The ryanodine receptor microdomain in cardiomyocytes

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Abstract

The ryanodine receptor type 2 (RyR) is a key player in Ca^{2+} handling during excitation-contraction coupling. During each heartbeat, RyR channels are responsible for linking the action potential with the contractile machinery of the cardiomyocyte by releasing Ca^{2+} from the sarcoplasmic reticulum. RyR function is fine-tuned by associated signalling molecules, arrangement in clusters and subcellular localization. These parameters together define RyR function within microdomains and are subject to disease remodelling. This review describes the latest findings on RyR microdomain organization, the alterations with disease, and presents novel technologies that guide future research to study and target RyR within specific microdomains.

1. Ryanodine receptors are organized in microdomains

In cardiomyocytes, the type 2 ryanodine receptor (RyR) is responsible for Ca^{2+} release from the sarcoplasmic reticulum (SR), which provides the major source of Ca^{2+} during excitation-contraction coupling (ECC). Opening of L-type voltage-gated Ca^{2+} channels (LTCC) on invaginations of the sarcolemma (SL) membrane, called transverse and axial tubules (TATS), during the action potential (AP) provides Ca^{2+} for activation and opening of RyRs on the underlying SR via a process called calcium-induced calcium release (CICR) [1]. Close proximity of LTCCs and RyRs in a dyad ensures efficient CICR. The resultant rise in free Ca^{2+} activates troponin C at the myofilaments to initiate contraction. Subsequently cardiomyocyte relaxation is brought about by removal of Ca^{2+} from the cytosol, mainly by re-uptake in the SR by Ca^{2+} ATPase (SERCA) but also by extrusion from the cell through sarcolemmal Na^{+}/Ca^{2+} exchanger (NCX), which balances the Ca^{2+} influx through LTCC (reviewed in [2,3]). As NCX is an electrogenic transport, it adds a depolarizing current to the late phase of the AP.

Dysfunction of RyRs during cardiac pathologies leads to alterations in Ca^{2+} dynamics that result in disruption of systolic contraction and promote arrhythmias associated with sudden cardiac death [4–6]. Central to these pathological consequences of RyR dysfunction is an increase in spontaneous RyR opening. The resultant Ca^{2+} leak reduces SR Ca^{2+} content, which leads to decreased contraction. In addition, substantial Ca^{2+} leak and spontaneous Ca^{2+} waves during diastole, causes delayed afterdepolarizations (DADs) and/or triggered AP through activation of the inward NCX current [7,8]. Genetic mutations in RyRs and RyR accessory proteins (e.g. calsequestrin (CSQ), Calmodulin (CaM)) in patients with catecholaminergic polymorphic ventricular tachycardia (CPVT) enhance spontaneous Ca^{2+} release explaining the susceptibility for cardiac arrhythmias in these patients [9].

Ca^{2+} release through RyRs is fine-tuned through functional modulation of RyR gating by associated signalling molecules, through the structural organization of RyR in clusters of variable size and configuration, and through variable location of RyR clusters within the cardiomyocyte [10–12]. These three elements combine in defining microdomains for Ca^{2+} release that shape the cellular Ca^{2+} transient and membrane potential. The advent of enhanced resolution imaging, refined molecular characterization and novel tools for probing local Ca^{2+} has advanced substantially our insights in these microdomains. Central to the functional assessment of RyRs in their microdomain, is the study of Ca^{2+} sparks as localized release events produced by a cluster of RyRs, usually in the absence of a LTCC trigger [13]. Amplitude and frequency of these Ca^{2+} sparks depend on the three elements that define the microdomain, i.e. local signalling, cluster organization and location.

The present interest in RyR microdomains stems from the growing evidence that disease-related contractile dysfunction and arrhythmias may relate to the emergence of RyR microdomains with altered properties, and to heterogeneity of microdomains within the cardiomyocyte. Therefore, this review focuses on the elements that define the RyR microdomain, summarizes the changes with disease that impact on RyR function and provides an overview of new tools and technologies that can guide future research.

2. The RyR as macromolecular complex for microdomain signalling

2.1 Binding sites on the RyR

RyRs are large homotetramer Ca^{2+} channels (2.2 MDa), which comprise four identical 560 kDa subunits extending from the junctional SR lumen into the cytosol. Each subunit is comprised of a large cytoplasmic region, six transmembrane regions and a smaller SR luminal region [14,15] (Figure 1).

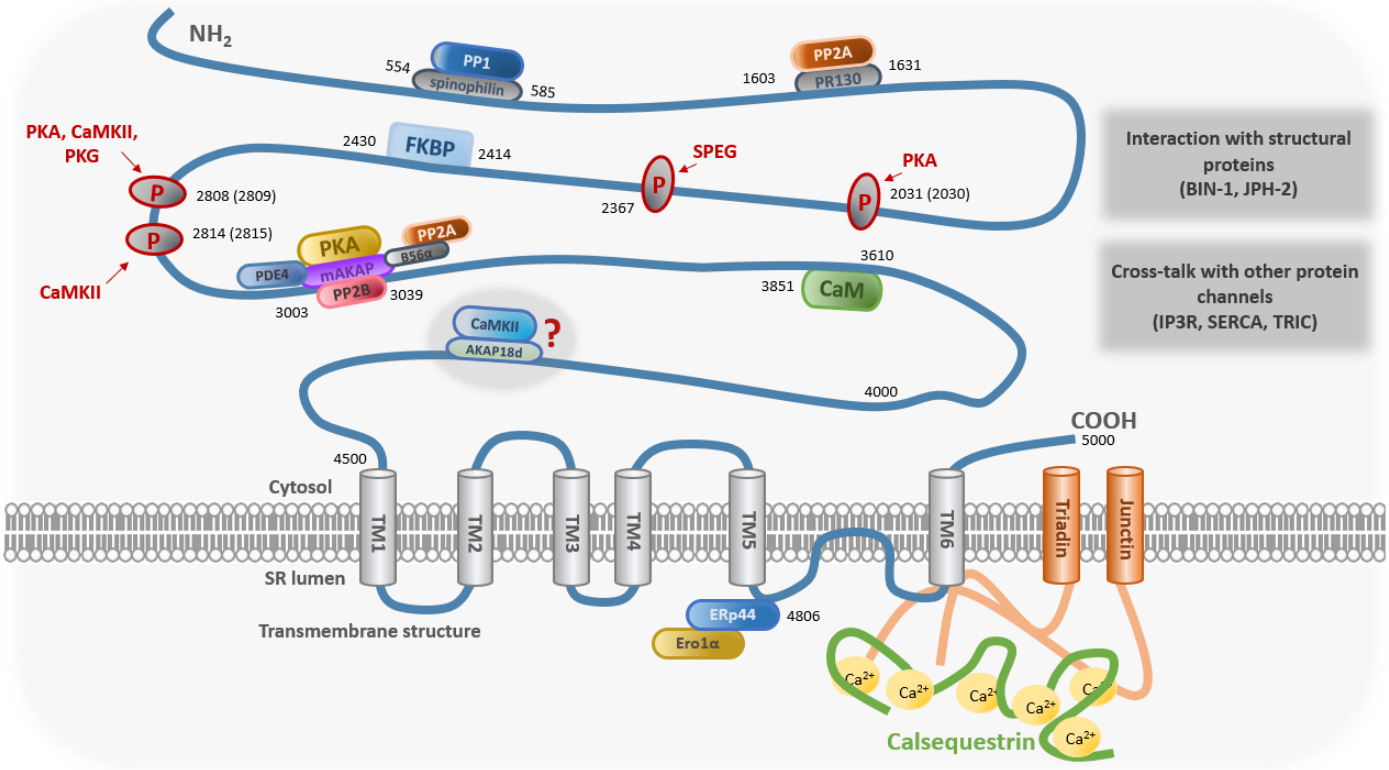

Figure 1. Schematic overview of the predicted RyR subunit structure and sites of interactions with regulatory proteins*. Each subunit consists out of a cytosolic, transmembrane and SR luminal segment. The N-terminal forms a bulky cytoplasmic domain that comprises 90% of the polypeptide chain. The remaining 10% of the sequence forms the transmembrane (TM) segments and channel-pore regions. Sites of interactions are shown at specific amino acids. Interactions with structural proteins and cross-talk with other channels have also been proposed. Phosphorylation sites for CaMKII and PKA are shown with different residue numbering depending on species.*

Ca^{2+} is the ligand for RyR activation. At low $[Ca^{2+}]_i$ of ~100-200 nM, RyRs are inactive with a low open probability (P_o). With increasing $[Ca^{2+}]_i$, RyR channel activity increases with P_o reaching its peak at $[Ca^{2+}]_i$ of around ~10 µM, as seen in lipid bilayers experiments (although modelling experiments predicts higher values in situ) [16–19]. At higher $[Ca^{2+}]_i$, channel activity is inhibited and P_o decreased, illustrating the channel's typical bell-shaped signature of regulation by cytosolic $[Ca^{2+}]_i$. Ca^{2+} also acts on the SR luminal side to modulate channel activity. Over the past decades, several **Ca^{2+}-binding sites** have been identified on the cytosolic and luminal sites of the channel, providing further insights into the channel's gating properties [14,20–23].

RyR channels serve as a large scaffold on which several regulatory proteins and enzymes can be assembled. The association and/or activity of these regulatory proteins with the extensive cytosolic domain or the intra SR region of the RyR provides an additional layer of regulation to that provided by Ca^{2+} serving to fine tune activity and sensitivity to other cell signalling pathways and cell state (Figure 1). On the cytosolic side of this macromolecular complex, a notable interaction occurs with **FKBP12.6**. The immunophilin FKBP12.6, also known as calstabin-2, was one of the first proteins considered to interact with and regulate RyRs. This protein tightly associates with each subunit of the RyR, stabilizing its closed conformational state and facilitating channel closure [24,25]. Dissociation of the FKBP12.6 protein from RyR was initially considered via phosphorylation of Ser2808 induced by PKA [26], although this mechanism for dissociation and whether the interaction is dynamically regulated is controversial. Different labs demonstrated that despite phosphorylation by PKA [27] or CaMKII [28], there is a continued association between RyR and FKBP12.6.

The archetypal Ca^{2+} signalling protein **calmodulin (CaM)** also interacts with and modulates RyR function. CaM binds to RyRs with nanomolar affinity and inhibits RyR function most effectively at $[Ca^{2+}]_i$ above 1 µM [29,30].

The RyR macromolecular complex further includes protein phosphatases and kinases which interacts either directly or via scaffolding proteins with the RyR. The association between **protein phosphatase 1** (PP1) and RyR occurs via PPP1R9B (spinophilin) [31,32] and PPP1R3A [32,33]. AKAP18delta (also known as AKAP7delta) anchors **CaMKII** with RyRs [34]. **PKA** associates with RyRs via the muscle-specific A-kinase anchoring protein (mAKAP) [35], that also serves as a scaffold to tether phosphodiesterases (PDE4D) [36], for local control of cAMP levels, and PP2A via B56α [37,38] and PP2B [39].

Sorcin is another accessory protein that binds to RyRs and inhibits the channel's P_o [40]. Linking metabolic state with RyR function, nucleotides (ADP, ATP and AMP), ions (Mg^{2+}) and metabolites (pyruvate, fatty acids and polyamines) can further modulate RyR function

[30,41]. Further, endoplasmic reticulum (ER) signalling pathways, such as through PERK (protein kinase RNA-like ER kinase) acting via calcineurin impacts upon RyR function [42].

At the SR luminal side, the C-terminus of the RyR is associated with **calsequestrin** (CSQ), **triadin** and **junctin**, which modulate RyR function via the SR lumen by acting as a luminal Ca^{2+} sensor [43]. Triadin and junctin directly interact with RyRs and act as bridging proteins that bind to CSQ [14]. CSQ is a low affinity Ca^{2+} binding protein that functions as the primary Ca^{2+} storage protein of the cardiomyocyte SR. It also serves to inhibit RyRs and dissociates from the RyR when luminal Ca^{2+} increases, thereby increasing RyR P_o. More recently, ER protein 44 (ERp44) was shown to covalently associate with RyRs on the luminal side (via cysteine 4806) to stabilize RyRs in a redox-dependent way [44].

Proteins involved in organising the complex TATS architecture of the cardiomyocyte, such as **junctophilin-2** (JPH-2) [45] and **bridging integrator 1** (BIN-1) [46], also interact with RyRs affecting its activity. Some of these proteins are important in RyR cluster formation and will be discussed in more detail in Section 3.

Cross-talk with other SR localised channels and pumps, such as inositol 1,4,5-trisphosphate ($InsP_3$) receptors (IP_3R) [47], SERCA [48,49] and trimeric intracellular cation (TRIC) channels [50] have also been demonstrated to modulate RyR function.

2.2 Post-translational modifications of RyR function

Phosphorylation

The activity and complex interplay between protein kinases (phosphorylation) and protein phosphatases (dephosphorylation) associated with the RyR determine the degree of RyR phosphorylation, under higher control of the sympathetic nervous system. The relationship between RyR function and phosphorylation status is V-shaped with RyR activity and SR Ca^{2+} release being the highest at very low or very high phosphorylation levels [51]. Protein kinase G (PKG), cAMP-dependent kinase (PKA) and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) phosphorylate specific serines on the cytoplasmic region of the RyR [52–54] (Figure 1), yet the location and physiological implication of some RyR phosphorylation sites are controversial. Ser2808 (or Ser2809 depending on species) was first proposed as the major PKA phosphorylation site, while others proposed Ser2030 (or Ser2031 depending on species) as a PKA target [55,56]. Ser2808 has also been found as a target for PKG [55] and CaMKII [57], while Ser2814 (or Ser2815 depending on species) seems exclusively be targeted by CaMKII [58,59]. Transgenic mouse models, lipid bilayer experiments and cellular Ca^{2+} signalling experiments all contribute to the general acceptance of increased RyR channel P_o and increased Ca^{2+} spark frequency upon phosphorylation of Ser2814 by

CaMKII. The mechanisms and consequences of PKA-induced phosphorylation are less clear. Various models show increased RyR channel P_o, increased Ca^{2+} spark frequency and dissociation of FKBP12.6 upon PKA phosphorylation, but the lack of these findings by others complicate the general picture of PKA phosphorylation and RyR activity. More recently, proteomic analysis of immunoprecipitated RyR and JPH-2 identified 'striated muscle preferentially expressed kinase', known as SPEG, as RyR kinase contributing to phosphorylation at Ser2367 [60,61]. In contrast to PKA- and CaMKII-mediated phosphorylation, SPEG phosphorylation of RyRs has been proposed to reduce SR Ca^{2+} leak [60].

RyR phosphorylation can be reversed by protein phosphatases 1 (PP1), 2A (PP2A) and 2B (PP2B, also known as calcineurin) [26] that are also present within the RyR macromolecular complex. Determining the specificity of the phosphatases for the various RyR phosphorylation sites is ongoing and remains incompletely understood. PP1 dephosphorylates Ser2814 and Ser2808 [31,51,62], while PP2A can dephosphorylate Ser2814 and Ser2030, but not Ser2808 [63]. Interestingly, PP1 not only dephosphorylates RyR Ser2814, but also can directly affect the autophosphorylation status of CaMKII [64]. While dephosphorylation is considered as a counteracting mechanism to maintain a basal RyR phosphorylation state during physiological conditions, excessive dephosphorylation of RyRs by PP1 leads to increased SR Ca^{2+} leak [62]. These findings further support the notion that RyRs require an intermediate level of phosphorylation to function under physiological conditions. RyR-associated phosphates have been reviewed in more detail by [39].

Redox modifications

RyRs also contain a number of amino acid residues (primarily at cysteines and methionines) that are sensitive to modification by reactive oxygen and nitrogen species (ROS and RNS) generated both during physiological and pathophysiological conditions in the heart. Under physiological conditions, low levels of ROS and RNS induce discrete and reversible amino acid modifications, whereas increased oxidative stress during cardiac pathologies generates more extensive irreversible modification to the RyR that is commonly associated with increased SR Ca^{2+} leak through hyperactive RyRs. ROS/RNS-dependent modulation of RyR function has been shown by NADPH oxidases (NOX) [65], xanthine oxidase (XO) [66], mitochondria [67] and nitric oxide synthase (NOS) [68] that can be counteracted by various cellular antioxidants. The modulation of RyRs by ROS/RNS therefore depends on the flux, lifetime and the local ROS/RNS scavenging capacity (reviewed by [69]).

RyR redox modifications are reversible in nature and increase the channel's sensitivity to intraluminal SR Ca^{2+} i.e. the channel remains open at lower Ca^{2+} such as in disease [70].

Disulfide bridge formation (inter-subunit and inter-channel cross linking), glutathionylation by glutathione (GSH), nitrosylation by nitric oxide (NO) and phosphorylation by ROS- and RNS-activated CaMKII can all contribute to an increased RyR channel activity [71–76].

Redox modifications of cysteine residues in the cytosolic domain of RyR have been most studied, although their specific location in the RyR sequence remain to be determined. Oxidation of the channel's cysteine residues has been generally suggested to cause RyR activation [77,78], yet many studies showed that the effect of oxidation highly depends on experimental conditions [79]. Low concentrations of oxidative agents activate RyRs, whereas prolonged exposure or high concentrations lead to irreversible inhibition of RyRs [80]. Redox modifications within RyR binding sites for CaM and FKBP12.6 interfere with protein-protein interactions [29]. Redox modifications are associated with an increased dissociation of CaM from the channel and in increased SR Ca^{2+} leak [81]. More recent work demonstrated the redox-dependent modulation on the luminal side of RyR via ERp44 and Ero1α [44].

Although most studies have examined post-translational modifications at a cellular level, evidence is emerging that these modifications may be restricted to RyR in microdomains determined by the structural organization, i.e. the formation of RyR clusters and the subcellular location of such clusters within the cardiomyocyte.

3. Structural arrangement of RyRs into clusters

The subcellular distribution of RyRs has been studied with EM and confocal microscopy with advanced image-processing methods, including deconvolution [82–84]. These studies estimated >100 receptors within a RyR cluster and provided the first evidence for cluster architecture and dimensions. Subsequent super-resolution technologies based on single molecule localization microscopy (SMLM), such as dSTORM, improved resolution by 10-fold (up to ~30-50 nm). These methodologies allow detection of single RyR clusters with increased sensitivity (~15-21 RyRs per cluster), which could not be resolved by conventional confocal imaging [85,86]. Further and relevant to their activation, edge-to-edge distances between clusters were found to be significantly smaller than initially predicted (<100 nm with super-resolution imaging vs. ~670 nm with confocal microscopy). However, arrangement of receptors within a cluster remain unresolved because dSTORM image processing manually assembles RyR into clusters with a centre-to-centre distance of ~30 nm (Figure 2E). The use of newer super-resolution imaging methodologies, such as DNA-paint and expansion microscopy or imaging equipment such as MINIFLUX from Abberior, have pushed resolution boundaries to a new level (10-15 nm precision or even lower) and make it possible to directly visualize receptor arrangements within a cluster [87–89].

Collectively, we now know that RyRs are organized into clusters of variable size and clusters can aggregate in supercluster formations, containing 3-4 clusters on average (Figure 2A). The average number of RyRs per cluster ranges from 8-22 near the SL [88,90] and from 8-100 in TATS regions [86,91–93]. When located within close proximity (<100 nm), these clusters are proposed to cooperatively operate as a Ca^{2+} release unit (CRU) that generate Ca^{2+} sparks (Figure 2A). In healthy adult ventricular cardiomyocytes, particularly in rodents, the largest fraction of RyR clusters are localized within the dyadic cleft. Transmission electron microscopy typically shows RyR clusters as electron dense structures, called 'feet', located between the junctional SR and SL that extend across most of the dyadic cleft space (≈ 10 nm) [82,94,95]. Recent work finds a larger fraction of superclusters in TATS and SL regions compared to non-junctional SR network regions [96] and improving techniques in high-resolution imaging continue to increase insight [97,98].

The relationship between RyR channel numbers and density within a cluster to Ca^{2+} release function as sparks, was mostly derived from separate measurements of RyR morphology through high-resolution microscopy in fixed cells and of sparks activity in live cells, with an important role for computational modelling to predict activity from clusters size [85,99,100] (Figure 2B-E).

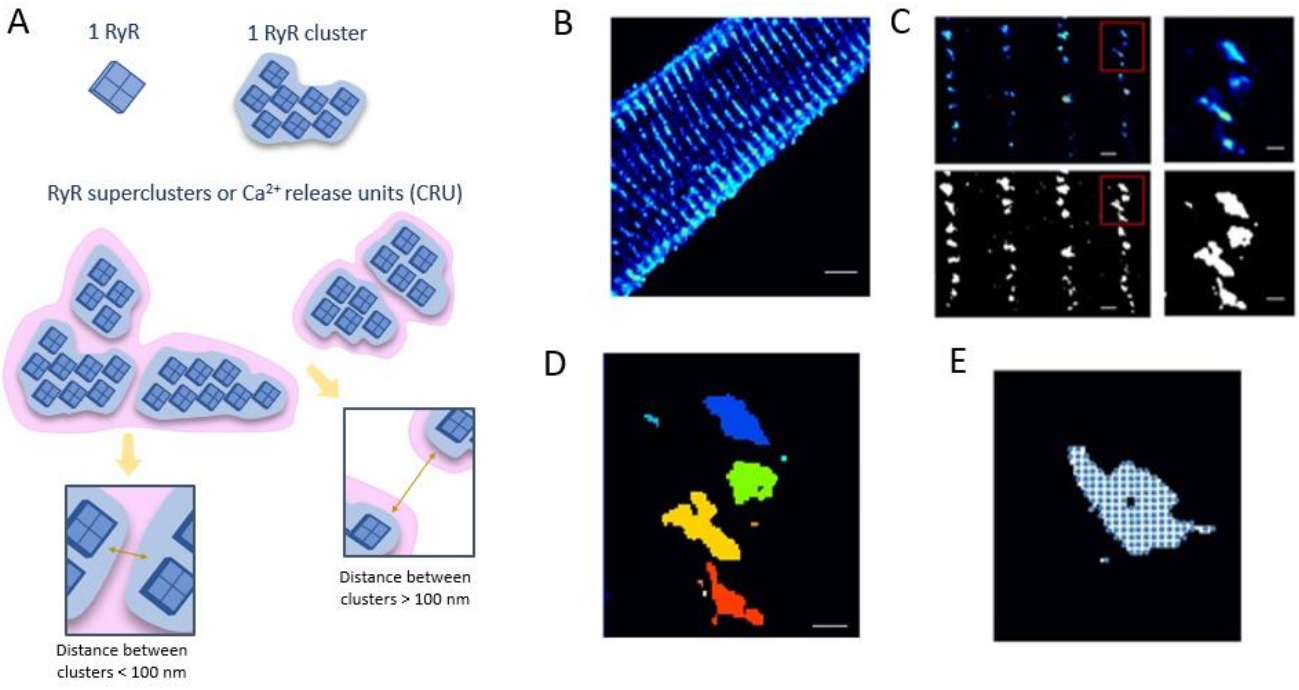

Figure 2. Organization of RyRs into clusters and superclusters. *(A) Schematic overview of hierarchical organization of RyRs into clusters (light blue) and superclusters (pink). Immunostained RyRs visualized with confocal microscopy, example shown in (B), appear as RyR clusters. RyR clusters that are in close proximity (within 100 nm of each other) are proposed to activate together and are referred to as RyR superclusters or Ca^{2+} release units (CRU). (B-E) Super-resolution imaging allows quantification of RyR arrangements within CRUs, after image processing and thresholding (C), with individual clusters identified (clusters are shown in different colours) (D). (E) Grid-based quantification method to count the number of RyRs within a cluster: a grid of single RyRs are superimposed on the thresholded image. (panel B-E modified from [85]).*

Novel imaging approaches with increased spatial and temporal resolution allow a more direct correlation [101]. It is now possible to correlate Ca^{2+} sparks imaged in 2D with the underlying RyRs visualised by super-resolution imaging of a photoactivatable red fluorescent protein incorporated into the RyR protein [97]. This analysis showed that the number of RyRs underlying a Ca^{2+} sparks is highly variable. Sparks were increased in magnitude under conditions of adrenergic stimulation through recruitment of additional RyR clusters [97]. Using a similar approach but involving RyRs engineered to contain a green fluorescent protein in its sequence to locate RyR clusters, and imaging the location of sparks using a red fluorescent Ca^{2+} indicator dye Rhod-2, the number of RyR clusters involved in generating a spark was analysed. In this analysis, sparks were generally found to comprise 1 cluster but under conditions of adrenergic stimulation, the numbers of clusters involved was increased giving rise to sparks with a greater signal mass [102].

4. Localization of RyR clusters in different subcellular compartments

RyR clusters on junctional SR membranes are primarily located in close apposition to the SL in transverse tubules, near axial tubules and at the cell periphery, where they associate with LTTCs in the dyadic cleft, forming couplons. This is the predominant organisation in ventricular cardiomyocytes of small mammals with high heart rates (e.g. mouse or rat) and dense TATS (Figure 3). Notwithstanding, RyR clusters also reside outside the dyadic cleft [93,103–109]. In ventricular cardiomyocytes of large mammals (e.g. pig, human) with less developed TATS, RyRs remain evenly distributed in the cell at sarcomeric Z-lines, resulting in a fraction of RyRs (~20%) that are not in close proximity of SL/TATS. These RyRs are thus not in dyadic junctions and are often referred to as ‘non-junctional’ RyRs. Based on ultrastructure data, others have used the term ‘orphaned’, while in our own work we mostly used the term ‘non-coupled’ in a more functional approach of not being coupled to LTCC activation. Depending on species, preparation and method of analysis, the nomenclature and identification of RyRs differs between studies, and measured distance of RyRs from SL/TATS can range up to several micrometres (Table 1). Confocal microscopy used in the listed colocalization studies have limited spatial resolution because of the diffraction of light (approximately ~300 nm), which is appreciably above the size of an average RyR complex. Therefore, the RyR subpopulations identified by the confocal colocalization approach can be an over-estimate of the spatial overlap between RyR and Cav1.2 proteins.

An example of such species differences is shown in Figure 3.

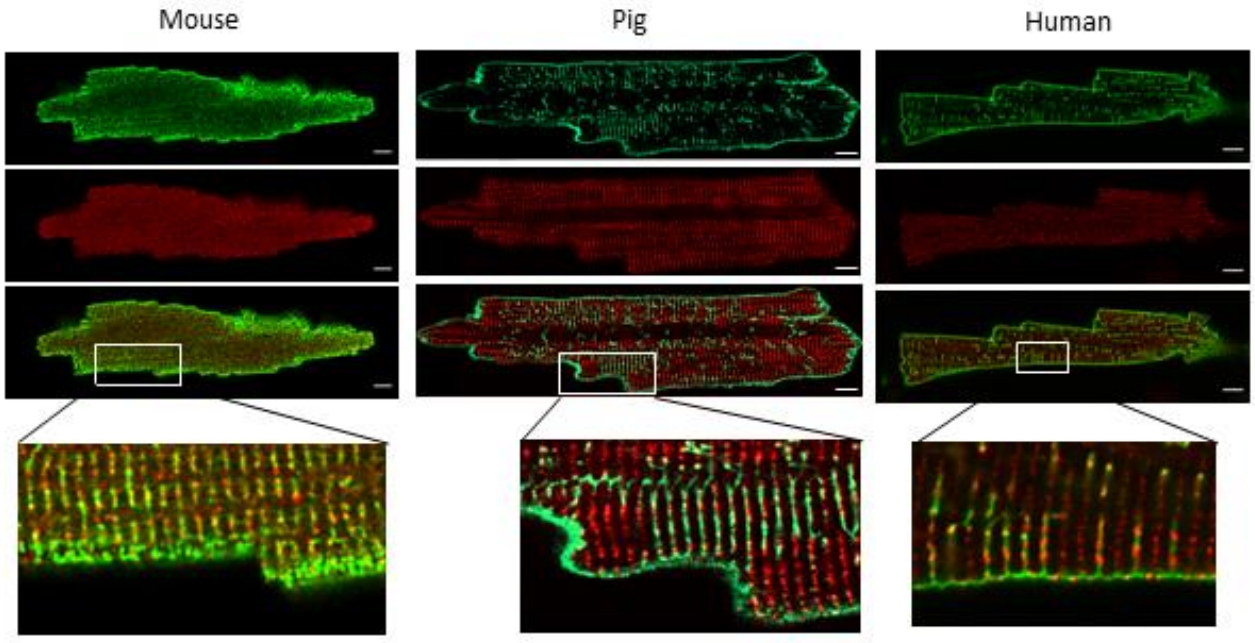

Figure 3. Proportions of RyR subpopulations differ amongst species. *Immunostainings are shown in ventricular myocytes from mouse, pig and human. Cardiomyocytes are labelled with NCX (green, as a surrogate to report on SL and TATS) and RyR (red). For each image, a zoomed in image is shown from the region indicated by the white box. In mouse ventricular myocytes, most RyRs co-localize with SL/TATS. In contrast, in ventricular cardiomyocytes from pig and human, not all RyRs coincidence with NCX labelling, indicating a large fraction of non-coupled RyRs (scale bar equals 10 µm).*

Table 1. RyR subpopulations in healthy left ventricular cardiomyocytes

Ref.	Species	Preparation	Identification method	Terminology	Fractions of RyRs	Distance to membrane
[110]	Human	Single cardiomyocytes (LV)	T_{F50} of CaT to identify distance to membrane	Coupled vs. Non-coupled	~ 70% coupled sites, ~ 10% non-coupled sites	Coupled RyRs < 0.5 µm, Non-coupled RyRs > 2 µm from membrane
[111]	Human	Tissue sections	% colocalization of RyR with Cav1.2	Coupled	~ 51% RyR cluster in couplon	-
[112]	Human	Tissue sections	% colocalization of RyR with TT (WGA-labelling)	-	~ 32% of RyR cluster colocalization with TT	-
[113]	Human	Tissue sections	% colocalization of RyR with TT (WGA-labelling)	Junctional vs. Non-junctional	~ 77% junctional sites, ~ 23% non-junctional sites	Junctional RyRs < 1 µm, Non-junctional RyRs > 1 µm from membrane
[107]	Human	Tissue sections	RyR distance histogram in relation to distance to SL membrane (immunostaining)	-	~ 41% of RyRs between 0-0.6 µm from SL ~ 15% of RyRs between 0.6-1 µm from SL ~ 13% of RyRs between 1-1.4 µm from SL ~ 31% of RyRs between 1.4-4 µm from SL	-
[104]	Pig	Single cardiomyocytes (LV)	T_{F50} of CaT to identify distance to membrane	Coupled vs. Non-coupled	~ 50% coupled sites, ~ 22% non-coupled sites	Coupled RyRs < 0.5 µm, Non-coupled RyRs > 2 µm from membrane
[114]	Pig	Single cardiomyocytes (LV)	T_{F50} of CaT to identify early and delayed release sites	Early vs. Delayed	~22% of delayed sites	-

[115]	Pig	Single cardiomyocytes (LV)	T_{F50} of CaT to identify early and delayed release sites	Early vs. Delayed	RyR fractions have not been quantitatively analysed	-
[106]	Pig	Tissue sections	RyR distance histogram in relation to distance to SL membrane (immunostaining)	-	~ 47% of RyRs between 0-0.5 µm from SL ~ 21% of RyRs between 0.6-1 µm from SL ~ 16% of RyRs between 1-1.4 µm from SL ~ 31% of RyRs between 1.4-4 µm from SL	-
	Pig	Single cardiomyocytes (LV)	T_{F50} of CaT to identify distance to membrane	Coupled vs. Non-coupled	RyR fractions have not been quantitatively analysed	Coupled RyRs < 0.5 µm, Non-coupled RyRs > 2 µm from membrane
[116]	Dog	Tissue sections	% of RyR in relation to distance membrane (WGA labelling)	Junctional vs. Non-junctional	~ 16% of non-junctional sites	Junctional RyRs < 1 µm, Non-junctional RyRs > 1 µm from membrane
[117]	Dog	Single cardiomyocytes (LV)	RyR distance histogram in relation to distance to SL membrane (WGA labelling)	-	~ 60% of RyRs between 0-0.5 µm from SL ~ 23% of RyRs between 0.5-1 µm from SL ~ 11% of RyRs between 1-1.5 µm from SL ~ 6% of RyRs between 1.5-4 µm from SL	-
[103]	Dog	Single cardiomyocytes	Median time to max. rate of rise of CaT	Coupled vs. Uncoupled	~ 46% coupled sites, ~ 48% uncoupled sites	Coupled < 1.2 µm, Uncoupled > 1.2 µm from membrane
[118]	Rabbit	Single cardiomyocytes	% of RyR in relation to distance membrane (Di-8-ANEPPS labelling)	Junctional vs. Non-junctional	~ 52% junctional sites, ~ 48% non-junctional sites	Junction RyRs < 0.5 µm, Non-junctional RyRs > 0.5 µm from membrane
[93]	Rat	Single cardiomyocytes	% colocalization of RyR with Cav1.2	Coupled vs. Extradyadic	~ 65% RyR clusters in couplon, 35% extradyadic clusters	-
[119]	Rat	Single cardiomyocytes (LV, RV)	T_{F50} of CaT to identify early and delayed release sites	Early vs. Delayed	~ 75% early sites, ~ 25% delayed sites in LV, ~ 60% early sites, ~ 40% delayed sites in RV	-
[120]	Rat	Single cardiomyocytes	T_{F50} of CaT to identify early and delayed release sites	Early vs. Delayed	~ 4% of delayed sites	-
[121]	Rat	Single cardiomyocytes	% colocalization of RyR with Cav1.2	Coupled vs. Orphaned	~ 55% RyR clusters in couplon	-
[122]	Mouse	Single cardiomyocytes	T_{F50} of CaT to identify early and delayed release sites	Early vs. Delayed	~ 9% of delayed sites	-

Notably, emerging data shows that the localisation of RyRs within a CRU or within a specific microdomain is not fixed. Phosphorylation and cytosolic levels of Mg^{2+} [123,124] as well as junctional proteins associated with the RyR channel can influence the arrangement of RyRs within a CRU or lead to cluster dispersion. The overexpression of JPH-2, a protein that anchors TATS to the SR and interacts with the RyR, is associated with larger RyR clusters [125]. Interestingly, BIN-1 another junctional protein associated with TATS growth and structure [126], is involved in the formation of CRUs [127] as well as in the rapid translocation of phosphorylated RyRs to the dyad minutes after exposure to adrenergic stimulation [46]. Therefore, it is the combination of different elements that will define RyR microdomains.

5. Location, cluster organization and post-translational modifications converge to form distinct RyR microdomains within cardiomyocytes

Evidence gathered by sophisticated imaging, functional and modelling studies support the concept that within cardiomyocytes, RyR function is dependent on the microdomain in which they reside.

Initially, the lack of specific tools to identify RyR populations during dynamic Ca^{2+} studies has limited the identification of specific RyR subpopulation within the same cell during live cell imaging. Coupled and non-coupled RyR subpopulations were at first studied solely by their temporal Ca^{2+} release [105,115,128,129]. Later imaging algorithms validated these approaches by concurrent TATS imaging and more accurately defined unique temporal and spatial Ca^{2+} release profiles identifying RyR subpopulations [103,104].

Comparative studies in atrial versus ventricular cardiomyocytes, and within ventricular cardiomyocytes from larger mammals, have revealed critical differences between coupled and non-coupled RyR function [105,130–133]. Non-coupled RyRs not only have delayed activation times, but also different kinetics of release and are considered to play a secondary role in the amplification of Ca^{2+} releases from coupled RyRs [105,106,108,118]. In the absence of a dyadic cleft that restricts diffusion, local Ca^{2+} may not rise to the same levels. Computational approaches have been major tools to assess RyR function in microdomains taking into account the dyadic environment as well as cluster size and configuration [134–136]. Modelling has provided insights into the relationship between distance between coupled and non-coupled receptor clusters and recruitment of non-coupled RyRs by Ca^{2+} release via coupled receptors [106]. More recently, genetically-targeted encoded probes allow for study of local signalling near RyRs within the dyad, including during ECC [47,137,138].

The actual [Ca^{2+}] in the dyadic cleft has been estimated to be at least 10 and up to 100-fold higher than in the bulk cytosol during RyR Ca^{2+} release [139,140]. This high dyadic [Ca^{2+}] is an important modulator of the local LTCC and NCX, contributing to the beat-to-beat variability of AP duration [141]. The presence or absence of dyadic Ca^{2+} microdomains also impacts on local signalling cascades. It is therefore anticipated that Ca^{2+}-dependent signalling proteins will be activated to a different extent in coupled and non-coupled RyRs. Indeed, increased activation of signalling proteins such as CaMKII is detected near coupled RyRs, where local $[Ca^{2+}]_i$ is higher than the bulk cytosol [104,139,142]. Work by Dries et al. showed that coupled RyRs in healthy ventricular cardiomyocytes are modulated by CaMKII, which is dependent on local Ca^{2+} and ROS microdomains [104,143] (Figure 4). Conversely, Belevych et al. reported opposite results with non-coupled RyRs being modulated by CaMKII in control ventricular cardiomyocytes from dogs [103]. The reasons behind these differences were not clear, but are most likely attributed to species- (pig vs. dog) and experimental-dependent differences (difference pacing conditions and identification methodology of RyR subpopulations). Similarly to CaMKII, direct effects of ROS on RyRs and/or RyR modulating proteins, are most likely restricted to a local microdomain as a consequence of low diffusion capacity of ROS and RNS [69].

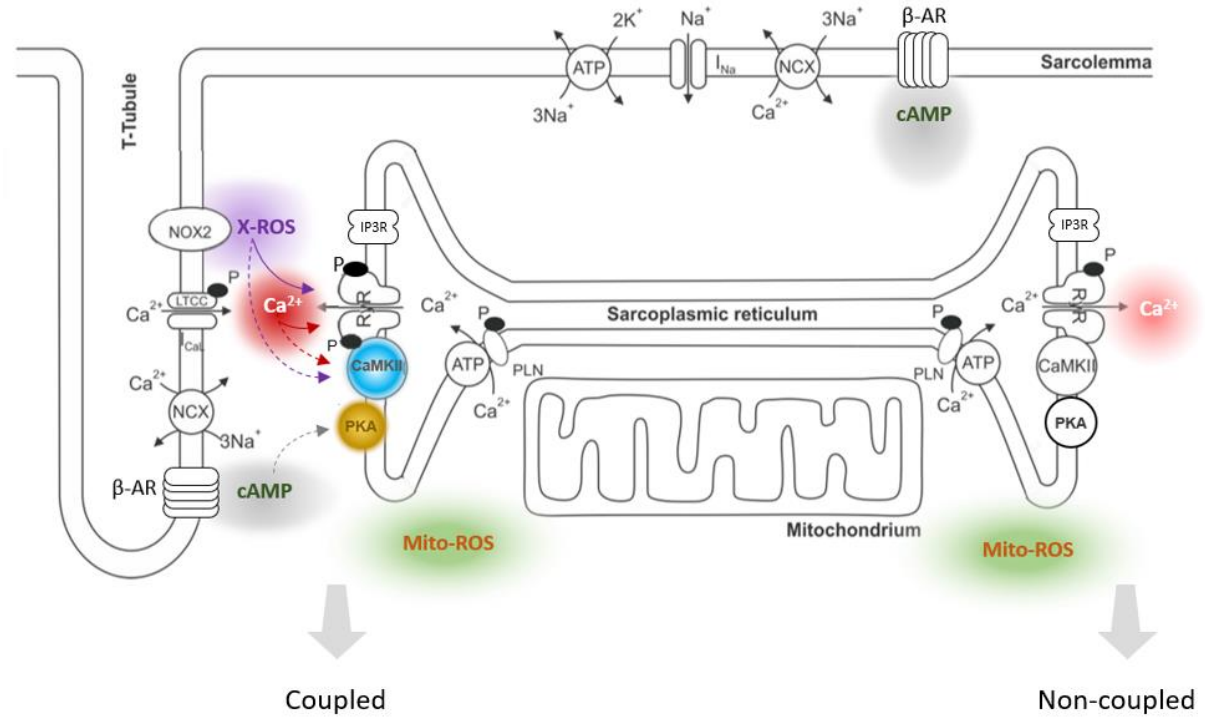

Figure 4. Schematic presentation of RyR microdomains within cardiomyocytes*. Coupled RyRs reside within the dyadic cleft near TATS, non-coupled RyRs are outside the dyadic cleft. The cellular ultrastructure defines local signalling within RyR microdomains. Local ROS (purple) by NOX2, high [Ca^{2+}] (red) and [cAMP] (grey) modulate coupled RyR function, either directly (solid arrows) or indirectly via kinase activation (dashed arrows) such as CaMKII (blue) and PKA (yellow) [104,143].*

A special subdomain of the TATS and SL location of RyR clusters, is the intercalated disc with its intensely folded membrane. Here RyR associate with connexin hemichannels, which are modulated by SR Ca^{2+} release by RyR but also influence RyR function [144].

As for many of the players involved in ECC, RyR activity is significantly influenced by cAMP generated downstream of β-adrenergic receptor (β-AR) stimulation. In this regard, cAMP acts via PKA to phosphorylate the RyRs increasing its P_o. Coupled RyR activity is particularly influenced by the local concentration of cAMP (Figure 4). Indeed, cAMP microdomains generated through the interplay between the production of cAMP and cAMP degrading enzyme (PDE) determines the local cAMP concentration and signalling near coupled RyRs. Emerging work revealed these localized cAMP compartments near coupled RyRs in healthy cardiomyocytes, depend on β-AR1 signalling [145].

Interfibrillar mitochondria are also in close proximity to the SR and RyRs [146,147] and can modulate RyR function through their uptake of Ca^{2+} via the mitochondrial Ca^{2+} uniporter (MCU). Functional MCU are enriched at the mitochondria-junctional SR interface and bring mitochondria within 40 nm of RyR clusters [148,149]. When overloaded with Ca^{2+}, mitochondria produce excessive ROS (i.e. mito-ROS) that can modulate RyR function directly by oxidizing cysteines or indirectly through RyRs phosphorylation via oxidized CaMKII. In the absence of adrenergic stimulation, coupled and non-coupled RyRs are not affected by mito-ROS [110], but in the presence of adrenergic stimulation, mito-ROS production as a result of increased energy demand will contribute to SR Ca^{2+} leak [67]. Moreover, subsarcolemmal and perinuclear mitochondrial function may also be regulated by the cross-talk with RyR and mitochondrial Ca^{2+} uptake during Ca^{2+} release.

It is thus evident that both cellular ultrastructure through generating physically compartmentalised cellular domains, and the macromolecular composition of molecules embedded in their restricted space, determine the modulation of RyRs and thereby microdomains. Conventionally, it was assumed that RyRs and their associated proteins are statically restricted within a microdomain. However, more recent studies demonstrate the mobility of RyR clusters and their modulators, which allows for local signals to be easily amplified and transduced across microdomains [46,150,151].

6. Altered RyR microdomains in heart failure

Heart failure (HF) is defined as dysfunction of the heart pump to provide blood to organs because of contractile failure. Most often, the contractile failure can be diagnosed by a reduced ejection fraction (HFrEF) and the most common cause of HFrEF is ischemic heart disease. In HFrEF, the role of altered ECC and Ca^{2+} handling is well established [152]. The

syndrome of pump failure predominantly due to ventricular filling problems in diastole, and with preserved EF (HFpEF), is increasingly prevalent and shows alterations in cell architecture and Ca^{2+} handling, but is less well studied with regard to RyR function [153]. While in HFrEF an increased diastolic SR Ca^{2+} leak leads to reduced SR content and lower Ca^{2+} transients in conjunction with reduced TATS, HFpEF features non-homogeneous impairments in Ca^{2+} handling, with either increased or preserved TATS dependent on etiology [153,154].

Here we use the term HF, in reviewing data obtained for HFrEF, for which alterations in RyR function have been studied in depth. Early reports referred to changes in expression at tissue level [155], but subsequent focus was on altered RyR function. In HF, cardiomyocytes are continuously exposed to oxidative stress and elevated sympathetic tone, which promote SR Ca^{2+} leak through hyperactive RyR channels [4,156]. As a result, luminal SR [Ca^{2+}] and SR Ca release during ECC are reduced, leading to reduced contractility. Such abnormal diastolic Ca^{2+} release also is involved in the increased incidence of arrhythmias in HF and the underlying RyR dysfunction can be attributed to both structural and functional adaptations.

6.1 Altered signalling and modulation of RyR activity

The elevated sympathetic tone in HF will alter phosphorylation of RyRs to increase SR Ca^{2+} leak, and many studies have correlated increased phosphorylation with RyR dysfunction at cell, tissue and organ level. Early studies in failing human hearts reported that PKA-induced hyperphosphorylation of Ser2808 was associated with the dissociation of FKBP12.6 from RyR with subsequent increased channel P_o [26], but this data has been contested [157]. Others found that Ser2808 was constitutively phosphorylated under basal conditions (i.e. in the absence of β-adrenergic stimulation or PKA activation) [56,158,159], or contested the importance of PKA phosphorylation at Ser2808 in cardiac dysfunction [160,161]. There is also possibly a role for Ser2030 phosphorylation [55]. Mice deficient in Ser2030 phosphorylation show reduced ECC gain and spark activity suggesting that PKA dependent phosphorylation of this site is required for the full effects of β-adrenergic stimulation [62]. Recent insights present PKA-dependent phosphorylation of Ser2030 as a regulator of CaM-dependent Ca^{2+} release termination and alternans induced during increased heart rate with β-adrenergic stimulation [162].

More consistent data have accumulated on the effect of CaMKII phosphorylation at Ser2814. Phosphorylation of Ser2814 has been commonly associated with an increase in diastolic SR Ca^{2+} leak, ventricular arrhythmias and HF [58,163–167]. Another mechanism is loss of SPEG inhibition of phosphorylation, which also increase RyR activity [60], yet a complete

understanding of SPEG-induced phosphorylation in pathophysiological conditions warrants further research.

Protein phosphatases associated with RyRs could be culprits as well as potential targets to modify RyR phosphorylation in HF. In animal HF models and in human HF, the expression and activity of PP1 is increased [168–171], yet the hyperphosphorylated state of RyRs in HF leaves the functional importance of increased PP1 expression unclear. Alterations in the regulatory subunit of PP1 impair the association between PP1 and RyR [63,172], leaving RyRs in a hyperphosphorylated state. Similarly for PP2A, reduced expression of the regulatory subunit B56α is associated with increased RyR phosphorylation and arrhythmogenesis [38]. Inhibitor-1, modulating phosphatase activity could be an indirect modulator of phosphorylation in HF [173,174].

In addition to phosphorylation, oxidative stress modulates RyR function. During HF, the redox state of the cell is disturbed because of altered signalling pathways to meet the increase in energy demand. This includes the switch from fatty acid to glucose metabolism, aberrant mitochondrial function with increased ROS production, the upregulation and uncoupling of NOS, the upregulation of xanthine oxidase and NOX as well as the decrease in glutathione [69,175–178]. Many labs demonstrate increased RyR activity when ROS are elevated, resulting in an increased Ca^{2+} spark frequency [65,179] and Ca^{2+} waves [176,180]. The dissociation of FKBP12.6 [181] or CaM [182,183] upon RyR oxidation is also proposed in HF cardiomyocytes as well as an indirect increase in RyR activity by the amplification of ROS-regulated CaMKII and PKA activation [184–186].

6.2 Altered distribution of RyR clusters define new microdomains

In HF, hypertrophy and remodelling of cardiomyocytes affects the TATS. While the TATS becomes lower in density and organisation, RyRs remain evenly distributed at the Z lines, resulting in a larger fraction of RyRs that are no longer in close proximity with LTCCs. An increase in ‘orphaned’ or non-coupled RyRs is seen in various animal models [110,117,121] and human [107,110,113], and can make up to 40-50% of all RyRs. This altered distribution leads to spatially and temporally dyssynchronous Ca^{2+} release, slower Ca^{2+} transients and contributes to contractile dysfunction in these cardiomyocytes [114,117,121,122,187,188].

Remodelling of the TATS also affects RyR modulation through proteins associated with RyR at the dyadic cleft. A growing body of evidence demonstrates a role for BIN-1 [126,189], JPH-2 [96] and more recently nexilin (NEXN) [190] and cardiomyopathy associated protein 5 (CMYA5) [191] in TATS remodelling that in turn affect RyR function in disease. Within the TATS and dyadic RyR populations, alterations in LTCC [192,193] and redistribution of β-adrenergic receptors [194] will affect coupled RyRs.

In human HF and post-MI pig cardiomyocytes we found that the normal phosphorylation and NO-dependent increased activity of coupled RyRs with higher heart rates was lost [110]. Facilitation of spontaneous RyR activity by Ca^{2+} released from proximal IP_3R, is a recent mechanism identified in post-MI and HF that affects differentially coupled and non-coupled RyRs [106,107] (Figure 5).

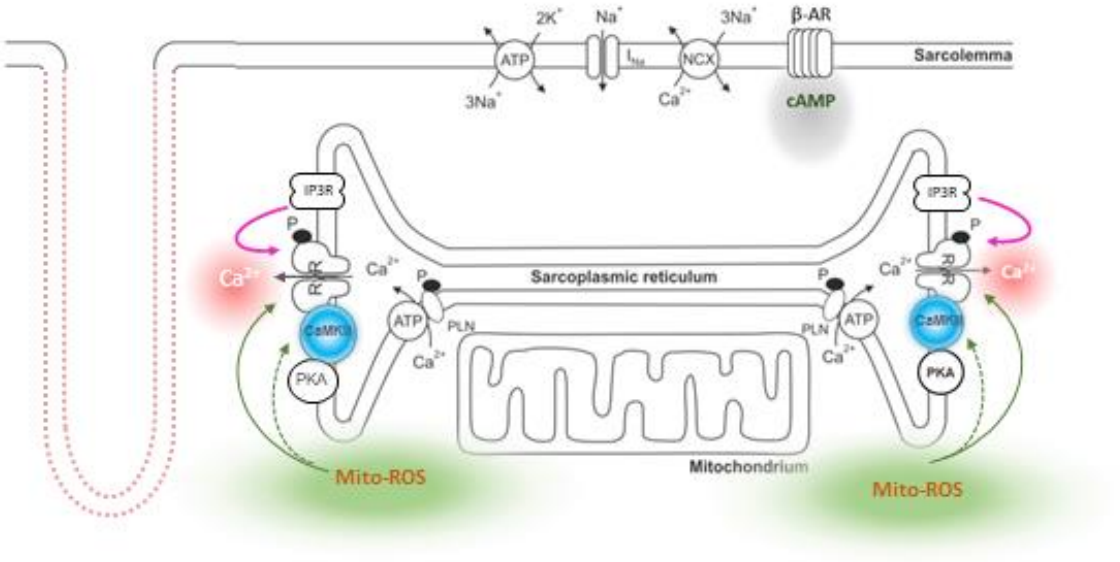

Figure 5. Structural remodelling of cardiomyocytes in cardiac disease. *Remodelling of the TATS and associated signalling molecules lead to an increase in uncoupled RyR with altered microdomain. Loss of the TATS (indicated in red dotted line) increases the fraction of non-coupled RyRs. The absence of the TATS is associated with altered local signalling: altered local [Ca^{2+}] and Ca^{2+} crosstalk with IP3R, shift in redox balance (loss of NOX2-mediated ROS and ROS scavengers and increased mito-ROS).*

Beyond the macroscopic organization of the TATS, with the consequent RyR re-distribution and with the alterations in associated proteins, there is a remodelling at the nanoscale of RyR clusters in HF. Electron tomography [123] and super-resolution microscopy [88,195] has identified changes in CRU organization. RyR cluster density can be reduced, with reduction apparent at junctional regions [96,111]. RyRs can become disorganized with irregular clusters of variable size and density [52,86,88,90,91,196]. Large RyR clusters (>75) have an increased probability of diastolic Ca^{2+} sparks [91], yet remodelling in HF results in dispersed RyR clusters, with increased silent Ca^{2+} leak and slowed Ca^{2+} sparks [196]. Dispersion contributes to more sparks that travel between RyR clusters [97]. Furthermore, acute exposure to adrenergic signalling can also modulate RyR cluster formation, with more widely spaced RyR tetramers having increased SR Ca^{2+} release [52], and Ca^{2+} dynamics in individual RyR clusters, giving rise to sparks with a greater signal mass [97,102]. Conversely, prolonged adrenergic stimulation results in a progressive dispersion of RyR clusters, with low spark fidelity, slow kinetics and reduced magnitudes [124]. The authors hypothesized that the dispersed RyR cluster arrangement could be a protective mechanism during prolonged

adrenergic activation as seen in HF with a reduced ability to trigger Ca^{2+} waves. Moreover, RyR cluster rearrangement upon phosphorylation is spatially restricted, with larger RyR clusters located near the surface membrane [52,124].

7. Is there a distinct microdomain harbouring arrhythmogenic RyRs?

The increase in number of non-coupled RyRs, together with a different regulation and cluster make-up of coupled and non-coupled RyRs, leads to the hypothesis that there are distinct RyR microdomains implicated in the increased susceptibility for triggered arrhythmias in HF. Yet, despite the large number of studies on RyR-induced arrhythmogenesis, the evidence remains limited, as most studies have used mouse and rat models that are not well suited to address this question. In cardiomyocytes from human HF and pig with post-MI remodelling, different RyR populations can be identified and studied as a source for Ca^{2+} waves and related after depolarizations [110]. Concomitant with a loss of TATS, non-coupled RyRs were primed by CaMKII to be at the origin of spontaneous Ca^{2+} release and waves, leading to DADs and triggered action potentials. Also Ca^{2+} mediated cross-talk between IP_3R and RyR was enhanced at non-coupled sites, which was associated with increased DADs and potential for arrhythmia generation [106]. These findings contrast with findings in a canine HF model in which TATS were preserved [103]. In this study, arrhythmogenic RyRs, primed by CaMKII, were located within the dyad, and are in line with the properties of coupled RyRs seen in healthy pig cardiomyocytes with preserved TATS. These results suggest that TATS remodelling is orchestrating location or properties of RyR modulators involved in the proarrhythmic-priming of RyR such as CaMKII. Whereas the dyad would form a site with limited diffusion for Ca^{2+} and consequently CaMKII activation, redox modification, including of CaMKII, in non-coupled sites may become more important in HF.

Intriguingly, cardiomyocyte cell types naturally devoid of TATS such as Purkinje cells exhibit profound proarrhythmic potential [197,198]. While TATS is absent or only rarely observed, SR Ca^{2+} release channels remain homogeneously distributed along the Z lines in Purkinje cells [199,200]. Immunostaining of these cells revealed a complex spatial organization of their SR Ca^{2+}-release channels in different subcellular compartments: IP_3R are directly located near the SL membrane, RyR3 and RyR2 are in the subsarcolemmal space and in the deeper cellular core devoid of SL/TATS membranes [200,201]. This unique structure in Purkinje cells implies that most RyRs are non-coupled. Interestingly, Purkinje cells are more prone to develop DADs [202] compared to ventricular cardiomyocytes [203,204]. Moreover, in Purkinje cells from MI animals, Ca^{2+} waves originated more frequently from the subsarcolemmal space and core - regions where non-coupled RyR2s and RyR3s are located. Together these data support a concept that in the absence of TATS, such as in

Purkinje cells and after MI remodelling, non-coupled RyRs may harbour an increased potential to initiate arrhythmias because they are more prone to spontaneous Ca^{2+} release. In addition, Ca^{2+} release at non-coupled sites may diffuse more easily to recruit additional RyRs and create cell-wide Ca^{2+} waves that elicit larger NCX currents and afterdepolarizations triggering AP. Lower levels of stabilizing K^+ currents will further facilitate this process, and may be of particular relevance during adrenergic stimulation, exacerbating arrhythmogenic RyR Ca^{2+} release [205].

Taken together, there is encouraging evidence to further explore the potential for microdomain-specific targeting of RyRs in the onset and perpetuation of arrhythmias.

8. Perspectives for future studies

Deeper investigation of RyR microdomains will require relevant models for study, ideally human and large animal models [206] as well as new and improved tools.

The gain in knowledge summarized above was possible due to new advanced imaging techniques and the development of specific biosensors. Ca^{2+} biosensors, such as GCaMP6f (and its more recent derivatives with more relevant kinetic properties [207]), targeted to junctional proteins (i.e. triadin, junctin) allow for selective visualization of nanodomain Ca^{2+} signalling in the dyadic space [47,137,138]. However, the overexpression of these transgenes typically relies on the fusion of the sensor with the target protein, which could affect local signalling environments, such as altered protein interactions and imbalanced stoichiometry. More direct study of signalling may become possible with the new class of biosensors called Fluorescent Sensors Targeted to Endogenous Proteins (FluoSTEPs) [208,209]. These biosensors can be exclusively reconstituted at an endogenously expressed protein of interest within a specific microdomain and could serve for the development of targeted ROS biosensors.

Another area of interest is the identification of currently unknown molecular targets that selectively characterize RyRs in specific microdomains. As a reference, membrane markers to identify coupled RyRs could be employed. Novel technologies, such as phospho-specific proteomics [210,211] and proximity proteomics [212–214] could help to unravel unique molecular signatures of different RyR subpopulations. Of interest is the novel technique of proximity-dependent biotinylation (BioID). This technique facilitates biotinylation of molecules in close proximity (up to 10 nm) to the protein of interest that is fused in frame to a biotin protein ligase. Such covalent labelling of biotin can survive through harsh lysing conditions which enable proteins from even poorly soluble intracellular compartments, such as membranes or the nuclear lamina to be purified and further identified by mass spectrometry

[215]. BioID revealed the composition of different protein complexes and the spatial organization of several membrane-bound organelles [216–219]. This technique characterised proximity-binding partners of JPH-2 in vivo [220], and identified new proteins involved in dyadic architecture [191]. Moreover, recent improvements allow for reduced labelling time, increased cell permeability, reduced toxicity and targeting of post-translation modifications (i.e. TurboID) [214,221], and provides a new strategy to delineate the unique molecular fingerprints of RyRs in various cellular compartments.

For live cell studies, fluorescence resonance energy transfer (FRET)-based biosensors are particularly suitable. Novel FRET biosensors with high sensitivity (CUTie, cAMPFIRE) [222,223] can be targeted to key proteins involved in ECC [223], in membrane fractions with different lipid compositions [224,225] as well as near SERCA [226] and RyR [227]. Likewise, visualization of activated CaMKII is reported by the FRET-based sensor Camui, which showed the highest activity at sarcomeric Z-lines in quiescent cells [228], that rapidly translocated to extradyadic spaces upon electrical stimulation [151]. More recently, a novel CaMKII biosensor (FRESCA) has been developed that allows visualization of endogenous CaMKII activity [229].

Further advances in microscopic imaging push study of subcellular structures to higher resolution. Serial block face scanning electron microscopy in 3D and new tissue preservation techniques unravel nanoscopic structure of the dyad and improved immunolabelling techniques combined with super-resolution or expansion microscopy, allow identification of RyR clusters with nanometre precision and at the single channel level [96,98,124,195]. The development of MINFLUX imaging will further advance super-resolution imaging with resolutions up to 1 nm precision [230].

Finally, computational modelling could assist in extrapolating experimental data to better understand the inter- and intra-connectivity of Ca^{2+} handling within subcellular compartments [106,134,231,232] as well as to suggest new hypotheses.

Summary and conclusions

The organization of RyR in clusters, at different subcellular location and with variable association with signalling and modulatory proteins, defines specific RyR microdomains in cardiomyocytes. These microdomains respond differently to environmental stress and remodelling in HF, where we see an increase in non-coupled RyR sites with higher arrhythmogenic potential. Reducing the number of non-coupled RyR sites, and their arrhythmogenic features thus emerge as areas of interest for translational studies.

Restoration of TATS has been demonstrated in diseased cardiomyocytes [117,233], but is less effective in advanced end-stage HF [234]. A better understanding of TATS biogenesis could assist in identifying critical mediators [235,236]. Similarly, the improved knowledge of RyR cluster generation, composition, and (re)arrangements will further assist these targeting strategies [96].

The aim of reducing genetically encoded arrhythmogenic RyR activity has stimulated efforts for designing anti-arrhythmic drugs that bind directly to RyR [237–239], but their use in HF with more heterogeneous RyR microdomains needs further study. For HF, targeting RyRs in specific microdomains is a strategy to be explored, as better tools for study become available.

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