failure.	Class I	No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation or dyspnoea.
Stage B	Structural heart disease without signs or symptoms of heart failure.	Class II	Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitation or dyspnoea.
Stage C	Structural heart disease with prior or current symptoms of heart failure.	Class III	Marked limitation of physical activity, Comfortable at rest, but less than ordinary activity results in fatigue, palpitation or dyspnoea.
Stage D	Advanced structural heart disease and marked symptoms of heart failure at rest despite maximal medical therapy.	Class IV	Unable to carry on any physical activity without discomfort. Symptoms at rest. If any physical activity is undertaken, discomfort is increased.

How Cardiac Contraction Works: the Excitation-Contraction Coupling

The contraction of ventricular myocytes is the result of the interplay of many molecular mechanisms belonging to the sarcolemma (the cellular membrane), the sarcoplasmic reticulum (SR*), and the cytosol. The cellular contraction begins with the depolarization of the sarcolemma and the following raise of cytosolic Ca^{2+} (from now on: $[Ca^{2+}]_{cyt}$). The calcium ion plays a central role in cellular contraction as changes in $[Ca^{2+}]_{cyt}$ determine contraction and relaxation through the reversible binding to myofilament proteins[24]. This process is called excitation-contraction (EC) coupling[25] and is summarized in figure 1. Derangements in the mechanisms involved in Ca^{2+} homeostasis constitute the molecular determinants of the impaired contractility observed in the later stages of heart failure.

The Calcium-Induced Calcium Release

The cellular contraction begins with the depolarization of the sarcolemma. The depolarization starts in the sinoatrial node and

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* Abbreviations used in the chapter: $[Ca^{2+}]_{cyt}$ - cytosolic concentration of Ca^{2+}; $[Ca^{2+}]_{SR}$ - intraluminal concentration of Ca^{2+}; CaM - calmodulin; CaMKII - Ca^{2+}-calmodulin kinase II; CICR - Ca^{2+}-induced Ca^{2+} release; cSR - corbular SR; CTS - cardiotonic steroid; DHPR - dihydropiridine receptor; EC-coupling - excitation contraction coupling; FKBP - FK-506 binding protein; jSR - junctional SR; $I_{Ca,L}$ - Ca^{2+} current mediated by L-type channels; IST - istaroxime; NCX - Na^+/Ca^{2+} exchanger; NKA Na^+/K^+ - ATPase; nSR - network SR; PLB - phospholamban; PMCA - plasma membrane Ca^{2+} ATPase; RyR - ryanodine receptor; SERCA - Sarco(Endo)plasmic Ca^{2+} ATPase; SR - sarcoplasmic reticulum; Tn - troponin

propagates through the conduction tissues (internodal tissue, atrioventricular node, His bundle and Purkinje fibers) until it reaches the working myocardium. The propagation of the depolarization is achieved by the flux of Ca^{2+} and Na^{+} ions through gap junctions connecting the cardiomyocytes and is regenerated within the single myocyte by the activation of Na^{+} channels (isoform $Na_v1.5$), whose opening initiates the cardiac action potential.

The sarcolemmal depolarization activates the Dihydropyridine Receptors (DHPRs), Ca^{2+} channels that allow Ca^{2+} entry into the cell, mediating the $I_{Ca,L}$ current. However, the amount of Ca^{2+} that enters the cell through the DHPRs during an action potential in not sufficient to induce myofilament shift and contraction to take place. Nevertheless, the Ca^{2+} that enters the cardiomyocyte can activate the Ryanodine Receptor (RyR) channels, Ca^{2+} channels on the SR membrane which open releasing Ca^{2+} from the SR, the main intracellular store for the ion, in a process termed Ca^{2+}-induced Ca^{2+} release (CICR)[26]. The Ca^{2+} transient generated by RyR opening raises $[Ca^{2+}]_{cyt}$ enough to allow the ion binding to the myofiament proteins and induce their activation.

The interplay between DHPRs and RyRs is made possible by the proximity of the two channels, which are juxtaposed in order to form a functional unit called couplon[27]. DHPRs are located on the T-tubules, invaginations of the sarcolemma that run deep into the cell. T-tubules greatly increase cell membrane surface, so that no region inside a ventricular myocyte is more than 1.2 μm from the cell membrane[28].

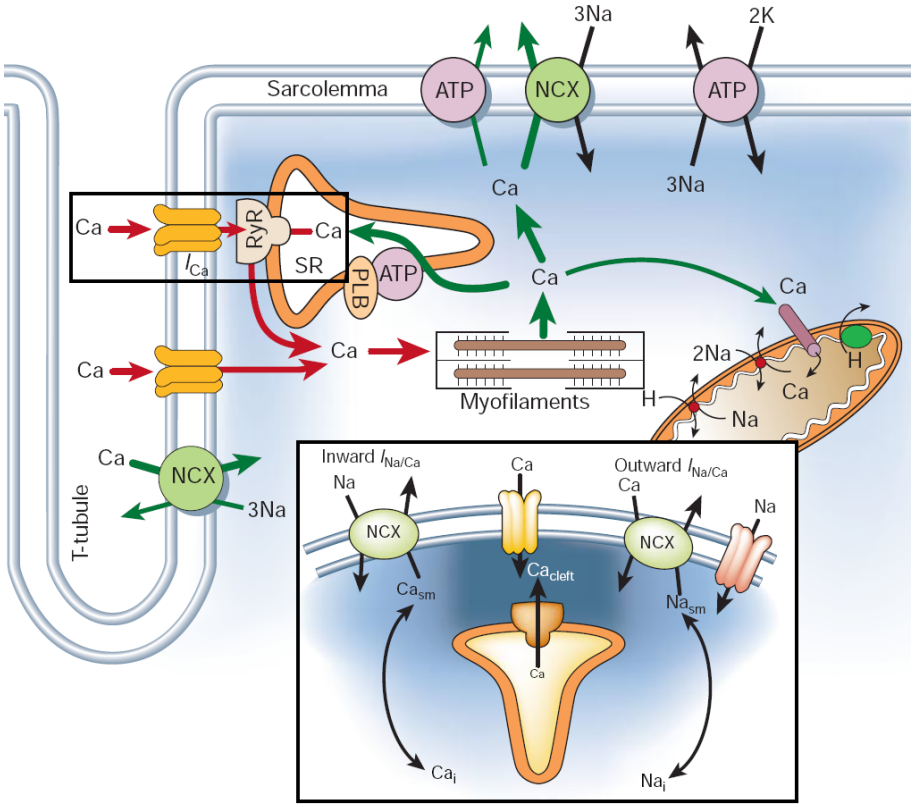

Figure 1 ***Schematic representation of the Ca^{2+}-induced Ca^{2+} release.*** The red arrows indicate the fluxes of Ca^{2+} that induce cell contraction while the green ones indicate the flux of the ion that allow cell relaxation. The black box outlines the dyad, magnified below. ATP - ATPase; NCX - Na^{+}/Ca^{2+} exchanger; PLB - phospholamban. Adapted form Bers[25].

The SR in turn forms sacs close to T-tubules, called terminal cisternae (which constitute the junctional SR or jSR), increasing the area of overlap between the two membranes[29]. The dyad, the region of the cell encompassing a T-tubule and the jSR in its proximity, is where the couplon forms (figure 1, inset). The couplon is constituted by 10-25 DHPRs on a T-tubule in register with a cluster of up to 300 RyRs on the jSR[27;30;31]. The cytosol spanning between the two membranes constitutes the dyadic cleft, a diffusionally limited space which grants that a small number of Ca^{2+} ions can produce a great change of their concentration[25]. In this way the relatively small number of Ca^{2+} ions that flows through a DHPR channel can easily activate a group of RyR channels in the cluster, allowing an high coupling efficiency (EC-coupling gain). The mechanism is highly redundant, since it is estimated that in the physiological setting the opening of a single DHPR is enough to trigger Ca^{2+} release from the jSR of a couplon[32]. Although a couplon contains hundreds of RyRs, only 10 to 25 of them per time activates simultaneously during the EC-coupling[31;33]. The RyRs which open in a dyad may vary from time to time[31].

The Dihydropyridine Receptor Channel

Two types of Ca^{2+} channels are present on the sarcolemma of ventricular myocytes, the L- (long lasting) and the T- (transient) type, the L-type being by far the predominant, although the T- to L-type current ratio varies among different species[24;34]. Notably, T-type currents have not been observed in human ventricular myocytes[35].

L-type Ca^{2+} channels are inactivated both by the membrane voltage and by Ca^{2+}, through the interaction with calmodulin (CaM). CaM mediates also $I_{Ca,L}$ facilitation[36]. Due to the diffusional constraints of the dyadic cleft, the Ca^{2+} that enters the cell during an action potential is sufficient to inactivate $I_{Ca,L}$ current, in addition to the inactivation induced by the Ca^{2+} released by the SR[37], limiting the amount of Ca^{2+} entry.

The channel is modulated by dihydropyridines, most of them acting as channel blockers or antagonists, some of them (like (-)-Bay K 8644) increasing the channel opening time and thus acting as agonists[38]. Also phenylalkylamines and benzothiazepines antagonize the channel, while Ni^{+} and Cd^{2+} block it[39-41].

The Ryanodine Receptor Channel

There are three RyR isoforms in mammals, the RyR2 being the one mainly expressed in the cardiac tissue. It is an homotetramer of big dimensions (each subunit weights about 565 kDa) with both a cytosolic and a reticular tail[42]. The RyR channel is present in the dyad, forming clusters of 100-300 units[27;30;31], although it has been hypothesized that not all RyRs in a cluster are functional[31]. In addition, clusters of RyR are present in the corbular SR (cSR), a portion of the SR far from the sarcolemma and the T-tubules[43;44]. Indirect evidence suggests that on the SR are present also non-clustered RyRs (rogue RyRs)[45;46].

Ca^{2+} Sparks

RyR opening is highly coordinated, with a group of 10 to 25 units within a cluster opening in a cooperative fashion[31;33;46-49]. The opening

of a group of RyRs generates a Ca^{2+} spark, a local rise of Ca^{2+} in the dyadic cleft[50] (for an extensive review of the subject, see ref. [51]). Although smaller releases of Ca^{2+} from the SR (Ca^{2+} quarks, releases of Ca^{2+} from a single RyR) have been characterized[45;52], in physiological conditions Ca^{2+} sparks constitute the elementary events of Ca^{2+} release in a cardiomyocyte. A Ca^{2+} spark lasts tens of milliseconds and is confined in an area of about 2 μm of diameter and 8 fl of volume[51]. The rising phase of a Ca^{2+} spark reflects the gating of a group of RyR channels, while the decay kinetics are largely determined by the diffusion of the ion from the dyadic cleft[53]. Ca^{2+} sparks can be seen in quiescent cells as spontaneously occurring events[50;54;55], or under stimulation in conditions that reduce the DHPR channel open probability or the Ca^{2+} flux through it (evoked sparks)[56-58]. Each macroscopic Ca^{2+} flux across the SR in a cardiomyocyte can be described by a temporal and spatial summation of Ca^{2+} sparks[51].

Modulators of RyR Activity

Many molecules affect RyR activity. Both cytosolic and reticular Ca^{2+} modulate RyR. The modulation by $[Ca^{2+}]_{cyt}$ is bell-shaped, with an increase of RyR activity from sub-micromolar $[Ca^{2+}]_{cyt}$ to about 100 μM Ca^{2+}, and a decrease at millimolar concentrations of the ion[59;60]. This raised the hypothesis that two Ca^{2+} binding sites exists on the cytosolic side of the RyR, one with high affinity which stimulates Ca^{2+} release and one with low affinity which inhibits Ca^{2+} release[61]. Intraluminal Ca^{2+} stimulates Ca^{2+} release[62-64] by grading the interaction of regulating luminal proteins with the channel rather than directly interacting with it[65-67].

Mg^{2+} and changes from physiological pH, which occurs during ischemia, depress SR Ca^{2+} release[59;68;69], while adenine nucleotides enhance it[59;60]. Ryanodine in the nanomolar range induces RyR opening stabilizing it in a semiconductive state, while at concentrations higher than 100 μM blocks it[70]. Caffeine is an agonist of the channel[60;71], and it is widely used at high concentrations (10 mM or more) to asses the SR Ca^{2+} content, as it makes all the RyRs on the SR to rapidly and reversibly open[72]. Conversely, local anaesthetics, like tetracaine, stabilize the channel in the closed state, reducing its basal activity (e.g., it reduces Ca^{2+} spark frequency)[73;74].

Many proteins interacts with the RyR regulating its activity[75]. Large macromolecular complexes are found on the cytosolic side of the channel, that for this reason is called RyR foot[42;75].

Ca^{2+}-CaM complexes are normally bound to RyRs, although the stoichiometry may vary according to the average $[Ca^{2+}]_{cyt}$ in the cell[76-78]. They shift RyR sensitivity to $[Ca^{2+}]_{cyt}$ towards higher values, stabilizing the channel at rest[79]. A similar action is exerted by sorcin, a protein that interacts with the RyR in a Ca^{2+}-dependent fashion, depressing the channel activity[80;81]. Being a soluble protein, sorcin dialysis may account for the increased Ca^{2+} spark frequency observed in permeabilized myocytes[80;82].

FK-506 binding proteins (FKBP), and particularly the cardiac isoform FKBP12.6, binds to the RyR giving it cooperativity[83;84]. An increased RyR cooperativity means lower activity of the channel at rest but higher Ca^{2+} release during a stimulated transient[85-87].

While in skeletal muscle the sterical interaction between DHPR and RyR is strong enough that a conformational change of DHPR channels

during their activation is sufficient to open RyRs independently from a Ca^{2+} influx, in cardiac myocytes a less strong interaction occurs. Nonetheless, conformational changes of DHPRs alter resting Ca^{2+} spark frequency even in the absence of extracellular Ca^{2+}, suggesting a loose modulation of the channel[88;89].

PKA, Ca^{2+}-calmodulin kinase II (CaMKII) and protein phosphatases PP1 and PP2A are also associated to RyRs, but their influence on the channel activity will be discussed later (see: Sympathetic modulation of EC-coupling).

On the luminal side, the main proteins interacting with the channel are calsequestrin, junctin and triadin. Calsequestrin is the main Ca^{2+} buffer inside the SR, while junctin and triadin are transmembrane proteins. The interactions among these proteins and the RyR are all Ca^{2+} dependent[90], and are probably involved in the modulation of RyR activity by intraluminal Ca^{2+} ($[Ca^{2+}]_{SR}$). The model proposed is the following: junctin and triadin normally interacts with the channel increasing its activity. However, at low sarcoplasmic concentrations of Ca^{2+}, calsequestrin binds to the two proteins exerting an inhibitory effect and reducing the overall activity of the channel. High $[Ca^{2+}]_{SR}$, instead, determine the dissociation of calsequestrin from the complex, relieving its negative modulation and promoting Ca^{2+} release[65-67;91].

Termination of Ca^{2+} Release

Given the auto-regenerative nature of CICR, the release of Ca^{2+} from the SR during EC-coupling should in line of principle end after complete SR depletion. Instead, there seems to be a threshold for the SR Ca^{2+} content (about 60% of the diastolic SR content) under which

Ca^{2+} release terminates[92;93]. Many mechanisms have been proposed so far to explain Ca^{2+} release termination, but no one had allowed the development of a model that could completely fit with the experimental observations reported. Although some of the processes certainly occurs during the termination of Ca^{2+} release, further work is required to clearly characterize the phenomenon.

Stochastic attrition is a mechanism that certainly participate to Ca^{2+} release termination. It represents the casual transition of a RyR receptor from the open state to the close one[94]. When a large number of RyRs are opened, the probability that all the channels close in a short period of time (shorter than the mean time for a channel to re-open) is very low, making stochastic attrition unlikely to be the driving mechanism for release termination. However, when other mechanisms start release termination, stochastic attrition may be account for the closing of the last opened channels[95]. The coordinated gating of RyRs is another mechanism that contributes to the last phases of the phenomenon, because when some RyRs in a cluster are closed, the closure of the others is highly favoured[83;84].

RyR inactivation may be induced by Ca^{2+} itself[59-61] or by other inactivating proteins, CaM[79] and sorcin[80;81] being the best candidates, since their inhibitory action is Ca^{2+} dependent. However, a direct inactivation of the channel by Ca^{2+} requires millimolar concentrations of the ion, which are higher than the ones normally achieved in the dyadic cleft[25]. On the other hand, CaM kinetics for RyR binding and dissociation are very slow (in the order of seconds or more)[76], and do not fit well with the kinetics of Ca^{2+} release termination, which are some order of magnitude below.

Finally, some contribution might derive from the depletion of the SR stores. One possibility is that the SR stores are diffusionally limited and that the depletion of a store induces the release termination in that site before Ca^{2+} can diffuse from other stores that did not participate to SR Ca^{2+} release (stores in non-junctional SR)[95]. However, the hypothesis of the SR as a diffusionally constrained space has been recently challenged[92;93]. A second mechanism is the binding of calsequestrin to junctin and triadin as intraluminal Ca^{2+} lowers and the consequent reduction of RyR activity[65-67]. Nevertheless, calsequestrin has a low affinity for Ca^{2+} (ref. [96]), and thus its modulation of RyR activity may account for large SR Ca^{2+} variations (e.g. it might affect RyR basal activity at low vs high SR Ca^{2+} contents) rather than for the local depletion of the SR during a Ca^{2+} transient.

Removal of Cytosolic Ca^{2+}

For cell relaxation to occur Ca^{2+} must be removed from the cytosol. Four mechanisms participate to cytosolic Ca^{2+} decay (see figure 1): the Sarco(Endo)plasmic Ca^{2+} ATPase (SERCA), the Na^{+}/Ca^{2+} exchanger (NCX), the sarcolemmal Ca^{2+} ATPase (PMCA: plasma membrane Ca^{2+} ATPase) and a mitochondrial uniporter. The relative contribution of each protein to Ca^{2+} decay varies among different species, but SERCA is always prevalent[25]: in rodents, this pump accounts for the 90% and NCX for 7% of cytosolic Ca^{2+} removal[97;98]; in other mammals and man SERCA accounts for about 70% and NCX for 28% of relaxation[24]. PMCA and the mitochondrial uniport are much more slower and usually contribute to cell relaxation only for 1-

2%, with the exception of ferret cardiomyocytes, where PMCA relative contribution is several times larger[99].

The Na^+/Ca^{2+} Exchanger

NCX is an antiporter that transports Na^+ inside the cell while extruding Ca^{2+} against its concentration gradient with a 3:1 stoichiometry[100]. It co-localizes with the Na^+ channel in the T-tubules, but in a discrete region from the DHPRs (it is not present within the dyad)[101]. Due to its stoichiometry, NCX is electrogenic, mediating an inward current. NCX can also work in the reverse mode, transporting Ca^{2+} inside the cell. Since the direction of ion transport depends on the equilibrium potential of the exchanger, which in turn is determined by the equilibrium potential of the two ions[24], the reverse mode is favoured by high intracellular Na^+ or in the early phases of the action potential[25].

It has been hypothesized that the influx of Ca^{2+} mediated by NCX at the beginning of the action potential could play a role in EC-coupling. Although in the absence of active DHPRs NCX can induce SR Ca^{2+} release[102;103], the relative contribution of the exchanger to the global Ca^{2+} entry is small, given that DHPR channels have a bigger flux with faster kinetics[24] and are closer to RyRs[101], while the SR Ca^{2+} release induced by NCX alone is not enough to activate myofilaments[103;104]. Instead, NCX has an indirect role in regulating diastolic Ca^{2+} release by setting the threshold for Ca^{2+} sparks[105], being the main regulator of $[Ca^{2+}]_{cyt}$ at rest[106;107].

NCX is allosterically modulated by Ca^{2+} and it is blocked by low pH, Ni^+ and Cd^{2+} (ref. [40;108;109]).

The Sarco(Endo)plasmic Ca^{2+} ATPase

SERCA is a P-type Ca^{2+} pump that mediates SR Ca^{2+} reuptake. It pumps two Ca^{2+} ions in the SR hydrolyzing an ATP molecule[110]. The cardiac isoform (SERCA2a) is located on the network SR (nSR), a portion of SR which wraps the myofilaments and nearby mitochondria[43;44]. Although thermodynamically unfavoured, the flux of the pump can be reversed (backflux), with concomitant synthesis of ATP form ADP and inorganic phosphate[111-113]. In ventricular myocytes, the pump can establish a maximum gradient $[Ca^{2+}]_{SR}$:$[Ca^{2+}]_{cyt}$ of 7000:1 at which there is a balance between the forward and the backward fluxes[114].

At variance with other isoforms expressed in skeletal and smooth muscles, SERCA2a activity is regulated by phospholamban (PLB), a reversible inhibitor[115]. PLB is a transmembrane protein which avidly binds to SERCA[116] and decreases the energetic efficiency of the pump (it favours the backflux mode)[117]. The phosphorylation of PLB by either PKA or CaMKII[118;119] relieves the inhibition by inducing the dissociation of the cytosolic domain of PLB from the pump[120]. On the SR membrane PLB is present in two states: monomeric, associated to SERCA and able to exert its inhibitory function, and pentameric[121]. The balance between the two states and the rate of exchange between them determines the efficiency of the inhibition[122].

SERCA activity is allosterically modulated by $[Ca^{2+}]_{cyt}$ (ref. [123]), and it is inhibited by thapsigargin[124] and cyclopiazonic acid[125].

The Myofilaments

In ventricular myocytes myofilaments occupy 45-60% of the cell volume[126;127]. Myofilaments are mainly composed by two proteins, myosin (thick filament) and actin (thin filament). Thin and thick filaments interdigitate displaying incomplete overlapping. Their disposition generates a pattern of bands clearly distinguishable by electron microscopy[128]. The ordinate progression of the bands led to the identification of the sarcomere, the fundamental unit of contraction[129]. The sarcomere is delimited by two z discs, from where thin filaments, anchored to the elastic components of the cytoskeleton, depart toward the center of the sarcomere. The thick filaments encompass the central region of the sarcomere, and the A band is the region where thin and thick filaments overlap[128;130].

Myosin is composed by a heavy and a light chain, the heavy one presenting a globular domain (myosin head) with the binding sites for actin. Myosin heads protrude from the thick filament, each one with a rotation of 120° from the previous head, in order to interact with the surroundings thin filaments[24]. Tropomyosin is a scaffold protein bound to the thin filaments, that allows the anchoring of troponin (Tn)[131]. Tn, the sensor for Ca^{2+}, interacts both with myosin and tropomyosin. Tn is constituted by three subunits, TnT, responsible for the interaction with tropomyosin, TnC, which binds Ca^{2+}, and TnI, which masks the binding sites for actin on the myosin head[132]. When $[Ca^{2+}]_{cyt}$ raises following SR Ca^{2+} release, the binding of the ion to TnC induces a conformational change of the protein able to displace TnI from the myosin head, allowing myosin-actin interaction[133].

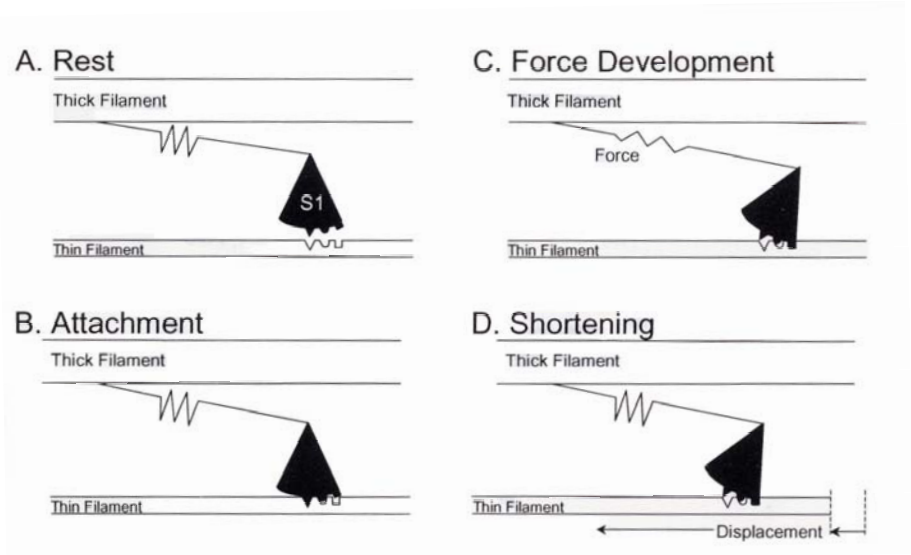

Figure 2 ***Mechanical model for thin and thick interaction and myofilament slide.*** A) myosin head (S1) is detached from actin; B) the myosin head interacts with actin; C) the sites for actin on the myosin head interact sequentially, developing force; D) the myofilments slide relative to one another. From Bers[24].

The hydrolysis of ATP by the myosin head induces the four binding sites to sequentially interact with actin, making the filaments to shift one on the other, resulting in sarcomere shortening[134] (see diagram in figure 2). The binding of a new molecule of ATP makes the myosin head to dissociate from actin and, if Ca^{2+} is still present, to start a new sequential interaction[135;136]. This allows force development and cell contraction. As $[Ca^{2+}]_{cyt}$ decays, TnI masks the binding sites again and cell relaxation takes place.

Macroscopic Ca^{2+} Fluxes in the Cytosol

Due to the number and the complexity of the mechanisms that participate to EC-coupling, different fluxes of Ca^{2+} can be seen in the cytosol, some of them being the consequence of Ca^{2+} mismanagement. Representative traces of these macroscopic fluxes recorded in mouse cardiomyocytes are reported in figure 3.

$I_{Ca,L}$ Current

$I_{Ca,L}$ current is the Ca^{2+} current that originates from DHPRs opening (L-type channels; fig. 3A). The current decreases rapidly due to voltage- and Ca^{2+}- induced inactivation of the channel[36;37], limiting the Ca^{2+} entry to 14 to 21 μmol per liter of cytosol[137;138]. The channel is activated at membrane potentials of -30 mV or more; the maximum current is observed at around 0 mV, further depolarizations reduce it because of decreased driving force for Ca^{2+} as the potential approaches the reverse potential of the ion[24;139-141].

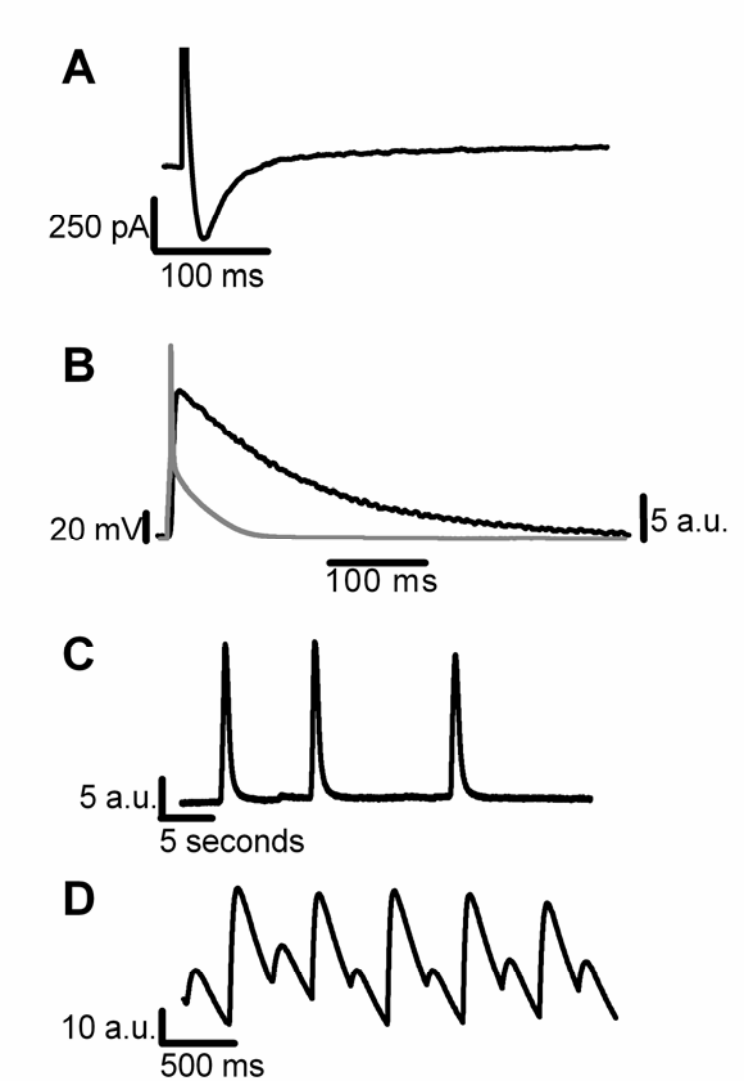

Figure 3 ***Macroscopic Ca^{2+} fluxes in the cytosol.*** Representative traces recorded in mouse ventricular myocytes. A) $I_{Ca,L}$ current induced by a step depolarization from -40 to 0 mV; B) evoked Ca^{2+} transient (black trace) superimposed to an action potential (gray trace) recorded at a frequency of stimulation of 2 Hz; C) Ca^{2+} waves recorded in a resting cell; D) Ca^{2+} alternans.

Ca^{2+} Transients

Ca^{2+} transients are the physiological consequence of EC-coupling. They are the result of the synchronous activation, induced by $I_{Ca,L}$, of the clusters of RyR belonging to almost all the couplons present in the cell[25]. Ca^{2+} transients lasts a few hundreds of milliseconds, a length of time comparable to the one of cardiac action potential, and begin soon after the upstroke of the action potential. The overlap of the two phenomena (shown in figure 3B) is extremely important: the plateau phase of the action potential delays the recovery of most of the Na^{+} and Ca^{2+} channels from inactivation, allowing $[Ca^{2+}]_{cyt}$ to decay and the cells to relax (which in the end results in the ventricle chambers refilling with blood), before a second contraction can be triggered[24]. The decay kinetics of a Ca^{2+} transient reflect the contributions of all Ca^{2+} remove systems[97].

The amplitude of Ca^{2+} transients determines the extent of sarcomere shortening (more myosin heads are recruited for the contraction) and thus the force developed by the cardiomyocyte[132]. It is influenced by $I_{Ca,L}$ and by the SR Ca^{2+} content, both positive modulators of SR Ca^{2+} release[26;62;64]. Also the frequency of stimulation affects the amplitude of the transient. The increase of frequency has two opposite effects on EC-coupling: it reduces the time available for recovery from inactivation of DHPRs and RyRs and it increases the Ca^{2+} entry into the cell (thereby increasing the SR Ca^{2+} content) by reducing the time for Ca^{2+} extrusion by NCX[142]. In rodents, where the SR is almost full even at low frequencies of stimulation, the former effect prevails,

reducing the efficiency of EC-coupling. Therefore, in these species a negative force-frequency relationship (negative staircase) is observed[143]. In upper mammals, the SR Ca^{2+} content largely depends on the frequency, and its elevation at high frequencies induces a larger release of Ca^{2+} during stimulation able to mask the lack of complete recovery of the channels, granting a positive staircase[143-145].

At steady state, the amplitude of Ca^{2+} transients is determined by the balance of sarcolemmal and sarcoplasmic fluxes: the amount of Ca^{2+} that enters the cell through the $I_{Ca,L}$ current is the same of the one extruded by NCX, and the Ca^{2+} released by RyR channels equals the one pumped by SERCA back into the SR[146]. Manoeuvres that simply alter one of these fluxes produce only transitory changes of the Ca^{2+} transient amplitude. For instance, low concentrations of caffeine, a positive modulator RyR activity[60;71], are able to induce an increase of Ca^{2+} transient amplitude (due to a higher sensitivity of the channel to the trigger) only during the first stimulations, because the increased RyR activity is not paralleled by a similar change of SERCA activity. Consequently, NCX can extrude a larger amount of Ca^{2+} lowering the SR Ca^{2+} content. The new SR content, by reducing RyR activity, offsets the effect of caffeine, bringing back the Ca^{2+} transient to the same amplitude that preceded caffeine application[147]. Similar results were obtained with low doses of tetracaine, a RyR inhibitor[148]. Thus, only those manoeuvres that increase both the influx and the corresponding efflux of $[Ca^{2+}]_{cyt}$ can stably affect Ca^{2+} transient amplitude[149].

SR Ca^{2+} Leakage

The basal activity of RyR channels results in an efflux of Ca^{2+} from the SR known as SR Ca^{2+} leakage. In addition to the efflux mediated by RyRs (Ca^{2+} sparks and the release of Ca^{2+} from rogue RyRs), also the backflux of SERCA contributes to Ca^{2+} leakage[150]. Procedures affecting RyR activity modify Ca^{2+} leakage and are paralleled by changes of the SR Ca^{2+} content[151]. An enhanced RyR activity increases in turn $[Ca^{2+}]_{cyt}$. At rest, SERCA activity is reduced, as the pump is approaching the thermodynamic balance[114], and small $[Ca^{2+}]_{cyt}$ changes are sensed primarily by NCX. Therefore, this Ca^{2+} efflux from the SR translates into a Ca^{2+} loss from the cell because of NCX extrusion[152].

It is less clear the net effect of SERCA stimulation on SR Ca^{2+} leakage, as the consequent increase of SR Ca^{2+} content positively modulates RyR activity. However, this effect is observable with each manoeuvre that produces an increase in the SR load, like increasing the frequency of stimulation[150]. Instead, the direct effect of SERCA stimulation on SR Ca^{2+} leakage seems to leave Ca^{2+} leakage unaltered (see Chapter 3), or even to reduce it, as the inhibition of SERCA lowers the threshold for spontaneous Ca^{2+} release[153].

Ca^{2+} Waves

Unlike SR Ca^{2+} leakage, which is always present at physiological conditions, a massive spontaneous release of Ca^{2+} from the SR is the result of altered Ca^{2+} handling. Ca^{2+} waves (fig. 3C) originates from the release of Ca^{2+} in one or a small number of couplons which is massive enough to let the ion to diffuse to nearby couplons and

activate them[154;155]. This anomalous Ca^{2+} release can thus regenerate itself by a CICR mechanism[156;157] and propagate from a site towards cell ends and even to adjacent cells by diffusion of Ca^{2+} through the gap junctions[155]. Recently, an alternative mechanism for Ca^{2+} waves propagation has been proposed: the reuptake of Ca^{2+} from the first release site increases $[Ca^{2+}]_{SR}$ locally, favouring the opening of RyRs of nearby sites. In this case, Ca^{2+} wave propagation is driven by SERCA, instead of by RyRs through CICR[158;159]. At the moment, however, it is not clear what mechanisms describes the propagation of Ca^{2+} waves.

There is a threshold of SR Ca^{2+} content above which Ca^{2+} leakage is so sustained that can generate Ca^{2+} waves[160]. Alterations of RyR activity modulate the threshold[148;161]. Ca^{2+} waves tend to lower the SR content as part of the Ca^{2+} released is extruded by NCX[162], until the SR content returns below the threshold[161]. Therefore, only in the presence of enhanced SERCA activity sustained spontaneous SR Ca^{2+} release can be observed[160;161].

Ca^{2+} waves induces aftercontractions (i.e. untriggered contraction) and membrane depolarizations (delayed afterdepolarizations) due to the inward current mediated by NCX[34]. It is still a matter of debate whether Ca^{2+} waves can underlie arrhythmias in the intact heart, since the correlation between abnormal Ca^{2+} cycling and arrhythmogenic oscillatory depolarizations was not reproduced by all groups[163-166] and the propagation of Ca^{2+} waves seems to be confined mostly to two or three cells[167]. Nevertheless, the occurrence of Ca^{2+} waves in the injured heart is increased and may have a role in the generation of triggered arrhythmias[168].

Ca^{2+} Alternans

Ca^{2+} alternans are beat to beat alternations of Ca^{2+} transients of high and low amplitudes and may cause ventricular fibrillation[169;170] (fig. 3D). The causes of Ca^{2+} alternans are still not clear and many hypothesis have been made[171;172]:

(1) A delay in Ca^{2+} being pumped back in the SR after a Ca^{2+} transient, because of slow diffusion from the dyadic cleft to the region where SERCA is expressed, can limit the amount of Ca^{2+} available for release during the following beat, reducing the amplitude of the second transient. By the time of the third beat, the Ca^{2+} has been pumped back into the SR and the transient is of high amplitude. Alternatively, the recovery from inactivation of RyR channels becomes too long, and at the second beat too many channels are still inactivated, limiting the amount of Ca^{2+} being released[171].

(2) If the action potential is prolonged, the diastolic interval before the next beat (provided a constant pacing) will be reduced, thereby limiting the recovery of ionic channels on the sarcolemma. The following action potential will be shortened, and the amount of Ca^{2+} entry through DHPR will be lowered, thus triggering a smaller Ca^{2+} transient[173].

(3) If the EC-coupling gain (amount of Ca^{2+} released from the SR for a given trigger) is very high, then a big Ca^{2+} transient will follow a membrane depolarization. However, this will enhance Ca^{2+} extrusion from the cell reducing the SR content. At the second beat lesser Ca^{2+} will be released from the SR, limiting Ca^{2+}-dependent inactivation of $I_{Ca,L}$ so that a greater Ca^{2+} influx will help refilling the SR stores[146;174].

Sympathetic Modulation of EC-Coupling

The sympathetic stimulation of the heart in normal conditions, by increasing the level of circulating catecholamines, induces an increase of the developed contraction (positive inotropic effect), accelerates heart relaxation (positive lusitropic effect) and increases the heart rate (positive cronotropic effect). The main isoform of the adrenergic receptor expressed on ventricular myocytes is the β one. Once bound to it, β-adrenergic receptor agonists induce a signalling cascade that affects many proteins involved in EC-coupling[25].

Basically, the modulation of EC-coupling during the sympathetic stimulation is achieved through the activation of two kinases, PKA and CaMKII. PKA is activated through the stimulation of a G-protein by the β-adrenergic receptor, the interaction of the G-protein with the adenylate cyclase and the raise of the levels of cAMP[13]. Instead, many pathways contribute to the activation of CaMKII. In addition to the increased $[Ca^{2+}]_{cyt}$ levels following PKA-mediated phosphorylation of PLB (described below) and the consequent activation of the kinase by the Ca^{2+}-CaM complex[175], Ca^{2+}-independent pathways (some of them still uncharacterized) are present in ventricular myocytes, like the cAMP-activated guanine nucleotide exchange factor Epac[176;177].

Although PKA and CaMKII share the same substrates (DHPR and RyR channels, the inhibitor PLB and the myofilament protein TnI), the effects of their phosphorylation are not equal. The phosphorylation of PLB by CaMKII, for instance, cannot modulate SERCA activity, while the PKA-mediated phosphorylation alone can accelerate the rate of decay of Ca^{2+} transients[178;179]. The role of either phosphorylation on RyR channel is still debated. PKA phosphorylation enhanced RyR

activity in single channel recordings, by inducing the dissociation of FKBP12.6 from the channel[180], but the same effect was not detectable in permeabilized cardiomyocytes[82], while CaMKII activation was able to promote RyR activity in the same setting[181]. β-adrenergic stimulation in cells expressing a mutant RyR where PKA phorsphorylation was not possible had identical consequences to that of cells expressing the wild-type channel[182;183]. Similarly, the enhancement of SR Ca^{2+} leakage by β-adrenergic stimulation was CaMKII-dependent but PKA-independent[176]. However, some groups reported that CaMKII depressed RyR activity, suggesting its involvement in the process of Ca^{2+} release termination[184-186]. Although the sympathetic stimulation clearly enhances RyR activity[187;188], the relative contributions of PKA and CaMKII to its modulation is still an open question.

Overall, the positive inotropic effect of the sympathetic stimulation is due to an increase of Ca^{2+} transient amplitude, because of the increased SR content (PLB phosphorylation[118;119]), the enhanced RyR activity[176] and the increase of $I_{Ca,L}$ current[189-191]. The positive lusitropic effect derives from the phosphorylation of TnI, which renders myofilaments lesser sensitive to Ca^{2+} (ref. [192]) and of PLB, the effect on PLB being by far the prevailing mechanism[193].

Alterations of EC-coupling Induced by Heart Failure

Heart failure deeply alters Ca^{2+} homeostasis by affecting both the expression profiles and the post-translational modifications of many proteins involved in EC-coupling[34]. To date, many animal models of heart failure have been developed and extensively characterized, and

also the Ca^{2+} homeostasis of human heart failure has been studied. Many techniques were employed to obtain the animal models so far developed, from genetic manipulation[194] to aortic constriction[195;196]. It is important to stress that among different models the features of heart failure vary, and no model faithfully reproduces human heart failure[197]. Given its wide aetiology, even human heart failure presents substantial subject-to-subject differences[15].

Molecular Alterations Induced by Heart Failure

A common feature of human and all the animal models of heart failure is the prolongation of the action potential duration[198-201], due to the downregulation of K^+ currents[196;202-204]. Abnormal action potential prolongation is potentially arrhythmogenic because it favours the reactivation of Na^+ and Ca^+ channels, leading to early afterdepolarizations[205]. DHPR levels greatly vary among the different models; the general trend is a decrease of the channel expression[206-208], although some exceptions have been reported[209]. However, structural remodelling of the cell architecture moves T-tubules toward the centre of the sarcomere and reduces their number[210;211], disrupting the structure of the dyad and leaving RyR channels uncoupled (orphaned RyR)[211]. In this way, even an unaltered $I_{Ca,L}$ profile would result in a reduced SR Ca^{2+} release, because of a less efficient coupling between DHPR and RyR[212;213].

Even if RyR levels change among the different animal models and human, varying from unaltered[212] to reduced expression[198;214-216], the channel is in an hyperphosphorylated state[214;217-219], its activity being enhanced. Also redox modifications of the channel[220], a reduced

interaction with phosphatases despite their increased expression[214;221], and with FKBP12.6 protein[214;222] contribute to its altered activity.

The expression of proteins responsible for Ca^{2+} decay greatly vary among species[195;198;223] and among different subtypes of human heart failure[15;224]. However, a general trend toward an increased NCX/SERCA ratio is always observed. Additionally, phosphorylated PLB is decreased, leading to further SERCA inhibition[115;214].

Heart failure alters myofilament proteins expression, particularly by inducing shifts in isoform expression rather than altering the relative stoichiometry[225]. There are also changes in the patterns of phosphorylation[226;227]. The net effect of these alterations seems to be an increased sensitivity of the myofilaments, which impairs complete relaxation (stiffness of the ventricular chambers), and a reduction of the ATPasic activity of myosin, which affects the maximal forced developed[225].

The β-adrenergic receptor is downregulated[228], mainly the β_1-subpopulation, so that the prevailing one becomes the β_2 (ref. [229]). The downregulation of the receptor decreases the response to circulating catecholamines, promoting further release of the neurohormones and the consequential worsening of the pathology. Moreover, the two receptor subpopulations interact differently with G proteins[230], and the stimulation of β_2-receptors (which results enhanced by the imbalance between the subpopulations) is arrhythmogenic because it induces SR Ca^{2+} overload[231].

Changes in Ca^{2+} Cycling

The consequences of the molecular rearrangement induced by heart failure on Ca^{2+} homeostasis are profound. The disruption of the dyad structure lowers the coupling efficiency between DHPRs and RyRs, causing spatial non-uniformities of the Ca^{2+} transient[211], and slows the raising phase of the transient[232]. The alteration of NCX and SERCA relative contributions to the transient decay slows the decay[151] and favours Ca^{2+} extrusion, thereby reducing the SR Ca^{2+} content[151;196;232]. The increased RyR activity translates into a larger Ca^{2+} leakage from the SR, which contributes to further SR depletion[151;214;233]. The final result is that Ca^{2+} transients have a lower amplitude[151;196;198;212;232] and in human the positive force-frequency relationship is reversed[145]. Together with the reduced ATPasic activity of myosin, the reduced amplitude of Ca^{2+} transients constitutes the major cause of the hampered contractility in heart failure.

Although SR Ca^{2+} leakage is greater during heart failure, changes in Ca^{2+} spark frequency have not been detected so far[198;212;234]. However, it is important to stress that Ca^{2+} sparks participate to Ca^{2+} leakage, but they are not its sole determinant (see Macroscopic Ca^{2+} fluxes in the cytosol). In fact, rogue RyRs may be important contributors of Ca^{2+} leakage, especially in the hyperphosphorylated state[46]. The elevated RyR activity favours abnormal Ca^{2+} release, like Ca^{2+} waves and alternans, particularly in the earlier stages of heart failure, where the SR Ca^{2+} content is not severely depressed.

Finally, rapid, massive Ca^{2+} releases from the myofilaments can take place following a rapid sarcomere shortening, a condition that ensues from spatial non-uniformities (like a scarred heart after myocardial

infarcion). This rapid Ca^{2+} release can trigger Ca^{2+} waves or, in the worst cases, induce potentially arrhythmogenic delayed afterdepolarizations through NCX mediated inward current[34].

Positive inotropic Interventions

Given the high mortality of heart failure, the primary endpoint for its management is the reduction of mortality both on the short and the long term, also through the prevention of further heart remodelling to avoid the worsening of the pathology. The relieve of the main symptoms, especially in the later stages, remains essential since fatigue, dyspnoea and frequent rehospitalization dramatically affect life quality[1]. The early diagnosis and the prevention of heavy remodelling are key points to reduce morbidity and mortality. For this reason, many pharmacological tools were developed to counterbalance the neurohumoral response. Angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, β-blockers and aldosterone antagonists are commonly employed for this purpose[1].

Since the hallmark of heart failure is hampered contractility, until the end of 1970s positive inotropic interventions constituted the core of heart failure therapy in order to restore the optimal cardiac output[235]. However, the chronic administration of positive inotropes was associated to an increased mortality, limiting their employment to conditions of severe cardiac impairment[236-238]. Nowadays, positive inotropic therapy is limited to patients with acute heart failure (first hospitalization or a general worsening of the cardiac performance that requires hospitalization) and insufficient end-organ perfusion, and

only as a bridge to more definitive therapies[235;239], or as a palliative care in end-of-life heart failure[240].

Available Inotropic Compounds

Cardiotonic Steroids

The first inotropic compounds to be used in the treatment of heart failure were cardiotonic steroids (CTS), particularly digitalis (digoxin)[241]. CTS increase the force of contraction by targeting the Na^+/K^+ ATPase (NKA)[242]. NKA is responsible for the maintenance of Na^+ and K^+ gradients across the sarcolemma. Its partial inhibition by CTS and related compounds increases intracellular Na^+ levels, thereby reducing NCX activity and promoting Ca^{2+} accumulation into the cell (Na^+-lag hypothesis)[243]. Part of this Ca^{2+} is internalized into the SR and part remains in the cytosol sensitizing RyRs and raising Ca^{2+} transient amplitude[243;244]. Some authors suggested additional mechanisms of action for CTS; this topic will be discussed in Chapter 4. Endogenous CTS are also present as hormones in human body and, by interacting with NKA, regulate gene expression in many different cell types[243].

Digoxin, when combined with angiotensin-converting enzyme inhibitors, showed to reduce the rate of hospitalization and the worsening of heart failure without altering overall mortality[245], at variance with other positive inotropes[236;238;246]. Although digoxin slows heart failure worsening, it tends to increase the incidence of sudden death, which offsets its beneficial effect in terms of mortality[247]. The mechanism associated to the pro-arrhythmic effect of the drug is still unknown. Nowadays, digoxin is employed mainly to

treat atrial fibrillation[1], for it increases the refractoriness of the atrioventricular node[248].

Milrinone and Dobutamine

Milrinone and dobutamine are the most commonly used positive inotropes. They both act increasing the cellular levels of cAMP, but with different mechanisms: milrinone is an inhibitor of phosphodiesterases, enzymes capable to hydrolyse cAMP, while dobutamine agonizes the β-adrenergic receptor thereby activating the adenylate cyclise enzyme. By activating PKA, cAMP increases the force of contraction. The two agents similarly enhance cardiac output and decrease cardiac filling pressure; milrinone has a more prominent vasodilatory effect, while dobutamine increases heart rate more[249]. Both agents increase oxygen consumption demand, a critical point due to the condition of starvation typical of the failing heart[21], and there is some concern about a possible worsening of the pathology after these drugs are discontinued, especially in those patients presenting ischemia or coronary artery disease[250;251].

Ca^{2+} Sensitizers

Ca^{2+} sensitizers enhance the sensitivity of myofilaments for Ca^{2+} improving the force developed by the sarcomere, without affecting the ATP consumption. A variety of mechanisms accounts for myofilament sensitization, from the enhancement of TnC affinity for the ion to the stabilization of the TnC-Ca^{2+} complex. Many Ca^{2+} sensitizers acts also as phosphodiesterase inhibitors, increasing the oxygen demand anyway and thus limiting their clinical employment[252].

Levosimendan is the Ca^{2+} sensitizer most extensively characterized and has recently been introduced in many countries in the acute heart failure therapy[253]. In addition to the myofilament sensitization, levosimendan induces vasodilation, reducing the cardiac workload. In the therapeutic range of doses, levosimendan does not increase myocardial oxygen consumption[252;254]. Although levosimendan use is associated to a better clinical outcome than dobutamine, its effect on mortality is controversial and it may reduce mortality only in some subsets of patients[255-258].

Novel Therapeutic Approaches

Due to the limitations of the inotropic agents currently available, there is a great interest in the development of new compounds that can exert their inotropic function with less side effects and possibly reduce the associated mortality. For this reason, new molecular targets are currently under investigation. The improvement of myosin ATPasic activity and the stimulation of SERCA constitute the most attractive topics in this field.

Cardiac myosin activators represent a new class of molecules that directly stimulates the ATPasic activity of myosin increasing the force developed without changes in $[Ca^{2+}]_{cyt}$ (ref. [259]). It is not known whether these compounds are associated to an increase in oxygen consumption. A myosin activator, CK-1827452, is currently in a phase 1 clinical trial[239].

The stimulation of SERCA activity can improve the force of contraction by increasing the SR Ca^{2+} content, which is partially depleted in heart failure, and speeds up the relaxation. With this kind

of manoeuvre the oxygen consumption is lowered, because SERCA can pump two ions by hydrolyzing a single molecule of ATP, while NCX, which relies on NKA activity, can extrude only one Ca^{2+} per ATP hydrolyzed by NKA[260]. Moreover, improved SERCA activity tends to reduce the action potential duration due to a major inactivation of $I_{Ca,L}$ by the bigger Ca^{2+} transient; therefore, SERCA stimulation might prevent early afterdepolarizations[261]. SERCA2a overexpression seems also to decrease or prevent cardiac hypertrophy[262-264] through a mechanism which is still not clear, but that might involve a reduced calcineurin activation[265]. The possible effect of SERCA stimulation on Ca^{2+} waves and delayed afterdepolarization is less clear, because the increased SR Ca^{2+} content, in the presence of enhanced SR Ca^{2+} leakage, may favour untriggered releases, and thus be pro-arrhythmic[160]. In this view, it has been hypothesized that a low SR Ca^{2+} content actually represents an adaptive response to offset the increased SR leakage[151]. On the other hand, SERCA lowers $[Ca^{2+}]_{cyt}$, which can induce Ca^{2+} waves by increasing RyR open probability[266]. This issue will be addressed in Chapter 4.

The improvement of SERCA activity can be achieved in two ways: gene therapy and pharmacologic intervention. SERCA gene transfection has been thoroughly investigated in animal models, both as a way to increase the expression of the endogenous protein[263;267] and as a possibility to introduce in the myocardium skeletal isoforms with an higher activity[268;269]. However, this procedure has practical limitations in terms of effectiveness of the transfection techniques and of regulation of the activity of the pump.

Istaroxime

Istaroxime (IST) represents the pharmacologic alternative to SERCA transfection. Originally designed as a pure inhibitor of NKA, in an attempt to dissociate the inotropy derived from the pump blockade from the side effects typical of CTS[270;271], IST showed to have a combined action on SERCA[244]. To date, IST is the only positive inotrope which has a marked positive lusitropic effect.

When compared to digoxin, a classical NKA inhibitor, IST showed to be less pro-arrhythmic[272]. However, this effect was not attributable to a shortening of the action potential duration, since digoxin reduced it to a similar extent[273]. IST succeeded also in increasing the SR Ca^{2+} content and in speeding up Ca^{2+} transient relaxation. The increase of the SR Ca^{2+} load had a positive effect on EC-coupling gain, too. Although digoxin similarly affected these parameters, IST did it to a greater extent, in accordance to a stimulation of SERCA that digoxin lacks[244].

At variance with milrinone and dobutamine, IST tends to slow the heart rate[272;274]. In many models of heart failure, IST improved the contractile and the relaxation parameters[195;272;275-277]. In men, IST confirmed its positive luso-inotropic effect with minor side effects during intravenous infusion. Additionally, IST decreased the heart rate[244;274;278]. Further analysis is needed to address important questions on IST, like the action of the drug in patients presenting acute heart failure with low cardiac output, where inotropic intervention is usually applied, or its effect on long-term survival (IST is currently under a phase 2 clinical trial)[279]. However, the unique pharmacological profile of IST and the differences observed with other positive inotropes

renders it an attracting alternative for the improvement of contractility in acute contractility.

Scope of the Thesis

The use of positive inotropic agents has been so far limited by the massive side effects of the compounds currently available, which tend to increase the overall mortality. Consequently, the demand for a positive inotrope associated to a reduced mortality, that could be used not only as a bridge to other therapies but as an everyday tool to improve the contractility of the failing heart, remains actual. IST might represent such an alternative. The lower pro-arrhythmic effect observed with IST, when compared to digoxin, seems promising, because digoxin, when associated to angiotensin converting enzymes, improved symptoms without altering overall mortality[245]. Even if this might not be the case, the understanding of the causes of the reduced pro-arrhythmogenic effect of IST will give insight into the cellular mechanisms which constitute the genesis of arrhythmias. The understanding of such mechanisms is fundamental for the therapeutic management of heart failure.

The work presented in this thesis goes in this direction. In Chapter 2 the effects of IST on Ca^{2+} handling are analysed in a guinea pig model of heart failure, to test whether mild SERCA stimulation (in opposition with the transfection of SERCA, that boost the pump activity but it does not allow its fine modulation) is capable of counterbalance the derangements induced by heart failure. In Chapter 3 the emphasis is on the effects the drug has, in comparison with digoxin, on SR Ca^{2+} leakage and resting $[Ca^{2+}]_{cyt}$, since they are

important factors for the genesis of Ca^{2+} waves. Moreover, being these latter experiments performed in mouse, possible species-specificities derived from a different regulation of Ca^{2+} homeostasis are taken into account.

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