Calcium in Arrhythmogenic Cardiomyopathy

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Arrhythmogenic cardiomyopathy (ACM) is an inherited heart disease characterized by sudden death in young people and featured by fibro-adipose myocardium replacement, malignant arrhythmias, and heart failure. The Ca2+ toolkit is heavily remodeled in cardiomyocytes derived from a mouse model of ACM defective of the desmosomal protein plakophilin-2. Furthermore, ACM-related mutations were found in genes encoding for proteins involved in excitation–contraction coupling, e.g., type 2 ryanodine receptor and phospholamban.

Keywords: arrhythmogenic cardiomyopathy ; Ca2+

1. Introduction

The heart is a highly specialized machine that requires fine regulation: intracellular changes and cell-to-cell communication disturbances could destroy this balance. Depending on the type of cardiac arrhythmia, a series of events may occur: intracellular dysfunction–tissue remodeling–impaired cell–cell connection–electrical dysfunction. Loss of cardiac tissue integrity could be both a cause and an effect of arrhythmic phenotype. A typical example is arrhythmogenic cardiomyopathy (ACM), a cardiac disorder in which mutations in proteins of the desmosome may be present, causing cell-to-cell discontinuity, arrhythmic events, and myocardial fibro-adipose substitution [1][2][3].

Intracellular calcium (Ca^{2+}) signals drive excitation–contraction (EC) coupling and are, therefore, necessary for the heart to effectively pump blood into the pulmonary artery and aorta. It is, therefore, not surprising that any defect in the Ca^{2+} cycling machinery or in the complex network of Ca^{2+}-related proteins that maintain cardiac Ca^{2+} homeostasis under tight control may severely compromise cardiac function. Alterations or mutations in the Ca^{2+} handling machinery have been associated with a number of severe cardiac diseases, including cardiac hypertrophy, dilated cardiomyopathy, and heart failure [4][5], and several inherited arrhythmia syndromes (i.e., Timothy syndrome, Brugada syndrome, and early repolarization syndromes) [6][7][8].

2. Calcium Signaling and Cardiac Arrhythmias: A Tight Connection

Cardiac contraction is triggered by a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by membrane depolarization according to a process known as EC coupling [6][7][9]. Briefly, as the action potential, triggered by an influx of sodium (Na^+) via the voltage-gated Na^+ channels, propagates along the T-tubules, membrane depolarization opens CaV1.2, which is the pore-forming α-subunit of L-type voltage-gated Ca^{2+} channels (VGCCs), thereby causing the influx of Ca^{2+} into the dyadic junction, which separates the sarcolemma from the closely apposed (~15 nm) junctional sarcoplasmic reticulum (SR) [10] in ventricular myocytes [9]. Extracellular Ca^{2+} influx causes a large increase in local $[Ca^{2+}]_i$, which activates a cluster (7–20) of type 2 Ryanodine receptors (RYR2) in a process known as Ca^{2+}-induced Ca^{2+} release (CICR) and gives rise to significant Ca^{2+} release events from the junctional SR (called Ca^{2+} sparks). The temporal summation of these local events by the propagating action potential results in a regenerative Ca^{2+} transient that is detected by troponin C to initiate the sliding of the thick (myosin) and thin (actin) filaments, cell shortening, and hence pressure development within each ventricle and ejection of blood into the circulation during cardiac systole [6][7][9]. Subsequently, $[Ca^{2+}]_i$ drops and mechanical force relaxes quickly to pre-systolic levels during cardiac diastole, which is essential to enable cardiac chambers to refill with blood. The increase in $[Ca^{2+}]_i$ is indeed short-lived as cytosolic Ca^{2+} is extruded across the plasma membrane by the Na^+/Ca^{2+} exchanger (NCX1) and the plasmalemmal Ca^{2+}-ATPase (PMCA) and sequestered back into the SR by the sarco-endoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) [6][7][9]. The Ca^{2+} affinity of SERCA2a is regulated by the phosphoprotein phospholamban (PLN), which increases or reduces the pumping rate of SERCA2a depending on its phosphorylation state. In the dephosphorylated state, PLN binds to SERCA2a and inhibits the Ca^{2+} transport ability of the pump by reducing its apparent affinity for Ca^{2+}. However, when PLN is phosphorylated, it dissociates from SERCA2a, thereby increasing the pump's affinity for Ca^{2+} and favoring relaxation [11]. PLN presents two sites of phosphorylation: Ser16 for protein kinase A (PKA) and Thr17 for

Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) and protein kinase B (AKT) [12]. Importantly, β-adrenergic stimulation exerts a profound impact on cardiac Ca^{2+} handling by modulating CaV1.2, RYR2 and SERCA2a activity. Accordingly, stimulation of β-adrenergic receptors, e.g., with isoproterenol, engages PKA and CaMKII. PKA, in turn, phosphorylates CaV1.2 and PLN, thereby increasing the amplitude of L-type voltage-gated Ca^{2+} currents and boosting SR Ca^{2+} uptake. Furthermore, Bovo and colleagues recently demonstrated that PKA-dependent phosphorylation also regulates RYR2-mediated SR Ca^{2+} release independently of PKA effects on SR Ca^{2+} load [13].

3. Arrhythmogenic Cardiomyopathy as Adhesion Disorder: Ca^{2+}-Dependent Desmosomes' Stability

Cell to cell junctions are essential to confer stability to tissues, especially those undergoing continuous mechanical stretch, such as the heart and skin. The myocardium tissue integrity is based on desmosomes, anchoring cell junctions that are assembled in strong and highly specialized complexes. Loss or mutations of cell junctions are associated with human genetic diseases and ACM [14][15][16][17]. Desmosomes consist of specific cadherins, i.e., DSC2 and desmoglein-2 (DSG2), which act like a bridge to join the lateral edges of neighboring cells. The desmosomal cadherins bind proteins of the armadillo family, PG and PKP2, that are anchored to desmoplakin (DSP), the main intracellular component responsible for the adhesion to the intermediate filament network [18].

Desmosomes undergo a transition from hyper- to low-adhesion states depending on extracellular Ca^{2+} levels. The Ca^{2+}-independent adhesive state of desmosomes is referred to as hyper-adhesion-state since it represents a higher-affinity and more stable binding ability both for desmosomes and adherens junctions. During tissue remodeling, wounding, and cell migration, a lower-affinity adhesive state is required and desmosomes adopt the Ca^{2+}-dependent state, losing their organized structure [19][20][21] (**Figure 1**). This transition does not involve protein composition rearrangement, but it can influence the cadherin packing. Based on the nature of molecules underlying the desmosomal adhesion, both the formation and disruption are Ca^{2+}-dependent mechanisms [22][23][24]. The desmosomal cadherins, DSC2 and DSG2, and the classical cadherin, E-cadherin, represent the Ca^{2+}-dependent components of desmosomes and adherens junctions, respectively [25]. In vitro, the Ca^{2+}-dependent mechanism is linked to the culture confluence state: in a confluent cell monolayer, desmosomes are Ca^{2+}-independent until the confluence is destroyed by wounding the cell sheet, thereby resulting in the propagation of Ca^{2+} dependence through the whole monolayer [19].

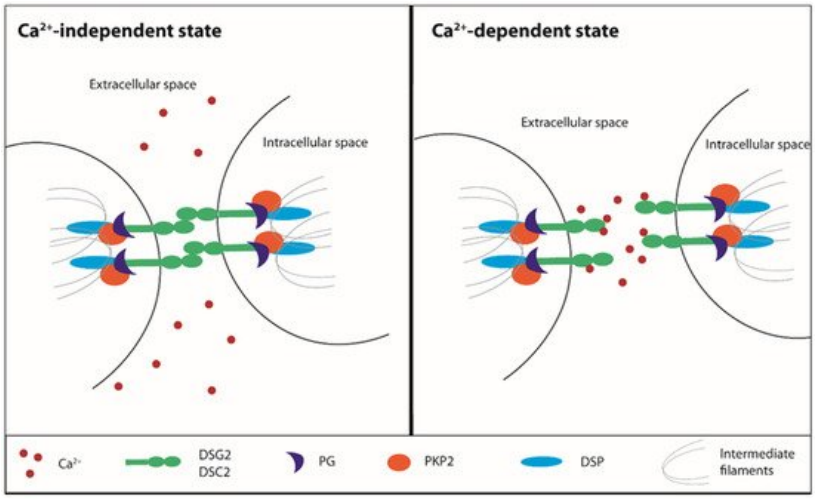

Figure 1. Ca^{2+}-dependent desmosomes stability. Ca^{2+}: calcium, DSG2: desmoglein 2, DSC2: desmocollin 2, PG: plakoglobin, PKP2: plakophilin 2, DSP: desmoplakin.

4. Desmosomal Mutations and Arrhythmogenic Cardiomyopathy: Derangement of the Ca^{2+} Toolkit as a Consequence Rather than a Pathogenic Defect

The mechanism that induces the reduction of adhesion during tissue remodeling is not completely clear, but desmosomes can be totally internalized by cells. After the internalization, they are degraded or disassembled in order to recycle their component proteins [26][27]. During the Ca^{2+}-dependence state, the desmosomes undergo a conformational rearrangement of the cadherin extracellular domains that induces a less organized structure and a more easily adhesive binding disruption [28]. The molecular basis of the adhesion between desmosomes involves the activation of protein kinase C (PKC) or inhibition of protein phosphatases [20][21]. The treatment with a low-Ca^{2+} medium induces loss of intercellular adhesion and the formation of half-desmosomes: the broken desmosomes are internalized by a PKC-dependent

mechanism and transported to the centrosome, where they undergo proteasomal and lysosomal degradation [27]. The central role of PKCα is supported by experiments performed on a mouse model lacking or overexpressing this kinase. Both in absence and following inhibition of PKCα, the switch to Ca^{2+}-dependence is blocked and hyperadhesive desmosomes lock the cells together, preventing cell motility and epithelial migration. On the contrary, in mice overexpressing a constitutively active PKCα, a rapid transition to a Ca^{2+} dependence state make desmosomes weakly adhesive, thus facilitating cell mobilization and promoting re-epithelialization [29].

Desmosomal mutations have been identified as a crucial determinant of ACM since the late 1980s [30]. A homozygous truncating mutation in the *JUP* gene, encoding for PG, was first identified through genetic linkage analysis as a typical syndromic form of right-sided ACM, known as Naxos disease [31]. Subsequently, genetic analysis revealed that another desmosomal gene, *DSP*, encoding for desmoplakin, harbored a homozygous truncating mutation in ACM in patients with a cardiocutaneous syndrome, named Carvajal syndrome [31][32]. These earlier discoveries sparked the search for causative mutations in additional desmosome genes in ACM patients and led to the identification of either missense or truncating mutations in the following desmosome genes: *PKP2* (encoding for plakophilin 2), *DSC2* (encoding desmocollin 2), and *DSG2* (encoding for desmoglein 2) (**Table 1**) [31]. It has been estimated that mutations in desmosomal genes are present in approximately half of ACM patients, with *PKP2* being the most commonly affected gene in adults [31]. The molecular mechanisms whereby *PKP2* mutations cause ACM are yet to be fully clarified, but no change in the Ca^{2+}-sensitivity of the desmosomal junctions has been reported [31][33]. Conversely, desmosomal mutations can affect the electrical activity of the heart by compromising the expression, localization, and/or function of other components of the 'connexome', such as NaV1.5, the α subunit of voltage-gated Na^{+} channels, and Cx43 (see above). Intriguingly, a recent report demonstrated that the Ca^{2+} cycling machinery is defective in a conditional *Pkp2* knockout mouse model [33].

Table 1. Lists of genes involved in ACM pathogenesis.

Gene	Coding Protein	Locus		Reference
desmosomal mutations	***JUP***	**Junction Plakoglobin**	**17q21.2**	[34]
	DSP	**Desmoplakin**	**6p24.3**	[35]
	PKP2	**Plakophilin 2**	**12p11.21**	[36]
	DSG2	**Desmoglein 2**	**18q12.1**	[37] [14]
	DSC2	**Desmocollin 2**	**18q12.1**	[38]
	TMEM43	**Transmembrane protein 43**	**3p25.1**	[39]
non-desmosomal mutations	***LMNA***	**Lamin A/C**	**1q22**	[40]
	DES	**Desmin**	**2q35**	[41]
	CTNNA3	**Alpha-T-catenin**	**10q21.3**	[42]
	PLN	**Phospholamban**	**6q22.31**	[43]
	TGFB3	**Transforming growth factor-3**	**14q24.3**	[44] [45]
	TTN	**Titin**	**2q31.2**	[46]
	SCN5A	**Sodium voltage gated channel alpha subunit 5 (NaV 1.5)**	**3p22.2**	[47]
	CDH2	**Cadherin C**	**18q12.1**	[48]
	RYR2	**Type 2 ryanodine receptor**	**1q42-q43**	[49] [50]

PKP2

PKP2, encoding for plakophilin-2, is the most frequently mutated gene in ACM patients [36]. It has also been linked to other inherited cardiac arrhythmia syndromes, such as Brugada syndrome [51], idiopathic ventricular fibrillation, hypertrophic cardiomyopathy, and dilated cardiomyopathy, even if with a low frequency [52]. PKP2 is expressed in both myocytes and non-myocytes [53]. The main function of PKP2 is to guarantee mechanical stability during the desmosomal intermediate filament assembly required for cell-to-cell contact. PKP2 is part of the so-called 'connexome' [54]. Different studies

highlighted the consequent novel role for PKP2 in the intracellular signaling regulation, electrophysiological and trafficking regulation, and the control of transcription processes [33].

Data from a cardiomyocyte-specific tamoxifen-activated *Pkp2* homozygous knockout (*Pkp2*-cKO) mouse model correlated the lack of PKP2 with transcriptional alteration of Ca^{2+} homeostasis [33]. This investigation reported the development of a cardiomyopathy of RV predominance that become evident at 21 days and then progressed into biventricular cardiomyopathy and HF. Transcriptome analysis showed that the transcripts of proteins involved in maintaining the intracellular Ca^{2+} concentration were downregulated in *Pkp2*-cKO hearts. The low level of transcripts relevant to Ca^{2+} cycling, i.e., *Ryr2*, *Ank2*, *Cacna1c*, and *Trdn*, accompanied a decreased expression of corresponding proteins and the impairment of the EC mechanism [33]. Accordingly, the downregulation of AnkB (encoded by *Ank2*) and triadin (encoded by *Trdn*), which contribute to maintaining the structural integrity of dyadic junctions [55][56], significantly reduced the distance between CaV1.2 and RYR2 [33]. In addition, *Pkp2*-cKO-derived cardiomyocytes showed decreased $I_{Ca,L}$ density and a slower rate of current inactivation, which is consistent with the reduced expression of *Cacna1c* [33]. It should, however, be pointed out that, although the peak Ca^{2+} current was decreased, the total Ca^{2+} charge (i.e., the total amount of Ca^{2+} entering the cell upon membrane depolarization) was unaltered as compared to wild-type cardiomyocytes because of the slower inactivation rate [33]. Notably, the loss of PKP2 was also correlated with a reduction in the SR Ca^{2+} leak due to the downregulation of both *Ryr2* and *Casq2* [33] (**Figure 1**). As a consequence, the SR Ca^{2+} content was remarkably increased in *Pkp2*-cKO cardiomyocytes, which exhibited an increase in the amplitude and frequency of spontaneous Ca^{2+} release events (due to RYR2 sensitivity to intraluminal Ca^{2+} levels) and were therefore more prone to release SR Ca^{2+} during the EC coupling [33]. Accordingly, when *Pkp2*-cKO cardiomyocytes were paced at increasing rates, they displayed both early and delayed after-transients, which were sufficient to generate ventricular arrhythmogenic events during β-adrenergic stimulation with isoproterenol [33]. A more recent investigation focused on the early events driving the remodeling of the Ca^{2+} handling machinery in RV-derived PKP2-cKO (*Pkp2*-cKO-RV) cardiomyocytes isolated 14 days after tamoxifen injection [57], i.e., when cardiomyopathy was not evident yet. This report revealed an increase in RyR2-dependent Ca^{2+} release and RyR2-mediated Ca^{2+} sparks due to the remarkable elevation in the SR Ca^{2+} load that was caused by a Cx43-dependent increase in membrane permeability [57]. Notably, uncoupled Cx43 hemichannels may provide an alternative pathway for extracellular Ca^{2+} influx [58][59][60] and may, therefore, contribute to refilling the SR Ca^{2+} store in a SERCA2A-dependent manner. In addition, RyR2's eagerness to release Ca^{2+} may be boosted by the observed phosphorylation in Thr2809, an amino acid residue near the consensus sequence for CaMKII and PKA [57]. These alterations were not detected in PKP2-cKO LV cardiomyocytes, thereby suggesting that this asymmetric dysregulation of the Ca^{2+} handling machinery precedes overt ultrastructural alterations and manifestations of ACM.

The relationship between PKP2 and Ca^{2+} machinery has also been highlighted by a recent bioinformatic approach that took advantage of a database containing transcriptomic information from human hearts, searching for coordinated transcription networks that are subjected to variations based on PKP2 abundance. The results were then validated with the information deriving from *Pkp2*-cKO murine hearts, thereby confirming the downregulation of *RYR2*, *ANK2*, and *CACNA1C* [61]. The results of the combined data supported the idea of a correlation between the PKP2 expression and the abundance of transcripts related to intracellular Ca^{2+} homeostasis. In this context, mathematical modeling confirmed that the PKP2-dependent downregulation of RYR2 and CASQ2 proteins is sufficient to cause the decrease in SR Ca^{2+} leak, which results in enhanced SR Ca^{2+} loading and EC coupling [33]. Accordingly, studies carried out on human iPSC-CM from a *PKP2*-mutated ACM patient showed that they present an abnormal Ca^{2+} handling capacity [62]. Of note, a recent investigation in iPSC-CM suggested that the Ca^{2+} handling machinery could also be affected by *DSG2* mutations. Accordingly, although the systolic and diastolic Ca^{2+} levels were similar, human ACM iPSC-CM exhibited spontaneous SR Ca^{2+} release and DADs in both the absence and the presence of β-adrenergic stimulation [63]. This investigation did not evaluate the molecular expression of Ca^{2+}-related proteins, but further supports the notion that desmosomal mutations may affect the cardiac Ca^{2+} toolkit in ACM.

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