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# Type 2 ryanodine receptor: A novel therapeutic target in myocardial ischemia/reperfusion

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## ABSTRACT

Cardiac pathologies remain the main cause of mortality worldwide. Among them the most common cause is cardiac ischemia. The rapid reperfusion after coronary occlusion has considerably improved the cardiac outcome, however reperfusion per se has deleterious effect also called reperfusion injuries. Cytosolic calcium overload is now well admitted as an essential pathophysiological mechanism involved in reperfusion injuries although the source and origin of calcium remain to be determined. Recent works have pointed out the potential defect of sarcoplasmic reticulum calcium release channels (ryanodine receptor, RyR) as a primary cause of calcium overload during ischemia-reperfusion. This finding opens new pharmacological perspectives in limiting reperfusion injuries since allosteric modulators able to restore and prevents RyR dysfunction have been developed during the last decade.

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## 1. Introduction

According to the World Health Organization, about 30% of deaths are due to cardiovascular disease (CVD). This represents  $\approx 17.3$  million people in 2008, and this may rise to almost 24 million of deaths in 2030. To date, coronary heart disease remains the main cause of cardiovascular deaths. Strategies to counteract the burden of CVD are, on the prevention side, the reduction of risk factor with adapted nutrition and diet, physical training and no tobacco smoking. On the therapeutic side, as soon as the CVD is clinically characterized, the best option available for the treatment of the cause and the consequences of the CVD has to be used. In the context of coronary heart disease, the rapid reperfusion of ischemic heart induced by coronary thrombosis has been the main strategy to improve outcomes. However, since the originals

**Abbreviations:** AKAP, A-kinase-Anchoring Proteins; ATP, Adenosine Triphosphate; ADP, Adenosine Diphosphate; Ca<sup>2+</sup>, calcium; CaM, Calmodun; CamKII, Calcium/calmodulin-dependent Protein Kinase II; CSQ, Calsequestrin; CVD, Cardiovascular Disease; CmC, 4-chloro- m-cresol (CmC); DAD, Delayed After Depolarization; FKBP, FK506-Binding Protein (also called calstabin); I/R, Ischemia Reperfusion; IPC, Ischemic Pre-Conditioning (IPC); mPTP, mitochondrial Permeability Transition Pore; NAD, Nicotinamide Adenine Dinucleotide; Po, open probability; PKA, cAMP-dependent Protein Kinase; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species; RyR, Ryanodine receptor; RyR1, Type 1 ryanodine receptor; RyR2, Type 2 ryanodine receptor; SR, Sarcoplasmic Reticulum; SERCA, SR Ca<sup>2+</sup> ATPase; TNF- $\alpha$ , Tumor Necrosis Factor- $\alpha$ .

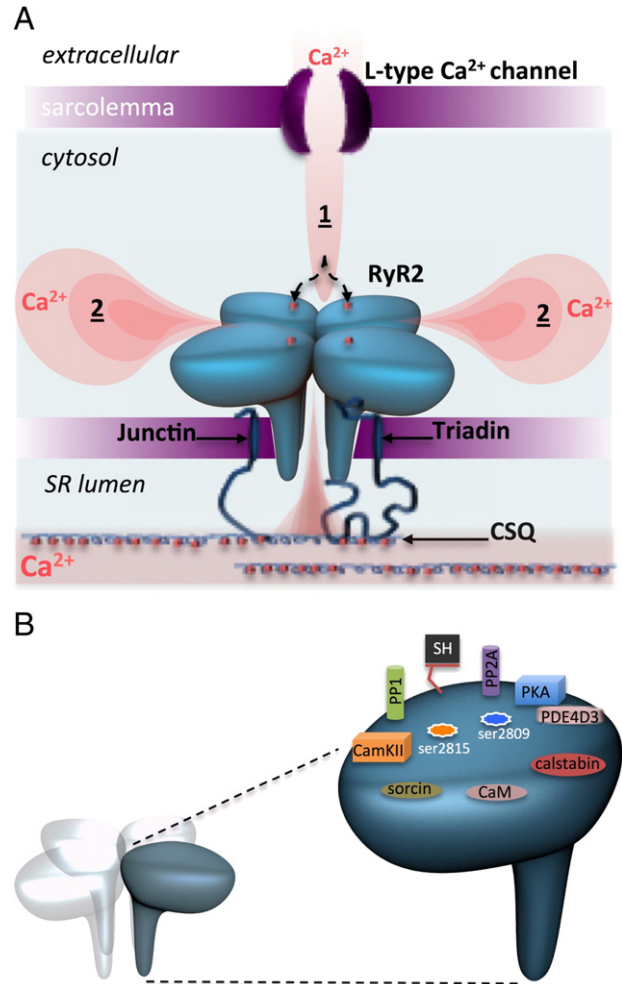
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works of Jennings (Jennings et al., 1960; Jennings & Reimer, 1983), several experimental and clinical studies have pointed out the deleterious effects of reperfusion per se. Restoration of cardiac circulation is accompanied by cell damages and death, reperfusion arrhythmias, myocardial stunning, vascular defects with the no-reflow phenomena (Hearse, 1977). Among all signaling pathways known to contribute to such reperfusion injuries, disturbances in both calcium ( $\text{Ca}^{2+}$ ) and redox homeostasis are ubiquitously observed. Intracellular  $\text{Ca}^{2+}$  overload that occurs at the onset of reperfusion favors vasoconstriction (Dimitrijevic et al., 2009), activates  $\text{Ca}^{2+}$  dependent proteases (Atsma et al., 1995), induces mitochondrial transition pore (mPTP) opening and triggers ventricular arrhythmias (Gomez et al., 2008). Elevation of intracellular  $\text{Ca}^{2+}$  level results from both transsarcolemmal  $\text{Ca}^{2+}$  entry and altered sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  cycling. Both L-type  $\text{Ca}^{2+}$  current and reverse mode  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) have been involved in the  $\text{Ca}^{2+}$  entry during acute ischemia (Talukder et al., 2009) whereas aberrant SR  $\text{Ca}^{2+}$  release through type 2 ryanodine receptor (RyR2) and altered SR  $\text{Ca}^{2+}$  uptake by the SR  $\text{Ca}^{2+}$  ATPase (SERCA2a) are known to be involved in genesis of  $\text{Ca}^{2+}$  overload. Although general overviews of  $\text{Ca}^{2+}$  overload in the myocardium following ischemia-reperfusion (I/R) have been already reported (Zucchi et al., 2001; Dong et al., 2006; Talukder et al., 2009), the present review will specially focus on the role of RyR2 during myocardial I/R.

## 2. The RyR2 in cardiac physiology.

RyR2 are homotetramer  $\text{Ca}^{2+}$  channels that form macromolecular complexes on the SR membranes. They are key components of the excitation-contraction coupling. In cardiac cells,  $\text{Ca}^{2+}$  entry through L-type  $\text{Ca}^{2+}$  channels during an action potential triggers synchronized openings of RyR2 allowing a rapid and transient release of  $\text{Ca}^{2+}$  from the SR (Fig. 1A). The subsequent intracellular  $\text{Ca}^{2+}$  elevation triggers myocytes contraction. Relaxation occurs when RyR2 closes,  $\text{Ca}^{2+}$  dissociates from C-troponin and is mainly taken back up into the SR by SERCA2a and partially extruded by the NCX. The amount of  $\text{Ca}^{2+}$  released by the SR during a cardiac cycle depends on the L-type  $\text{Ca}^{2+}$  current time course, the RyR2  $\text{Ca}^{2+}$  sensitivity and the SR  $\text{Ca}^{2+}$  load. Activation of RyR2 is highly sensitive to  $\text{Ca}^{2+}$  concentration, and intracellular  $\text{Ca}^{2+}$  is the main modulator of RyR2 function in the heart. To date, the mechanism responsible for local  $\text{Ca}^{2+}$  release termination remains unclear (Cheng & Lederer, 2008). RyR2 open probability ( $P_o$ ) is regulated by other endogenous factors such as  $\text{Mg}^{2+}$  ions, ATP (Copello et al., 2002) and cyclic ADP-ribose (Lee, 1997), pH and redox state (Marengo et al., 1998). Furthermore, on its large N-terminal cytosolic side, RyR2 is associated and regulated by several proteins including FK-506 binding protein 12.6 (FKBP12.6 also called calstabin2) (Timerman et al., 1993; Lam et al., 1995), calmodulin (Balshaw et al., 2002), sorcin (Meyers et al., 1995; Lokuta et al., 1997), homer (Pouliquin et al., 2006, 2009), S100A1 (Kettlewell et al., 2005), protein kinase A (PKA) (Marx et al., 2000) and his anchoring protein mAKAP (Kapiloff et al., 2001), calmodulin kinase II (Rodriguez et al., 2003), phosphatase 1, phosphatase 2A (Bandyopadhyay et al., 2000; Marx et al., 2000) and phosphodiesterase 4D3 (Lehnart et al., 2005) (Fig. 1B). These proteins dynamically control RyR2 stability, coupled gating (i.e. for calstabin2),  $\text{Ca}^{2+}$  sensitivity and  $P_o$ . From the luminal side, RyR2 are directly linked to triadin, junctin and calumenin (Guo & Campbell, 1995). These proteins sense SR  $\text{Ca}^{2+}$  content especially through their interactions with calsequestrin (CSQ), which affects RyR2 channel gating (Kawasaki & Kasai, 1994). Independently of the classical  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release mechanism, spontaneous  $\text{Ca}^{2+}$  released may occurs when SR  $\text{Ca}^{2+}$  load reaches a certain threshold. This process also called store-overload-induced  $\text{Ca}^{2+}$  release (SOICR) generates cytosolic  $\text{Ca}^{2+}$  waves and subsequent delayed after depolarization (DAD). Mutation of the RyR2 or the associated proteins on the luminal side may affect intra-store  $\text{Ca}^{2+}$  sensitivity and reduced SOICR threshold (Priori & Chen, 2011).



**Fig. 1.**  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release in cardiomyocytes and RyR2 macromolecular complex. **A.** SR  $\text{Ca}^{2+}$  release is initiated by  $\text{Ca}^{2+}$  entry into the cell through the activation of L-type  $\text{Ca}^{2+}$  channel (1).  $\text{Ca}^{2+}$  then binds to RyR2 and activates the channel.  $\text{Ca}^{2+}$ -induced RyR2 opening releases  $\text{Ca}^{2+}$  from the SR to the cytosol (2), which allows myocytes contraction. The amount of SR  $\text{Ca}^{2+}$  released depends on both the  $P_o$  of RyR2 and the amount of  $\text{Ca}^{2+}$  stored in the SR. CSQ is a luminal  $\text{Ca}^{2+}$  binding protein. CSQ interacts with RyR2 via triadin and junctin on a  $\text{Ca}^{2+}$  dependent manner and thus senses SR  $\text{Ca}^{2+}$  content during the excitation-contraction coupling cycle. **B.** The RyR2 macromolecular complex is composed by four identical RyR2 subunits. Each subunit is associated with regulatory proteins (calstabin2, sorcin, calmodulin (CaM)), kinases (protein kinase A (PKA), camodulin kinase II (CamKII)), as well as phosphatases (phosphatase 1 (PP1) and phosphatase 2 (PP2)) and phosphodiesterase 4D3 (PDE4D3). This macromolecular complex is regulated by post-translational modification such as phosphorylation of the serine 2815 (CamKII phosphorylation site) and/or 2809 (PKA phosphorylation site), or by oxidation/nitrosylation of sulfhydryl group of cysteine (SH group). Hyperphosphorylation and oxidation/nitrosylation of RyR2 subunits is associated with the dissociation of calstabin2.

## 3. Pharmacological modulation of RyR2.

Several pharmacological compounds target RyR2 activity and are common tools for analyzing RyR2 function either through direct effects on the channels or indirectly through a modulation of accessory proteins (Table 1). As the name imply, RyR2 binds with a high affinity, the plant alkaloid: ryanodine. In the nanomolar range, ryanodine locks RyR2 in a sub-conductance open state, which induces a continuous SR  $\text{Ca}^{2+}$  leak (Boutjdir et al., 1990; Hayashi et al., 1996). When applied at micromolar concentrations ryanodine inhibits RyR2 channels. Ruthenium red is a polycationic compound that decreases  $\text{Ca}^{2+}$  release rate at concentrations ranging from 1 nM to 20  $\mu\text{M}$  and the RyR2  $P_o$  by producing prolonged channel closings (Rousseau et al., 1996). Ruthenium red is thus a potent RyR2 inhibitor despite its effect on mitochondrial  $\text{Ca}^{2+}$  transporter (Miyamae et al., 1996). Procaine and tetracaine also

inhibit RyR2 channel activity (Volpe et al., 1983; Antoniu et al., 1985) however these local anesthetics are potent antagonists of Na<sup>+</sup> voltage-gated channels. Tetracaine inhibits RyR2 with IC<sub>50</sub> ≈ 50 μM by inducing long closing state. Similarly procaine increases long closing state indicating that this anesthetic inhibits the RyR2 in its close configuration (Zahradnikova & Palade, 1993; Xu et al., 1998). One other commonly used inhibitor of RyR is dantrolene especially in the context of malignant hyperthermia (MacLennan & Phillips, 1992). Although its specificity for skeletal RyR1 isoform is established, RyR2 inhibition with dantrolene appears to depend on the conformational state of the channel (Wang et al., 2011). Dantrolene stabilizes interdomain interactions between the NH<sub>2</sub>-terminal and central regions of RyR, and, on cardiac RyR2, these sites are poorly accessible (Paul-Pletzer et al., 2005; Wang et al., 2011). Inversely, digitalis glycosides such as digitoxin have been reported to activate RyR2, but not RyR1, in the nanomolar range (1–10 nM; (McGarry & Williams, 1993)). Caffeine and 4-chloro-m-cresol (CmC) increase channel opening presumably by enhancing Ca<sup>2+</sup>-dependent activation (Rousseau & Meissner, 1989; Iaizzo et al., 1999). Caffeine is a purine derivative used as a potent RyR2 modulator. At low concentrations (i.e., micromolar ranges), caffeine increases RyR2 Po by increasing the frequency of opening without affecting the durations of open states. At millimolar concentrations, caffeine triggers an increase of both RyR2 open channel duration and Po, allowing the channels to open at diastolic Ca<sup>2+</sup> concentrations (Santonastasi & Wehrens, 2007). Caffeine is commonly used in preclinical studies however, it is also a potent phosphodiesterase inhibitor (Butcher & Sutherland, 1962; Beavo, 1995) and an adenosine receptors antagonist (Fredholm, 1995). Additional pharmacological agents increase RyR2 activity such as halothane a volatile anesthetic (Herland et al., 1990). Halothane activates RyR2 by increasing the Po and the duration of open events in a dose dependent manner without affecting the channel conductance (Connolly & Coronado, 1994). Similarly anthracyclines, such as doxorubicin, increase acutely the open state of the channel, either through a direct effect on a specific binding site or by increasing the Ca<sup>2+</sup> sensitivity of RyR2 (Pessah et al., 1990; Saeki et al., 2002). This acute effect is fully reversible, whereas long term anthracyclines treatment may affect the redox state but also the expression level of the RyR2 (Pessah et al., 1992). Antidepressants such as nortriptyline or trifluoperazine, have also been shown to affect RyR2 function as antagonists of CaM and by decreasing CSQ Ca<sup>2+</sup> binding capacity (Vandonselaar et al., 1994). In addition at high concentration (>1 μM) these drugs increase RyR2 Po independently of CSQ and CaM (Zima et al., 2008). At low concentration, Po increases due to an elevated open frequency and for high concentration antidepressants increase the duration of mean open events (Zima et al., 2008). More recently, two well established pharmacological compounds the β-blocker carvedilol (Zhou et al., 2011) and class 1 antiarrhythmic flecainide (Watanabe et al., 2009), have been reported as potent RyR2 blockers with promising therapeutic perspectives in SR dependent ventricular arrhythmias. Although carvedilol has been shown to prevent SOICR (Zhou et al., 2011), the exact mechanism of these pharmacological agents leading to RyR2 induced SR Ca<sup>2+</sup> leak is still not fully understood.

Modulation of accessory proteins may also be used to affect RyR2 function. FK506 and rapamycin, two clinically used immunosuppressant, disrupt calstabin2-RyR2 interaction causing diastolic SR Ca<sup>2+</sup> leak (Kaftan et al., 1996). Calstabin-FK506/rapamycin complexes inhibit calcineurin (Sewell et al., 1994) and mTOR signaling pathway respectively (Panwalkar et al., 2004). JTV519 (K201), and derivative (S107), antagonized these effects by stabilizing Calstabin2-RyR2 interaction (Yano et al., 2003; Bellinger et al., 2008; Lehnart et al., 2008; Andersson & Marks, 2010). K201 is not selective and especially inhibits SERCA pumps and may affect other ion channels such as L-type calcium channels (Loughrey et al., 2007). Such side effects were absent with JTV519 derivative, S107 (Bellinger et al., 2008). At physiological concentration (50–100 nM) the Ca<sup>2+</sup>-calmodulin complex has been shown to activate directly RyR2 and increase SR Ca<sup>2+</sup> leak (Wu & Bers, 2007;

Sigalas et al., 2009), whereas high concentration (1 μM) of Calmodulin inhibits RyR2 (Xu & Meissner, 2004). Suramin and its derivate modulate such effects by competing with CaM binding site. Most notably, the suramin increase the channel conductance and the Po in concentration dependent manner (Hill et al., 2004).

#### 4. RyR2 in ischemic Ca<sup>2+</sup> overload: general concept

The involvement of SR Ca<sup>2+</sup> release in I/R Ca<sup>2+</sup> overload is the consequence of a concomitant altered RyR2 function and SR Ca<sup>2+</sup> uptake (Fig. 1). During the ischemic phase, the drop of the ATP synthesis reduces SERCA activities and thus SR Ca<sup>2+</sup> uptake capabilities (Zucchi et al., 2001). This will progressively reduce SR Ca<sup>2+</sup> content and would therefore be responsible for a progressive reduction in SR Ca<sup>2+</sup> leak since RyR2 Po depends on SR Ca<sup>2+</sup> content (Gyorke & Terentyev, 2008). However, this reduced ATPase activity also affects sarcolemmal ATPase such as Ca<sup>2+</sup> ATPase and Na<sup>+</sup>/K<sup>+</sup> ATPase (Griese et al., 1988). Inhibition of Na/K ATPase will be partially compensated by the extrusion of sodium via the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger in the reverse mode (Tani & Neely, 1989). Thus calcium entry through the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, reduced sarcolemmal and SR Ca<sup>2+</sup> ATPase together with a reduction of mitochondrial Ca<sup>2+</sup> uptake. This leads to an initial increase in cytosolic Ca<sup>2+</sup>, which increases RyR2 Po by a Ca<sup>2+</sup>-induced Ca<sup>2+</sup>-release mechanism and thus favors SR Ca<sup>2+</sup> leak during the early phase of ischemia. Prolonged ischemia and metabolic inhibition, the decrease in cytosolic pH, the increased Mg<sup>2+</sup> level and NADH/NAD<sup>+</sup> ratio (Zima et al., 2004) together with the reduction in SR Ca<sup>2+</sup> content will decrease RyR2 Po and SR Ca<sup>2+</sup> leak (Gyorke & Terentyev, 2008). However, in the mean time, metabolic alterations during the ischemic phase favor the induction of posttranslational alteration of RyR2 rendering the RyR2 potentially leaky (Fauconnier et al., 2011). The net result at the end of the ischemic phase is a new steady state with a redistribution of the Ca<sup>2+</sup> from the SR to the cytosolic space. The level of cytosolic Ca<sup>2+</sup> overload will thus depend on the initial level SR Ca<sup>2+</sup> load at the beginning of the ischemia and the duration of the ischemic phase. At reperfusion, the restored blood flow is accompanied by increase oxygen supplied and the reestablishment of oxidative phosphorylation. This will inevitably generate reactive oxygen (ROS) and nitrogen (RNS) species (Raedschelders et al., 2012). The production of superoxide anion (O<sub>2</sub><sup>-</sup>) during the first seconds of reperfusion exceeds the antioxidant defense capacity (Zweier et al., 1987). Furthermore, O<sub>2</sub><sup>-</sup> has parent free radicals triggering a cascade of events that affect redox state of key proteins involved in cellular homeostasis (Pacher et al., 2007). For instance, nitric oxide (NO<sup>•</sup>) and O<sub>2</sub><sup>-</sup> may interact together to form peroxynitrite (ONOO<sup>-</sup>) and affect cardiac excitation-contraction coupling through post-translational modification of proteins involve on both Ca<sup>2+</sup> signaling and contraction (Zima & Blatter, 2006). RyR2 is highly sensitive to redox state due the presence of several sulfhydryl groups of cysteine in the proteins. The burst of mitochondrial ROS production that occurs at the reperfusion contributes to posttranslational modifications of RYR2 that increase RyR2 Po (Fauconnier et al., 2011). In parallel, at the reperfusion, the metabolic inhibition is released and ATP synthesis restarts. Direct consequences are the reactivation of SERCA activity and release of RyR2 inhibition. Thus the reperfusion is accompanied by a rapid and massive SR Ca<sup>2+</sup> re-uptake. Both the increase in luminal Ca<sup>2+</sup> and posttranslational RyR2 modifications may contribute to an increase in RyR2 Po that synergistically induces transient cytosolic Ca<sup>2+</sup> oscillations until new steady states occurred between the different compartments. Cytosolic Ca<sup>2+</sup> oscillations have been involved in reperfusion arrhythmias. Indeed, SR Ca<sup>2+</sup> leak plays a role in triggering arrhythmias during the early phase of reperfusion (Fauconnier et al., 2011). Pharmacological normalization of RyR2 function prevents numerous ventricular extra-systoles and sustained ventricular tachycardia that occur during the first 12 h of reperfusion. More generally the extent of

**Table 1**

Most common pharmacological agents known to activate (+) or antagonize (–) type 2 ryanodine receptor.

| Name chemical structure                                       | Effect | Concentration range | Use mechanism   |
|---|--------|---------------------|---|
| Caffeine Methylxanthine, purines derivative                   | +      | mM                  | Increases RyR2 Ca <sup>2+</sup> -sensitivity  |
| Digoxin Digitalis glycosides                                  | +      | nM to μM            | Increases number of openings without changes in open time duration  |
| Suramin   | +      | μM                  | Competes with CaM binding site. Increases the channel conductance and the Po  |
| Nortryptiline, Tifluoperazine Antidepressants                 | +      | nM to μM            | At <1 μM: antagonizes CaM binding and decreases CSQ Ca <sup>2+</sup> binding capacity, increase open frequency.<br>At > μM: increases RyR2 duration of mean open events independently of CSQ and CaM. |
| Doxorubicin Anthracyclines                                    | +      | μM                  | Increases the Ca <sup>2+</sup> sensitivity Affects oxidation states of thiols at an allosteric site   |
| Volatile anesthetics (Halothane, isoflurane)                  | +      | μM                  | Increase the duration of open events. No effect on channel conductance  |
| 4-Chloro-m-cresol (CMC) Chlorinated phenol                    | +      | μM to mM            | Enhances Ca <sup>2+</sup> -dependent activation (non permeable compound))   |
| IpTx Peptide Toxines  | +      | nM                  | Induces the formation of conductance substates  |
| Dantrolene Hydantoin derivative                               | –      | μM                  | Stabilizes inter-domains interactions   |
| Tetracaine, lidocaine, Procaine Local anesthetics Amino ester | –      | μM to mM            | Inhibits the RyR2 in its close configuration  |
| Ruthenium Red Polycationic dye                                | –      | nM to μM            | Prolonged channel closings  |
| Ryanodine Plant Alkaloid                                      | +/-    | nM to μM            | At nM range: locks RyR2 in a sub-conductance open state.<br>At μM range: Inhibits RyR   |
| Flecainide (class Ic antiarrhythmic agent)                    | –      | μM                  | Blocks channel open state   |
| Carvedilol (Nonselective beta blocker)                        | –      | μM                  | Reduces open RyR duration and prevents SOICR  |
| FK506, rapamycin Macrocyclic compounds                        | +      | μM                  | ROS scavenger, prevents RyR interdomain destabilization<br>Dissociates calstabin/RyR complex  |
| "Rycals": JTV519, K201, S107 1-4-Benzothiazepines derivatives | –      | μM                  | Stabilizes calstabin/RyR complex  |

cytosolic Ca<sup>2+</sup> overload and SR dysfunction are key determinants in reperfusion injuries.

## 5. RyR2 post-translational modification and ischemia reperfusion

As mentioned above, RyR2 is a macromolecular complex subject to several post-translational modifications that might affect the function of the channel. During I/R such modifications occur which favor SR Ca<sup>2+</sup> leak and subsequent reperfusion injuries. The following sections will summarize the data regarding RyR2 phosphorylation, redox modification and degradation.

### 5.1. RyR2 phosphorylation

Phosphorylation of RyR2 is a key regulator of the channel function and dysfunction upon pathophysiological conditions. In ischemic HF a hyper-phosphorylation of the channel on the protein Kinase A dependent sites has been originally described (Marx et al., 2000). Catecholamine-induced protein activation of PKA was demonstrated to dissociate the RyR2–calstabin2 complex, and this was suggested as a leading mechanism underlying heart failure (Marx et al., 2001; Reiken et al., 2001). RyR2 PKA phosphorylation as a pathophysiological mechanism remains controversial (Houser, 2010). Indeed, different groups were either unable to detect PKA-phosphorylation of RyR2 or unable to report pathophysiological consequences of RyR2 PKA-phosphorylation. More recent data strongly suggest that this PKA phosphorylation requires other translational modification of RyR2 such as oxidation (Eschenhagen, 2010). This may in part explain such controversy. In addition, an increase in the CaMKII phosphorylation of the RyR2 has also been reported (Said et al., 2011). CaMKII enhances RyR2 conductance causing significant SR Ca<sup>2+</sup> leak via RyR2. Previous work in isolated cardiomyocytes and one report analyzing the properties of RyR2 incorporated in planar lipid bilayers suggest that CaMKII-induced Ca<sup>2+</sup> leak is not susceptible to calstabin2 (Seidler et al., 2011). Although some controversies remain according to which kinase and RyR2 serine are effectively relevant pathophysiological situation, it is admitted that RyR2 phosphorylation leads to asynchronous RyR2

openings, diastolic SR Ca<sup>2+</sup> leak and arrhythmia in ischemic HF. In the context of acute ischemia, there is a consensus on the absence of PKA-dependent phosphorylation of RyR2, and an increase in CamKII activity induced by acidosis and cytosolic Ca<sup>2+</sup> levels at the end of ischemia. CamKII phosphorylates phospholamban, which releases its inhibition on SERCA activities and favors SR Ca<sup>2+</sup> reuptake (Mattiuzzi et al., 2004; Vittone et al., 2008). Regarding RyR2 CamKII phosphorylation, although no change in RyR2 phosphorylation ratio was originally described, an increase in the phosphorylation of RyR2 was then clearly shown at the onset of reperfusion (Said et al., 2011). A significant decrease in reperfusion arrhythmia has been observed using a knock-in mice model with the non-phosphorylatable Ser2814A on RyR2 (Chelu et al., 2009; Said et al., 2011). CamKII appears to play an early role in Ca<sup>2+</sup> induced reperfusion injury, even though CamKII induced phosphorylation of RyR2 dysfunction remained to be confirmed.

### 5.2. Redox alteration of RyR2

RyR2 contains several cysteine residues highly sensitive to redox modification. ROS and/or RNS as superoxide anion, nitric oxide, peroxynitrite, and hydrogen peroxide may activate RyR2 to promote SR Ca<sup>2+</sup> leak (for review see (Donoso et al., 2011)). Redox modification and sulfhydryl oxidation of RyR2 also affect Ca<sup>2+</sup> sensitivity of the channels. At the onset of reperfusion there is an abrupt and large increase in oxygen and metabolic supply in the ischemic area that is accompanied by a massive production of ROS. In addition, reperfusion is accompanied by an increase in inflammatory process where circulating level of pro-inflammatory cytokines such as TNF-α increase within the first hours of reperfusion (Meldrum, 1998). TNF-α, through caspase-8 activation, leads to mitochondrial O<sub>2</sub><sup>-</sup> production associated with an increase in NO level (Fauconnier et al., 2011). Simultaneous ROS and NO production would affect RyR2 function through peroxynitrite formation and S-nitrosylation (Zima & Blatter, 2006; Pacher et al., 2007) resulting in a diastolic SR Ca<sup>2+</sup> leak (Yano et al., 2005; Zima & Blatter, 2006). S-nitrosylation and diastolic SR Ca<sup>2+</sup> leak are associated with calstabin2 depletion from the channel complex, which is prevented by pharmacological inhibition of caspase-8

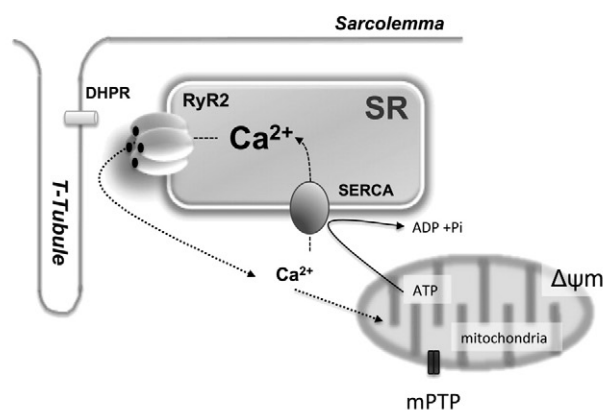
or with S107 treatment. Of note only caspase-8 inhibition prevented both calstabin2 depletion and S-nitrosylation (Fauconnier et al., 2011). This suggests that changes in the redox environment of the channel may lead to calstabin2 depletion and increased RyR2 channel activity in I/R as previously shown in HF (Yano et al., 2003; Fauconnier et al., 2010). Oxidation of RyR leading to SR  $\text{Ca}^{2+}$  leak has been recently reported in skeletal muscle in aging. According to this report, mitochondrial ROS production affects RyR1 function by dissociating the calstabin1/RyR1 complex (Andersson et al., 2011). In cardiac pathophysiology, RyR2 oxidation has also been reported after ischemic HF in addition to S-nitrosylation and PKA phosphorylation (Shan et al., 2010). Interestingly, this study suggests that deleterious RyR2 PKA-dependent phosphorylation requires oxidation and/or nitrosylation of the channel. During acute ischemia, RyR2 S-nitrosylation mediates a rapid SR  $\text{Ca}^{2+}$  leak independently of channel phosphorylation (Fauconnier et al., 2011). RyR2 may also be oxidized during ischemia-reperfusion (Zucchi et al., 1998). However, each RyR2 monomer contains 80–100 sulfhydryl residues per monomer. Only one fourth are free for covalent modifications and therefore for sensitive to reduction, oxidation and/or nitrosylation (Dulhunty et al., 2000), but the identity of the residues affected during ischemia reperfusion is not yet determined. The use of sulfhydryl reagents has indeed reveal the existence of critical thiols that could play a role in a normal RyR2 function or contribute to abnormal SR calcium release in pathophysiological situations (Dulhunty et al., 2000; Salama et al., 2000). It is admitted that a reducing or an oxidizing environment favor respectively close and open state of RyR. In normal situation, the redox potential is maintained at negative value mainly by the presence of a large excess of reduced glutathione (Pessah & Feng, 2000; Salama et al., 2000). During ischemia reperfusion, the subsequent oxidative stress may favor the oxidation of hyperactive sulfhydryl moieties. There are therefore different classes of thiol residues that can either activate or inhibit RYR2 opening depending on the redox potential of the cardiomyocytes (Dulhunty et al., 2000).

### 5.3. RyR2 degradation and expression

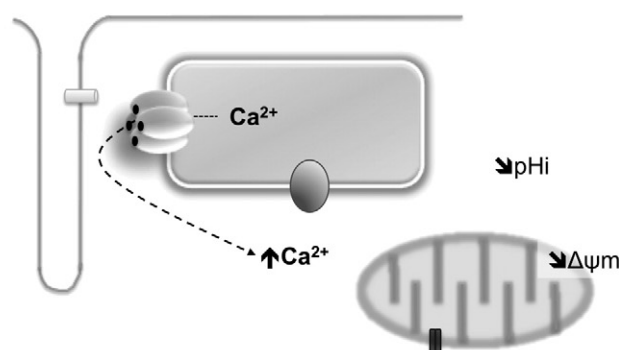
Proteases regroup all enzymes and systems that control proteins and cell homeostasis, protein turnover and degradation. During I/R, activation of proteases such as caspases and calpains has been extensively studied. These proteases have multiple targets and their activation is critical for extend of reperfusion injuries. Among all proteases, the  $\text{Ca}^{2+}$ -dependent cysteine proteases, calpains, are activated upon reperfusion. Calpains are inactivated by low pH during the ischemic phase, and restoration of normal pH at the reperfusion releases calpain inhibition in addition to increase cytosolic  $\text{Ca}^{2+}$  level. Among proteins targeted by calpain activation, cleavage of RyR2 has been observed (Müller et al., in press; Singh et al., 2012). RyR2 content may decrease by half without any change in mRNA. In addition to calpain, the proteasome system has also been involved in RyR2 degradation in simulated I/R. Interestingly, inhibition of proteasome prevents simultaneously RyR2 degradation and calpain activation in this model suggesting that activation of the proteasome pathways is required for calpain mediated RyR2 degradation (Pedrozo et al., 2010). In this study they also showed that inhibition of autophagy increases RyR2 degradation suggesting that some other proteolysis pathways are regulated by autophagy (Pedrozo et al., 2010). Knowing that caspase activation may interfere with RyR2 function (Fauconnier et al., 2011) the balance between autophagy and caspase activation is questionable in RyR2 degradation. The impact of RyR2 proteolysis on the channel function per se and reperfusion injury is not clear. In vitro, calpain releases a cytoplasmic portion of the channel which caused a prolonged open state of the  $\text{Ca}^{2+}$  release channel suggesting that calpain may digest a portion of channel involved in its inactivation (Rardon et al., 1990). Increased open state in the context of I/R may participate in  $\text{Ca}^{2+}$  overload and reperfusion injury. The impact of RyR2 proteolysis and

the sequence involved in this process remain to be specified since RyR2 degradation is not systematically reported (Mattiuzzi et al., 2004, 2008).

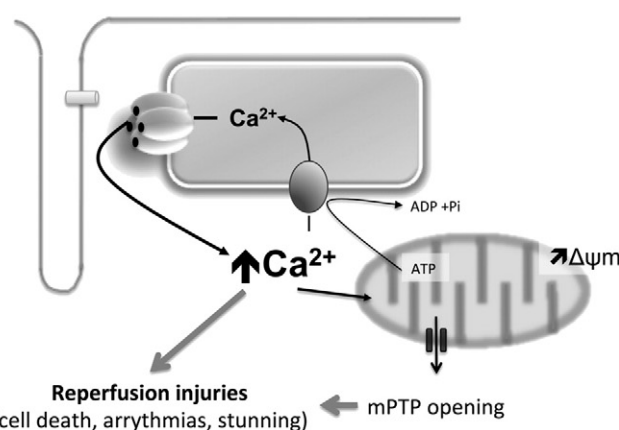
#### A/ Physiological diastolic situation



#### B/ During myocardial ischemia



#### C/ At the reperfusion



**Fig. 2.** Diastolic calcium cycling in cardiomyocytes. Calcium ions ( $\text{Ca}^{2+}$ ) are stored in the sarcoplasmic reticulum (SR). Both an efflux via spontaneous opening of SR  $\text{Ca}^{2+}$  channels (ryanodine receptors, RyR2) and an influx via the  $\text{Ca}^{2+}$ -ATPase (SERCA) equilibrate the SR  $\text{Ca}^{2+}$  content. Mitochondria are also able to uptake  $\text{Ca}^{2+}$  to maintain ATP synthesis. In physiological conditions, this steady state diastolic  $\text{Ca}^{2+}$  cycling is discrete (A). During an ischemia, the mitochondrial impairment resulting in the loss of ATP production reduces the uptake to the SR by SERCA. Therefore, this SR  $\text{Ca}^{2+}$  cycling is impaired contributing to an increase in the diastolic  $\text{Ca}^{2+}$  concentration (B). At the myocardial reperfusion (C), the cardiomyocytes re-oxygenation, restores SERCA function due to mitochondrial energetization and ATP production. In parallel, reperfusion is accompanied with defective RyR2 function (i.e. RyR2 mediated SR  $\text{Ca}^{2+}$  leak). Together this leads to an unstable diastolic  $\text{Ca}^{2+}$  cycling equilibrium with the generation of  $\text{Ca}^{2+}$  waves contributing to reperfusion injuries.

## 6. Role of RyR2 in SR-mitochondria interaction

The strong implication of mitochondrial permeability transition pore (mPTP) opening in the establishment of the reperfusion injury has been clearly demonstrated (Javadov et al., 2011). The interplay between the SR and mitochondria appears critical for mPTP opening as it controls mitochondrial  $\text{Ca}^{2+}$  overload (Fig. 2). Prevention of SR  $\text{Ca}^{2+}$  oscillations and inhibition of SR  $\text{Ca}^{2+}$  release with ryanodine have been shown to decrease mPTP opening and cell death (Ruiz-Meana et al., 2009; Abdallah et al., 2011) without affecting the global  $\text{Ca}^{2+}$  overload. These results suggest that SR  $\text{Ca}^{2+}$  oscillations are critical for mPTP opening and cell death. The close vicinity of the SR with mitochondria is essential in this process where the destabilization of the cytoskeleton prevents SR  $\text{Ca}^{2+}$  release-induced mitochondrial  $\text{Ca}^{2+}$  overload (Ruiz-Meana et al., 2009). In addition, normalization of RyR2 function either through inhibition of CamKII-mediated RyR2 phosphorylation or decrease S-nitrosylation of the channel and/or normalization of calstabin2/RyR2 ratio prevents reperfusion injury (Fauconnier et al., 2011; Said et al., 2011). This clearly indicates that RyR2 are involved in the exchange of  $\text{Ca}^{2+}$  from the SR to mitochondria presumably via contact points (Boncompagni et al., 2009). Although inhibition of SR  $\text{Ca}^{2+}$  release prevents mPTP opening in simulated I/R, inhibition of mitochondrial  $\text{Ca}^{2+}$  uptake or mPTP opening also reduces  $\text{Ca}^{2+}$  oscillations (Abdallah et al., 2011). Similarly, decrease mitochondrial  $\text{Ca}^{2+}$  uptake during ischemic phase raises cytosolic  $\text{Ca}^{2+}$  levels and reinforce cell death upon reperfusion (Ruiz-Meana et al., 2006). Moreover, the duration of ischemia appears critical in the settings of mPTP opening at the onset of reperfusion, which indicates a sensitization of mPTP to  $\text{Ca}^{2+}$  overload (Ruiz-Meana et al., 2011). Consistently, inflammatory process that occurs at reperfusion, increases mitochondrial ROS production through caspase-8 activation, and contributes to S-nitrosylation of RyR2 and calstabin2 dissociation. Therefore, preventing caspase-8 activation or calstabin2 dissociation reduces reperfusion injury (Fauconnier et al., 2011). This suggests the potential involvement of a dual detection mechanisms sensitizing mPTP opening (Pinton et al., 2001; Jacobson & Duchon, 2002; Hajnoczky et al., 2006). Accordingly, caspase-8-induced mitochondrial depolarization alone, without SR  $\text{Ca}^{2+}$  leak will not be sufficient to trigger cell death but would require a concomitant  $\text{Ca}^{2+}$  concentration oscillation. Discrete modification of the SR  $\text{Ca}^{2+}$  leak may thus be sufficient to prevent large scale swelling and allowing functional recovery of the mitochondria (Pinton et al., 2001; Jacobson & Duchon, 2002; Hajnoczky et al., 2006). Although the exact role of RyR2 in the dynamic regulation of SR-mitochondria  $\text{Ca}^{2+}$  exchange are poorly understood, the establishment of a vicious circle between SR  $\text{Ca}^{2+}$  release and mitochondrial  $\text{Ca}^{2+}$  uptake appears as key events in reperfusion injury. Interestingly, it has been reported that a type 1 like RyR could be expressed at the inner membrane of the mitochondria (Pan et al., 2011). Although this information remains controversial, this channel is thought to contribute to mitochondrial calcium uptake. One can therefore speculate that it could contribute to mitochondrial  $\text{Ca}^{2+}$  overload under pathophysiological situations but this remains to be demonstrated.

## 7. RyR2 in cardioprotection

### 7.1. Pre- and post-conditioning

Reduction of reperfusion injury has become challenging over last decade. Inhibition of mPTP opening using cyclosporine A appears to be clinically relevant (Piot et al., 2008) despite the fact that the duration of ischemia may influence the role of mPTP during reperfusion injury (Ruiz-Meana et al., 2011). In the mid 80's, Murry et al. (1986) applied short sequence of I/R before a prolonged acute ischemia. This so-called ischemic preconditioning (IPC) was shown to be greatly protective against reperfusion injury (Murry et al., 1986). Since this original work,

several signaling pathways involved in IPC have been described and most of them converged to the mitochondria and the mPTP (for review see (Otani, 2008)). Later, other types of preconditioning such as tachycardia, exercise or pharmacological preconditioning (Domenech et al., 2003; Zaugg et al., 2003; Sanchez et al., 2008; Frasier et al., 2011) have been shown to reduce ischemic damages. In general, preconditioning improves  $\text{Ca}^{2+}$  handling during reperfusion with a decrease in  $\text{Ca}^{2+}$  overload. During IPC, the brief episode of ischemia increases diastolic and decreases systolic  $\text{Ca}^{2+}$  level progressively (Smith et al., 1996). In addition, IPC decreases ryanodine binding on RyR2 due to thiol oxidation of the channel (Zucchi et al., 1998). Consistently, increased NADPH oxidase activity in the vicinity of the RyR2 induces S-glutathionylation of the channels (Aracena et al., 2005; Sanchez et al., 2008). Redox modification of the RyR2 enhances RyR2-mediated  $\text{Ca}^{2+}$  release and may produce the observed increase in cytosolic  $\text{Ca}^{2+}$ . Altered  $\text{Ca}^{2+}$  release would decrease SR  $\text{Ca}^{2+}$  content and prevent  $\text{Ca}^{2+}$  overload during sustained ischemia and reperfusion (Donoso et al., 2011). Whether other post-translational modifications of the RyR2 such as S-nitrosylation, RyR2 CamKII phosphorylation and/or calstabin2 depletion occurred during preconditioning remain to be clarified. However inhibition of CamKII blunt the protective effects of IPC on SR  $\text{Ca}^{2+}$  uptake (Osada et al., 2000). In addition, late preconditioning, defined by a second window of cardioprotection several hours after IPC, is associated with an increase in calstabin2 expression (Lucats et al., 2007). In overall, preconditioning-induced decrease in SR  $\text{Ca}^{2+}$  content seems cardioprotective. This is reinforced by studies showing that application of nanomolar ryanodine prior to ischemia, which locks the channel in sub-conductance open-state, decreases reperfusion injuries (Meldrum et al., 1996; Zucchi et al., 2001). For a clinical point of view, the feasibility and the efficiency of IPC are limited to some specific cases (Crisostomo et al., 2006). However, the post-conditioning, which consists of brief ischemic sequences prior the complete reperfusion after the ischemic insult, is also cardioprotective (Zhao et al., 2003). As for IPC, post-conditioning activates several signaling pathways (Hausenloy et al., 2011) that converge towards mitochondria (Boengler et al., 2011) and prevention of mPTP opening (Argaud et al., 2005). Whether post-conditioning affects RyR2 function or not is currently unknown and few data are available on  $\text{Ca}^{2+}$  cycling. The reduced mPTP opening was associated with decrease in  $\text{Ca}^{2+}$  overload, mitochondrial ROS production and an increase mitochondrial  $\text{Ca}^{2+}$  retention capacity (Argaud et al., 2005; Sun et al., 2005; Argaud et al., 2008). Finally, prevention of aberrant  $\text{Ca}^{2+}$  release either through a decrease in pre-ischemic SR  $\text{Ca}^{2+}$  load or normalization of RyR2 function at the reperfusion time is cardioprotective (Fauconnier et al., 2011).

### 7.2. Pharmacological modulation of RyR during I/R

Evidence of a pathophysiological implication of RyR2 in reperfusion injury has been defined using pharmacological agents able to modulate the channel function (Zucchi et al., 2001). Although the beneficial effects of ryanodine application at the onset of reperfusion are controversial depending on its RyR2 inhibitory effects and the consequence on oxygen consumption, application of ryanodine prior to a sequence of I/R decrease reperfusion injury. This cardioprotective effect was attributed to a pre-ischemic decrease in SR load preventing subsequent  $\text{Ca}^{2+}$  overload (Akita et al., 1993). For example, low dose of ryanodine (1-100 nM) or application of caffeine (10 mM) prevents ventricular arrhythmias during acute myocardial ischemia and reperfusion (Thandroyen et al., 1988). Similarly, increasing intracellular magnesium concentration also prevents reperfusion-induced ventricular fibrillation through a decrease in diastolic  $\text{Ca}^{2+}$  level (Miyoshi et al., 2000). In line with this, anesthetics compounds such as sevoflurane and/or remifentanyl at low dose decrease SR  $\text{Ca}^{2+}$  leak and reperfusion injury (Zaugg et al., 2012). Dantrolene applied before the onset of ischemia has also been shown to be protective at micromolar range (Yu et al., 2000), suggesting a conformational change of RyR2 during I/R (Wang

et al., 2011). A conformational change could be attributed to RyR2 post-translational modification that occurs during I/R (see below). Such post-translational modification is associated with a dissociation of calstabin2 from the channel, which participate in SR Ca<sup>2+</sup> leak. The 1,4-benzothiazepine derivative JTV519, which stabilizes calstabin2 binding to RyR2, as been shown to reduced I/R induced Ca<sup>2+</sup> overload (Inagaki et al., 2000). More recently, its more specific derivate S107, has also been shown to prevents calstabin2 dissociation, SR Ca<sup>2+</sup> leak and reperfusion injuries (Fauconnier et al., 2011). Despite that an hyper S-nitrosylation of RyR2 appear to be deleterious in the setting of reperfusion injury, administration of NO donors as been shown to improve post-ischemic function especially through their vasorelaxant effects (Hein et al., 2003). However, l-arginine administration may also prevent calpain induced RyR2 degradation and SR function (Chohan et al., 2006). A more specific approach using small interfering RNA of RyR2 showed a significant cardioprotection in simulated I/R (Guo et al., 2008).

One other approach would consist to prevent SR Ca<sup>2+</sup> overload at the setting of the reperfusion. Thus, inhibition of SERCA using thapsigargin or CPA reduced reperfusion induced Ca<sup>2+</sup> oscillations and ventricular fibrillation (du Toit & Opie, 1994). The intraluminal control of RyR2-dependent SR Ca<sup>2+</sup> release during I/R appears to play a key role in such oscillation as the ablation of triadin and junctin, which participates in the control of the luminal dependence of Ca<sup>2+</sup> release, exacerbate reperfusion injury (Cai et al., 2012). However, overexpression of SERCA, which accelerates SR Ca<sup>2+</sup> reuptake, reduced myocardial infarction (Talukder et al., 2007) indicating that a fine-tuning between SR Ca<sup>2+</sup> release and uptake is crucial in the setting of reperfusion injury.

### 7.3. RyR2 in cardioprotection: impact of co-morbidities and confounding factors

It is important to consider that cardioprotective strategies are strongly influenced by co-morbidities and confounding factors such as hypertension, diabetes, hypercholesterolemia, obesity, ageing and/or sex (Sack & Murphy, 2011). This may in part explain the discrepancy between the clinical trials in human and the cardioprotective results obtains by different approaches on homogeneous animal models (Bolli et al., 2004). Posttranslational modifications and/or alteration in RyR2 function are highly influenced by co-morbidities and confounding factors.

Metabolic syndrome is clinical disorder combining hypertension, insulin resistance, impaired glucose tolerance, obesity, and dyslipidemia. Patients with metabolic syndrome have significantly increased risk of cardiovascular disease including myocardial infarction, congestive heart failure, and arrhythmia. It has been suggested that elevated level of circulating catecholamine during metabolic syndrome could lead to PKA-dependent phosphorylation of RyR2 and SR Ca<sup>2+</sup> leak (Dincer, 2012).

Hypertension by itself is one of the major causes of cardiac hypertrophy. It has been shown that RyR2-dependent SR Ca<sup>2+</sup> leak activates the calcineurin/NFATc pathway, in cardiomyocytes, under conditions of pressure overload to induce cardiac hypertrophy (van Oort et al., 2010; Zou et al., 2011). It is to note that disrupting the calstabin1/2/RyRs complex in endothelial cells also results in an intracellular Ca<sup>2+</sup> leak, which may contribute to endothelial dysfunction and hypertension (Long et al., 2007).

Diabetes mellitus is also a major risk factor for cardiovascular complications. In this pathophysiological context RyR2 have been shown to be highly sensitive to redox changes, either directly and indirectly through kinases (PKA, PKC, CaMKII) activation (Turan & Vassort, 2011).

Mortality and morbidity in very elderly patients with ST-segment elevation myocardial infarction are significantly higher than in younger patients (Renilla et al., 2013). In skeletal muscle, RyR1 present a leaky behavior with ageing contributing to sarcopenia (Andersson et al.,

2011). Due to the strong homology between skeletal muscle isoform RyR1 and cardiac muscle isoform RyR2, one can speculate a higher susceptibility of RyR2 to become leaky with ageing but this remains to be determined.

Finally, females have been shown to exhibit endogenous cardioprotection (Sack & Murphy, 2011). This gender-based difference in cardiovascular function and cardioprotection may be in part due to estrogen (Mendelsohn & Karas, 1999). Interestingly, it has been initially reported that although SR dependent Ca<sup>2+</sup> release was similar in male and female calstabin2 deficient mice, estrogen would protect female against Ca<sup>2+</sup> overload mediated cardiac hypertrophy (Xin et al., 2002). In contrast, it was most recently reported a gender difference in SR Ca<sup>2+</sup> release events (i.e. Ca<sup>2+</sup> sparks) reflecting RyR2 function was different in males and females. In type 2 diabetes, defects in RyR2 (i.e. interaction with calstabin2 and S2808 phosphorylation) would be more pronounced in male than in female during diabetes (Yaras et al., 2007).

## 8. Conclusion

During acute ischemia there is now a consensus to conclude that both mitochondrial impairment and calcium homeostasis are impaired. There is also now a growing number of evidence to link defects in both mitochondrial and Ca<sup>2+</sup> homeostasis. There is now also a consensus that origin and source of Ca<sup>2+</sup> in this pathophysiological situation is the SR. The use of pharmacological agent like ryanodine aimed at preventing a massive SR Ca<sup>2+</sup> release has demonstrated for many years a potent cardioprotective effect suggesting the importance of the SR. Nevertheless, the implication of the SR could be the consequence of primary mechanisms able to trigger a massive SR Ca<sup>2+</sup> release. It has been recently demonstrated that RyR2 structure and function were rapidly impaired during acute ischemia, resulting in a diastolic Ca<sup>2+</sup> leak (Fauconnier et al., 2011). Therefore, defective RyR2 function appears as one of the primary pathophysiological mechanism involved during ischemia reperfusion. This is confirmed by the use of pharmacological agents able to prevent RyR2 dysfunction that induced a significant reduction in reperfusion injuries. As stated above, the post-translational mechanism by which RyR2 function is impaired in cardiac pathophysiology remains controversial and although S-nitrosylation of the channels has been observed after I/R one cannot exclude other mechanisms accounting for RyR2 impairments. Nevertheless, reducing RyR2-dependent SR Ca<sup>2+</sup> leak in I/R appears as a relevant therapeutic approach. In this context, the use of specific allosteric modulators of RyR function (Andersson & Marks, 2010), reducing diastolic Ca<sup>2+</sup> leak without altering excitation-contraction coupling, opens important pharmacological perspectives.

## Conflict of interest

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