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Ca^{2+} signaling of *h*uman pluripotent stem cells-derived cardiomyocytes as compared to adult mammalian cardiomyocytes

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Abstract

Human induced pluripotent stem cells derived cardiomyocytes (hiPSC-CMs) have been extensively used for *in vitro* modeling of human cardiovascular disease, drug screening and pharmacotherapy, but little rigorous studies have been reported on their biophysical or Ca^{2+} signaling properties. There is also considerable concern as to the level of their maturity and whether they can serve as reliable models for adult human cardiac myocytes. Ultrastructural difference such as lack of t-tubular network, their polygonal shapes, disorganized sarcomeric myofilament, and their rhythmic automaticity, among others, have been cited as evidence for immaturity of hiPSC-CMs. In this review, we will deal with Ca^{2+} signaling, its regulation, and its stage of maturity as compared to the mammalian adult cardiomyocytes. We shall summarize the data on functional aspects of Ca^{2+} signaling and its parameters that include: L-type calcium channel (Cav1.2), I_{Ca}-induced Ca^{2+} release, CICR, and its parameters, cardiac Na/Ca exchanger (NCX1), the ryanodine receptors (RyR2), sarco-reticular Ca^{2+} pump, SERCA2a/PLB, and the contribution of mitochondrial Ca^{2+} to hiPSC-CMs excitation-contraction (EC)-coupling as compared with adult mammalian cardiomyocytes. The comparative studies suggest that *qualitatively* hiPSC-CMs have similar Ca^{2+} signaling properties as those of adult cardiomyocytes, but *quantitative* differences do exist. This review, we hope, will allow the readers to judge for themselves to what extent Ca^{2+} signaling of hiPSC-CMs represents the adult form of this signaling pathway, and whether these cells can be used as good models of human cardiomyocytes.

Keywords

hiPSC-CMs; mammalian cardiomyocytes; Ca^{2+} signaling; mitochondrial Ca^{2+}

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Conflict of interests

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Introduction

The development of inducible pluripotent stem cells (iPSC) from human somatic cells [1] and the procedures to differentiate them into cardiac myocytes [2] could potentially become a milestone in advancing basic and translational cardiovascular research. Creating patient specific human cardiac myocytes from patient's own tissue biopsy may also serve as a cell-source for *in vitro* modeling of human inherited cardiac pathologies, its pharmacotherapy, and drug screening [3–8]. There is nevertheless considerable concern as to the level of maturity of these cells and whether hiPSC-CMs can serve as a good model for primary human cardiomyocytes. Often cited examples of cellular immaturity are that hiPSC-CMs express atrial-, ventricular-, and SA-nodal-type action potentials, reflecting possibly their mixed ancestral origins [6, 9–11], and that these myocytes lack t-tubular network, have disorganized sarcomeric structures, inconsistent adrenergic responses, and beat rhythmically in cultures unlike adult cardiomyocytes [12, 13]. Since Ca^{2+} signaling plays a central role in initiation of action potential, regulation of pacemaking and cardiac contractility, evaluating the level of maturity of Ca^{2+} signaling pathway of hiPSC-CMs, as compared to primary mammalian cardiomyocytes, is crucial in determining whether these cells can serve as appropriate physiological models of adult human cardiomyocytes.

The studies reported thus far on Ca^{2+} signaling of hiPSC-CMs are mostly confined to measurements of intracellular calcium transients, without detailed analysis of the parameters of the calcium signaling pathway. Here, we shall examine the Ca^{2+} signaling parameters of hiPSC-CMs that include: biophysical properties of calcium channel (Cav1.2), the expression levels of I_{Ca}-induced Ca^{2+} release (CICR and its parameters: fractional release, gain, Ca^{2+} spark characteristics, the magnitude and source of calcium stores), the expression levels and regulation of cardiac Na/Ca exchanger (NCX1), the ryanodine receptors (RyR2), sarcoplasmic reticular Ca^{2+} pump, SERCA2a, and the contribution of mitochondrial Ca^{2+} in hiPSC-CMs excitation-contraction (EC)-coupling. To assess the level of maturity of Ca^{2+} signaling pathway in hiPSC-CMs, we shall compare their Ca^{2+} signaling properties to those of adult ventricular, atrial and neonatal rat cardiomyocytes. To this end and to facilitate comparisons of EC-coupling events between hiPSC-CMs and adult cardiomyocytes, we have chosen first to present an overview of Ca^{2+} signaling data of the adult mammalian cardiomyocytes in Section I, before discussing the published data on hiPSC-CMs in Section II. We hope this approach will allow the readers of this review to judge for themselves to what extent Ca^{2+} signaling of hiPSC-CMs represents the adult form of this signaling pathway.

I. Ca^{2+} signaling in primary mammalian cardiomyocytes

1.1. A historical retrospective of Cardiac EC-coupling

1.1a. EC-coupling and Ca^{2+}-induced Ca^{2+} release.—Cardiac EC-coupling encompasses the molecular events that couple the depolarization of surface membrane to mechanical events of myofilament contraction. First attempts to determine the role of action potential in regulation of contraction in the mammalian heart, showed that the first 20–50ms of the action potential triggered over 70% of the contractile force, while the remaining 300ms of the action potential regulated the remaining fraction of the contraction [14]. These

first voltage clamp studies in cat ventricular strips when compared to similar studies in the frog heart [15], suggested two types of regulatory mechanism for cardiac Ca^{2+} homeostasis. In the mammalian heart the action potential initially triggers and then in graded manner regulates the development of tension while in the frog heart the action potential continuously and linearly mediates the entry of Ca^{2+} into the myocytes that directly determine the magnitude and duration of contraction [15, 16]. This unique mammalian cardiac specific mechanism coincides with the activation of Ca^{2+} channels in the initial phase of the action potential [17], and suggests the possibility that the influx of calcium in early phase of the action potential triggers the release of intracellular Ca^{2+} causing the rapid component of phasic tension in the initial 50ms of the action potential [14, 18]. Despite such findings, significant controversy continued to exist about the mechanisms that coupled membrane depolarization to initiation of contraction. One popular idea was that depolarization of cardiomyocyte directly triggers the release of Ca^{2+} in a manner similar to skeletal muscle where the depolarization-induced capacitive charge movement in the surface membrane transfers the activating signal to the SR membrane, triggering the release of calcium [19]. Another view, consistent with 1830s frog heat reports of Sydney Ringer, held that cardiac contraction required extracellular calcium [15, 16], where influx of calcium either directly activated the contraction as in the frog heart, or triggered the release of larger Ca^{2+} from intracellular pools in the mammalian hearts. Two seminal reports in 1980s finally settled this issue: 1) in skinned mammalian cardiac muscle strips rapid applications of small Ca^{2+} concentrations caused larger releases of cytosolic Ca^{2+}, a process called calcium induced calcium release, CICR [20–22] and 2) in intact voltage-clamped rat cardiomyocytes, 1–2 seconds of rapid withdrawal of extracellular Ca^{2+} prior to activation of calcium channels that fully suppressed I_{Ca}, but allowed large influx of Na^{+} to permeate through the Ca^{2+} channels, failed to trigger the release of Ca^{2+} from fully loaded SR stores, suggesting that influx of Ca^{2+} through the Ca^{2+} channels was mandatory for the release of Ca^{2+} [23]. This I_{Ca}-gated Ca^{2+} release mechanism showing that direct entry of Ca^{2+} and not membrane depolarization was responsible for the release of Ca^{2+} from the SR, was further strengthened by findings that the voltage dependence of intracellular Ca^{2+} release mirrored the bell-shaped voltage-dependence of I_{Ca} [24–26]. In sharp contrast, in skeletal muscle the bell-shaped voltage dependence of I_{Ca} was accompanied by a sigmoid voltage-dependence of Ca^{2+} release and developed tension, reflecting the voltage-dependence of charge movements associated with activation of Ca^{2+} channel, and consistent with a voltage-gated Ca^{2+} release mechanism [19]. Further, cardiac Ca^{2+} release could be triggered equally well by depolarizing pulses, or by repolarizing pulses (from positive potentials to resting potentials) that re-activated I_{Ca} “tail currents” [24, 26]. It was therefore concluded that mammalian cardiac EC-coupling is mediated by influx of Ca^{2+} through the L-type Ca^{2+} channels that triggers the release of 10–20 times larger Ca^{2+} concentrations from sarcoplasmic reticulum (SR) through the type-2 ryanodine receptor (RyR2) sufficient to activate the myofilaments.

In a purely CICR controlled mechanism, however, only the charge carried by calcium channel would determine the magnitude of released calcium independent of the activating voltage of the Ca^{2+} channel. That is, there should be no voltage-dependence to the efficacy or gain of CICR. Several labs including ours [27], however, have found significant voltage-dependence to the gain of CICR at voltages between −30 to +10mVs [27, 28], suggesting

that other mechanisms may also contribute to the release process. In our experiment CICR gain was ~40 at +10 to 30mVs (the plateau range of voltages of cardiac action potential) but increased to values exceeding 100 at −30mVs [27], inset fig.1. It has been suggested that the voltage dependence of CICR gain may arise from the greater efficacy of calcium release unites that are activated by the larger unitary I_{Ca} at −30 mV compared to +10 mV, despite the lower open probability of the channel at −30 mV. This possibility was tested by Bers and colleague who evaluated the role of open probability of the channel and its magnitude in the voltage dependence of CICR gain, and found that the decrease in the open probability of the channel rather than the magnitude of the current at −40mVs was responsible for the higher gain of CICR. They attributed this to the redundant openings of Ca-channels at +10 mV that would lower gain of CICR at the plateau range of potentials [29]. Another possible mechanism that could render voltage-dependence to the gain of CICR at negative potentials is a direct interaction of carboxyl tail of CaV1.2 with the RyR2 that would sensitize RyR2 to increase its spontaneous Ca^{2+} sparks frequency, resulting in enhanced I_{Ca}-gated Ca-transients at −30 to −40mVs [30–32]. This possibility may also underlie the often observed small differences in voltage dependence of I_{Ca} and I_{Ca}-triggered Ca-transients at voltages between −40 to −10mVs.

The released Ca^{2+} initiates contraction as Ca^{2+} binds to the myofilaments, allowing the cross-bridges to initiate their “rowing” action, allowing the sliding of actin over the myosin filaments and shortening the sarcomere. Cardiac relaxation begins as the released Ca^{2+} is sequestered by ATP-dependent Ca^{2+} uptake(SERCA2a) into the SR [33, 34], the activity of which is regulated, in turn, by phospholamban (PLB) phosphorylation [35–37] by protein kinase A/beta adrenergic agonists [38] or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [39], thus increasing the affinity of SERCA2a to Ca^{2+} and enhancing the rate of Ca^{2+} uptake and relaxation of contraction, see figure 1.

1.1b. CICR and its inactivation—Fabiato et al. were first to suggest that influx of Ca^{2+} through CaV1.2 first activates the SR release mechanism, triggering the larger release of Ca^{2+} from the SR that in turn inactivates the release mechanism [20], somewhat similar to the L-type Ca^{2+} channel’s calcium-dependent inactivation mechanism, CDI [20]. This idea, when tested in intact myocytes by photo-releasing calcium from caged calcium compounds, at different times following the activation of release process by I_{Ca}, failed to show any evidence of inactivation or termination of Ca^{2+} release. Photo-released Ca^{2+} only enhanced the rise of cytosolic Ca^{2+} irrespective of its release time following I_{Ca}-gated Ca^{2+} release, i.e. photo-released Ca^{2+} failed to suppress or terminate the I_{Ca}-triggered Ca^{2+} release [40]. Studies in isolated cardiac SR vesicles expressing RyR2 further confirmed that Ca^{2+} release from the SR vesicles could only be suppressed by large un-physiological concentration of Ca^{2+}. In skeletal muscle, while RyR1 is inhibited by ~1mM Ca^{2+}, about 10-fold higher concentrations of Ca^{2+} are required to inhibit RyR2 channel [41, 42]. In canine vesicles of cardiac SR also, 1mM or higher Ca^{2+} concentrations were required to inhibit Ca^{2+} release in the presence of Mg^{2+} and adenine nucleotides [43]. Despite large differences in Ca^{2+} concentration required for inactivation of RyRs in skeletal and cardiac muscle *(IC_{50} of RyR1, ~1mM, and RyR2 >10mM)*, the Ca^{2+} activation of Ca^{2+} release channels occurred with EC_{50} values of about 1μM.

Possible roles of other accessary proteins associated with RyR2, such as calmodulin, calsequestrin, triadin and junctin, in the inactivation of the release mechanism have also been considered [44,45]. Gyorke et al, for instance, suggested that the binding of Ca-free calsequestrin to the RyR/Junctin/Triadin complex may decrease the calcium sensitivity of RyR2 when the SR Ca^{2+} content decreases after SR release [46]. Considered also in the inactivation of RyR2 mechanism, was the possibility that the dyadic release couplons become fully emptied of their Ca^{2+} by the triggering I_{Ca}, thereby obviating the inactivation of Ca^{2+} release, and thereby suppressing also the inherent positive feedback of CICR [47]. This idea is consistent with the findings that the frequency of occurrence of activated Ca^{2+} sparks, but not their size, depended on the magnitude of activated I_{Ca} [48]. Considering the various likely mechanisms proposed and tested for the inactivation of the release mechanism, it is likely that multiple molecular processes may be involved in the Ca^{2+} dependent inactivation of the dyadic Ca^{2+} release.

1.1c. Dyadic Calcium release and RyR2 microdomains.—As it became generally accepted that I_{Ca} gates the release of calcium, ultrastructural and live cellular imaging were used to study the focal characteristics of dyadic Ca^{2+} release and its microdomains (20–50nm). Studies in voltage-clamped myocytes dialyzed with high concentrations of EGTA to buffer the global cytosolic Ca^{2+} concentrations showed that gating of dyadic Ca^{2+} release by I_{Ca} was resistant to 14–20mM intracellular EGTA concentrations, as if accessibility into the subsarcolemmal space surrounding the dyads was limited to the Ca^{2+} influx through the channel [49]. This and similar findings suggested that RyR2 proteins of dyadic junctions may have privileged access to dihydropyridine receptors of the surface membrane [49, 50]. These conclusions were later supported by confocal imaging data on intact cardiomyocytes, showing the development of spontaneous focal Ca^{2+} releases, sparks, associated with the dyadic structures that had durations in excess of 100ms and covered sarcomeric area of 1–2μm [51]. Since the morphology and time course of focal Ca^{2+} releases could be significantly altered by binding kinetics and affinity of the Ca^{2+} sensitive fluorescent dyes and cellular diffusion of the dye/Ca^{2+} complex, the measured sparks were unlikely to represent accurately the time course of dyadic Ca^{2+} release. In support of this possibility, rapid (240f/s) 2-D confocal imaging of 100s of sparks in patch-clamped rat ventricular myocytes, dialyzed with combination of EGTA and Fluo3 to limit the diffusion of dye/calcium complex, showed that spontaneous or triggered sparks had an average duration of ~7ms, carrying ~100,000 Ca^{2+} ions [48]. This finding and the fact that the sparks occurred at the same cellular location on repetitive activation of Ca^{2+} release ruled out that Ca^{2+} sparks represented the stochastic activity of a single RyR2, but rather the activation of single dyadic structure, with multiple RyR2s [48]. These studies also showed that the magnitude of Ca^{2+} sparks remained mostly constant, irrespective of the magnitude of I_{Ca} activated at different voltages, but the frequency of occurrence of sparks varied with the magnitude of I_{Ca}.

1.1d. Molecular determinants of Ca^{2+} signaling: Ca^{2+} and caffeine binding sites.—Ca^{2+} influx through the channel and its binding to the high affinity Ca^{2+}-binding site on the ryanodine receptor is the initial step for the activation of RyR2 and Ca^{2+} release from the SR [43]. Two classes of Ca^{2+}-binding sites have been identified on RyR protein: a high-affinity *Ca^{2+} activation* and a low-affinity *Ca^{2+} inactivation* site [41, 52]. Early studies

of MacLennan group, using truncated forms of rabbit RyR1 with $^{45}Ca^{2+}$ overlay [53, 54] and RyR1 site-specific antibodies purified by immobilized synthetic peptide [55], identified multiple Ca^{2+} regulatory domains in RyR1. Using site-directed mutagenesis in recombinant rabbit RyR3 expressed in HEK293, Chen et al showed that a glutamate-to-alanine mutation of E3885A in the M2 transmembrane sequence (now considered to be in cytosolic domain in high resolution structural model) resulted in decreases of both the caffeine response and the sensitivity to activation by Ca^{2+}, implicating RyR3-E3885 as the potential calcium sensor residue [56]. The corresponding E3987A mutation in mouse RyR2 was later reported by the same group to markedly reduce the sensitivity of the channel to activation by Ca^{2+}, indicating that the glutamic acid residue is the common Ca^{2+} activation site in all RyR isoforms [57]. Data from 4.3Å cryo-electron microscopic structure of RyR1, using 30 μM Ca^{2+} as activating stimulus, appears to implicate 5 amino acids E3893(3847), E3967(3921), T5001(4930), Q3970(3925) and H3895(3850) of RyR1/RyR2, located at the core domain just above the transmembrane domain to form the protein's putative Ca^{2+}-binding site [58, 59]. The site-directed mutagenesis of the recombinant RyRs and structural mapping of formerly identified E4032 of RyR1 (corresponding to E3987 of RyR2), not only showed that these residues were critical for Ca^{2+}-dependent activation of RyRs, but also that E4032 may form an interface with carboxyl terminal that includes T5001, suggesting that mutation of E4032 could destabilize the Ca^{2+} binding conformation. In this model, the neighboring amino acid W4716 of RyR1 (corresponding to W4645 of RyR2), just below the Ca^{2+}-binding site, was identified to be the putative caffeine-binding site. Our recent studies on human stem cell derived cardiomyocytes have confirmed some of these findings and further suggest possible interaction between the caffeine and Ca^{2+} binding sites, such that mutations of either Ca^{2+} or caffeine binding sites may alter each other's sensitivity to caffeine binding and Ca^{2+}-induced activation [60, 61]. Other studies using mutational analysis have shown that mutation or deletion of residues Δ4274–4535 of rabbit RyR1 leads to altered caffeine and Ca^{2+} sensitivity of RyR1 channel, suggesting that caffeine and Ca^{2+} activation sites may not be located within these residues [62]. In addition, several residues outside of these domains in RyR carboxyl terminal were found to be involved in Ca^{2+}-dependent inactivation, which included two EF hand type Ca^{2+} binding sites (EF1: RyR1 aa 4081–4092; EF2: 4116–4127), with milli-molar affinity to Ca^{2+}[42, 62], consistent with biochemical studies [63]. Gomez et al, in addition, showed that EF1 and the second transmembrane segment (S2) are critical for isoform-specific Ca^{2+}-dependent inactivation of RyR [64]. Since EF hand domain and cytoplasmic loop between the second and the third transmembrane (S2-S3) loops are in close proximity of low affinity Ca^{2+} binding site [65], EF1 may transmit the signal for inactivation to the pore through S2-S3 loops. High resolution structural mappings of low affinity Ca^{2+} binding site may clarify this issue, but such studies remain to be carried out.

1.2. Physiological modulation of EC-coupling

1.2a. NCX1 and the modulation of CICR.—To achieve Ca^{2+} homeostasis, the additional per beat entry of Ca^{2+} through the Ca^{2+} channels is extruded by the sarcolemmal Na^{+}-Ca^{2+} exchanger (NCX1), which exchanges one calcium for three sodium ions, generating an inward current [66–68]. Since NCX1 protein can function both in Ca^{2+} influx or efflux modes, a number of reports in 1990s suggested that during early phases of the

plateau of the action potential Ca^{2+} could enter the cell via the Ca^{2+} influx, reverse mode, of the NCX1 and thereby trigger Ca^{2+} release from the SR [69, 70]. Such findings were, somewhat, controversial as other studies that examined Ca^{2+} influx on NCX1 failed to show significant role for the entry of Na^{+} or the exchanger in release of Ca^{2+} under physiological conditions [71]. Considering the stoichiometry, kinetics and voltage-dependence of NCX1, it is also unlikely that NCX1 could operate in the Ca^{2+} influx mode within the first 10ms of depolarization of the action potential, because Ca^{2+} concentrations delivered by the Ca^{2+} channel into the nano-domains of dyadic RyR2, in the first few ms of action potential, would reach micro-molar levels, thereby shifting the electro-chemical potential for NCX1 ($E_{Na\text{-}Ca}$) to potential positive to +30mVs, forcing the exchanger to operate in its Ca^{2+} efflux mode. In support of this idea, high-resolution immunofluorescence imaging of NCX1, CaV1.2 and RyR2, showed that while CaV1.2 and RyR2 were co-expressed in the same microdomains in the t-tubules, there was little co-expression of NCX1 and RyR2 in the same microdomains [72, 73]. Since the proximity of RyR2 proteins to CaV1.2 in dyadic junctions is paramount for effective activation of RyR2 to release Ca^{2+}, the absence of NCX1 from the dyadic microdomains would further minimize the role of the exchanger in gating Ca^{2+} release from the SR, under physiological conditions. The sub-cellular microarchitecture, where dihydropyridine receptors and RyR2s are co-expressed in the same microdomains, and are surrounded by clusters of NCX1 proteins in the inter-dyadic spaces, would tend to minimize the diffusion of Ca^{2+} from neighboring dyads that could trigger Ca^{2+} release, thus suppressing the inherent positive feedback of CICR.

1.2b. The evolutionary role of NCX1 in cardiac EC-coupling—The role of NCX1 in Ca^{2+} homeostasis of mammalian myocardium, where there is both triggered release and entry of Ca^{2+}, appears to have diverged significantly from that expressed in hearts of species that have not developed Ca^{2+} uptake and release components of the reticulum. In *Rana pippins frog* and *Squalus acanthus shark* hearts, where SR is poorly developed and dyadic junctions and caffeine-triggered pools are absent, NCX1 appears to mediate both the influx of Ca^{2+} for contraction and extrusion of Ca^{2+} mediating the relaxation [74–77]. Surprisingly, in frog heart, which lacks SERCA2a/PLB entities of SR [78, 79] that mediate the adrenergic enhancement of Ca^{2+} uptake and relaxation in the mammalian hearts, beta-adrenergic agonists still produce both their positive inotropic and relaxant effects. In these hearts, calcium enters the myocardium during the action potential continuously via the Ca^{2+} channels and Na/Ca exchanger and relaxation is mediated by efflux of Ca^{2+} on the exchanger [15, 80]. Fan et al, were first to report that the beta-adrenergic induced enhancement of relaxation was mediated by adrenergic modulation of NCX1 activity [76], where NCX1 has an additional P-loop with Walker A sequence [81], which endows the protein with PKA-mediated regulation. Consistent with this possibility, while the Na^{+}-dependent Ca^{2+} uptake in oocytes expressing the frog exchanger was enhanced by cAMP, this effect was absent in the dog exchanger, or the recombinant frog exchanger lacking the p-loop [81]. Further, insertion of the p-loop in the dog exchanger conferred the cAMP-dependent regulation to HEK cells expressing the chimera NCX1 expressing the frog exon [82]. The molecular data, therefore, suggests that the 9-amino acid of P-loop containing the Walker A nucleotide binding motif maybe responsible for the observed cAMP-dependent functional differences between the frog and the mammalian heart NCX1 [81].

In shark heart too, reduction of $[Na^+]_i$ suppressed while elevation of $[Na^+]_i$ enhanced a tonic component of contraction as it activated an outward current reflecting the influx of Ca^{2+} via the electrogenic Na^+-Ca^{2+} exchanger, thus regulating the contraction/relaxation cycle [77]. A bimodal adrenergic regulation of NCX1 appears to have evolved in the shark heart, where adrenergic agonists suppress greatly the Ca^{2+} influx on NCX1 as they enhance the efflux mode of the exchanger [83]. Cloning and sequencing of shark NCX revealed an insertion of A/P rich region-2 domain, not present in the mammalian NCX1 [84]. The deletion of this domain removed cAMP mediated bimodal regulation of the shark exchanger, suggesting that it mediates the beta-adrenergic modulation by PKA phosphorylation. The bi-modal beta-adrenergic/PKA mediated phosphorylation of NCX1 may have evolved to satisfy the evolutionary needs for accelerated contraction and relaxation during a fight or flight response in hearts of animals missing the SR Ca^{2+} release and uptake molecular machinery. The development Ca^{2+} release and uptake stores of SR and their regulation by PKA phosphorylation of PLB/SERCA2a protein complex, appears to have resulted in pruning the molecular motifs that were evolved for adrenergic regulation of NCX1. This evolutionary pruning provides the cell with only one regulated Ca^{2+} efflux or uptake mechanism. With the development of SR expressing SERCA2a/PLB, beta-adrenergic phosphorylation of PLB enhances the sequestration of Ca^{2+} into the SR leading to enhancement of the Ca^{2+} store, the contractile force, and relaxation, obviating the evolutionary needs of the heart to have PKA-regulation of NCX1.

1.3. Transgenic mice with deletions or overexpression of NCX1, ICa, PLB, CSQ, and their pathophysiological consequences

Creation of transgenic mice with overexpression or deletion of Ca^{2+} signaling pathway proteins have provided unexpected insights into pathophysiology of Ca^{2+} signaling.

1.3a. Na/Ca exchanger, NCX1.—Over expression of cardiac NCX1, for instance, while producing 3–5 times larger exchanger current on releasing the SR Ca^{2+} pools by caffeine, did not produce significant hypertrophy or cardiac pathology in transgenic mice [85, 86]. In myocytes isolated from mice hearts overexpressing the exchanger, the intracellular Ca^{2+} pools of SR remained either unchanged or were slightly enhanced. The myocytes also showed no changes in their I_{Ca} density, Ca-transients magnitude, level of expression of SERCA2a, phospholamban or calsequestrin [28, 85–87]. Wang et al confirmed significantly larger SR Ca^{2+} content and no cardiac hypertrophy in NCX overexpressing myocytes [88]. The enhanced SR calcium content is unexpected in the light of larger Ca^{2+} efflux on the exchanger, suggesting possible compensatory mechanisms involving enhanced SERCA2a calcium reuptake into the SR. Homozygous overexpression of NCX1, on the other hand, caused 40% cardiac hypertrophy with contractile dysfunction and clinical evidence of heart failure [89]. Homozygous knock out of NCX1 was embryonically lethal. Heart tubes at 9.5 day post coitum had normal Ca^{2+} transients but were unable to maintain Ca^{2+} homeostasis when subjected to Ca^{2+} challenges [90]. Conditionally-deleted NCX1 mice appeared to live into adulthood, had normal cardiac functions at seven-weeks and somewhat higher CICR gain [91]. In sum, although deletion of NCX1 is embryonically lethal, either its conditional deletion or overexpression in adult mice appears not to produce significant cardiac pathology.

1.3b. L-type Ca^{2+} channel.—Over expression of α1C subunit of calcium channel is reported to generate a slowly developing cardiac hypertrophy within 8 months and 20% increase in cell capacitance at 4 month [92, 93]. While I_{Ca} density and its inactivation kinetics increased in 2–8 months, Ca^{2+} transients increased significantly only in 4-month-old mice. Although the larger Ica of overexpressed hearts triggered larger Ca^{2+} release from the SR, the caffeine sensitive Ca^{2+} store, diastolic Ca^{2+} levels and Ca^{2+} spark properties remained mostly unchanged. The enhanced I_{Ca} entry and SR Ca^{2+} release appeared to have been compensated for by upregulation of the Na-Ca exchanger activity. Somewhat unexplained was the finding that the inotropic and lusitropic responses of isoproterenol and forskolin were significantly reduced in I_{Ca} overexpressing myocytes [92–94].

1.3c. Calsequestrin, CSQ2 the SR Ca^{2+}-binding and storage protein.—Overexpression of calsequestrin in mice hearts leads to marked cardiac hypertrophy, arrhythmias and heart failure. Electrophysiologically, myocyte action potentials were prolonged and I_{Ca}, I_{to}, and I_{K1} were suppressed [95]. CSQ2 overexpressing mice hearts with 10-fold higher levels of calsequestrin survived into adulthood, but had severe cardiac hypertrophy. The impaired Ca^{2+} signaling in isolated myocytes appeared to result from structural uncoupling of dyadic couplons from the sarcolemma, resulting in suppressed I_{Ca}-gated Ca^{2+} release and marked reduction in the rate of inactivation of I_{Ca}, even though caffeine-triggered Ca^{2+} release was markedly enhanced [96]. Since the Ca^{2+} storage capacity of SR was increased by overexpression of CSQ2, caffeine releasable Ca^{2+} pools and the resultant exchanger currents were also highly enhanced. Ca^{2+} channel gated Ca^{2+} release, however, and the frequency of spontaneously occurring sparks were suppressed despite the high Ca^{2+} content of SR, implicating structural deformation of dyadic/sarcolemmal couplings [95, 96].

CSQ2 null mice *myocytes*, showing significantly decreased triadin and junction levels, unexpectedly displayed normal caffeine-triggered Ca^{2+} release and unchanged contractile function under basal conditions [97]. In another study, CSQ2 knockout mice showed accelerated intra-SR Ca^{2+} refilling kinetics, reduced refractoriness of Ca^{2+} release and reduced rate-dependent Ca^{2+} alternans in intact mouse hearts [98]. The loss of SR Ca^{2+} release refractoriness appeared to have resulted from impaired Ca^{2+}-CaM-dependent inactivation of L-type Ca^{2+}current [99]. Catecholamines appears to increase the diastolic SR Ca^{2+} leak in CSQ2 knockout myocytes, triggering premature spontaneous Ca^{2+} releases and aberrant contractions [97]. Heterozygous CSQ2 deleted mice, with 25% decrease in protein levels and no significant decrease in junctin and triadin-1 levels, had unchanged field-stimulated Ca^{2+} transients, L-type Ca^{2+} currents, and SR volume [100]. Adult rat myocytes transfected with adenoviral gene expressing antisense for canine calsequestrin cDNA, reduced CSQ2 levels by >50% and caffeine-releasable pools by 30%. These myocytes had markedly reduced I_{Ca}-gated Ca^{2+} release [101], supporting the idea that the most critical Ca^{2+} signaling defect of either suppression or overexpression of CSQ2 is the defective signaling between CaV1.2 and RyR2, most likely caused by structural disruptions between t-tubules and SR membranes. It is likely that the observed increase in SR Ca^{2+} leak is responsible for the reported triggered ventricular arrhythmias in patients expressing CSQ2 mutations [100]. It should be noted that although it has long been known that CSQ2 was the

major Ca^{2+} storage protein of the SR, the creation of transgenic mice made it clear that CSQ2 in addition critically regulates CICR and the normal function of the heart. It still remains somewhat puzzling why the deletion of CSQ2 in mice does not reduce the magnitude of Ca^{2+} release from the SR as it does in CSQ antisense experiments, and whether the increased volume of SR in CSQ2 null mice compensate for the lack of this protein in the SR.

1.3d. Phospholamban (PLB).—Phospholamban is the key inhibitory protein associated with the cardiac Ca^{2+} pump (SERCA2a) that regulates the rate of Ca^{2+} uptake into the SR under baseline conditions. On beta-adrenergic stimulation, PLB is phosphorylated decreasing its affinity to bind and inhibit SERCA2a, thus enhancing the rate SERCA2a sequestration of Ca^{2+}, thereby enhancing the rate of decay of Ca^{2+} transients and relaxation.

Mice hearts overexpressing PLB show significant suppression of electrically triggered Ca^{2+} transients, cardiac contractility and rate of relaxation with no hypertrophy or heart rate changes, resulting from suppressed SERCA2a activity [102–104]. In contrast, genetic deletion of PLB causes enhanced myocardial contractility, increased Ca^{2+} ATPase activity and Ca uptake resulting in larger Ca^{2+} content of SR [105–108]. As expected PLB-deficient myocytes lose or respond poorly to β-agonist stimulation [107]. Phospholamban deletion, using CRISPR/Cas9 technique, appeared to improve the mortality of mice suffering from heart failure caused by CSQ-overexpression [109]. Recent finding in saponin-permeablized cardiomyocytes isolated from wild-type or PLB-knockout mice suggest that PLB is also highly expressed in the nuclear envelope where it regulates the nuclear Ca^{2+} handling and IP_3R-mediates nuclear Ca^{2+} release [110]. PLB engineered mice appear to reflect the functional consequences of altered SERCA2a activity on Ca^{2+} uptake and Ca^{2+} content of SR.

1.4. Ca^{2+} signaling in Atrial myocytes

Atrial myocytes are significantly smaller than ventricular myocytes averaging in diameter (5–10 vs 20–40 um), are often devoid of t-tubules, especially in smaller mammals [111–113]. Electrophysiologically, also there are differences in expression levels of K^+ and chloride channels, resulting in shorter atrial action potentials. Generally, atrial Ca^{2+} signaling is initiated by activation of I_{Ca} that triggers a rise in cytosolic Ca^{2+} in cell periphery that diffuses to cell center activating release of Ca^{2+} from the corbular SR and giving rise to the global Ca-transients [111, 114, 115]. Such a Ca^{2+} diffusion-dependent signaling pathway and sparse expression of t-tubules requires time delays for development of Ca^{2+} transients and generation of contraction, consistent with the finding that action potential induced Ca^{2+} releases in cat atrial myocytes are spatially inhomogeneous [116]. Rapid 2-D (240 f/s) confocal imaging also shows two groups of Ca^{2+} sparks in rat atrial myocytes, those near the surface membrane co-localized with dihydropyridine receptors (DHPRs), having significant voltage-dependence to the frequency of their occurrence, and those centrally located (not associated with DHPRs), showing no voltage-dependence [117]. Peripheral sparks were brighter, flatter, had ~5-fold higher frequency of occurrence, ~2 times faster diffusion coefficient, and extinguished abruptly. Central sparks, in contrast,

occurred less frequently, were elongated along the cellular longitudinal axis, and dissipated slowly [117]. Interestingly, the magnitude of Ca^{2+} sparks was about 3 times larger in rat atrial cells, ranging ~300,000 Ca^{2+} ions, as compared to rat ventricular cells. Surprisingly, central sparks and peripheral sparks appeared often to activate within 4ms of each other (2-D confocal imaging at 240 frames/s), even in myocytes dialyzed with 5mM EGTA, suggesting that either expression of vestigial t-tubular system mediates the rapid activation of central sparks, or an as yet unknown signaling pathway couples the activation of central sparks to calcium channel current. A sparse t-tubular network was also reported in dog hearts, in both right (<25%) and left (~12.5%) atria [118], but despite the sparsity of t-tubules the rate of developed tension in dog atrial strips was faster than ventricular strips [119], suggesting that either an alternate, Ca^{2+} signaling pathway is expressed in the dog atria, or that atrial myofilament sensitivity to Ca^{2+} is significantly higher than in the ventricles.

There seems to be little qualitative difference in the fundamental steps that regulate Ca^{2+} signaling pathway when comparing the atrial and ventricular EC-coupling. Variability in size of Ca^{2+} sparks and their morphology in atria seems to be related to the location of SR Ca^{2+} release structures (couplons), vis-à-vis the sarcolemma or vestigial t-tubules, and the consequence of calcium dependent inactivation of sarcolemmal CaV1.2. Development of t-tubules seems to be strictly cell size dependent, as larger mammals (sheep, pigs, etc.) with larger hearts appear to have a higher fraction of their atrial cells expressing t-tubular network [120–122]. Despite these differences dictated by ultrastructural differences of atrial myocytes, the fundamental aspects of CICR, the gain, the fractional release, the magnitude of SR calcium stores and their sensitivity to caffeine and adrenergic agonists are nearly identical to those of ventricular myocytes. Physiologically, however, atrial roles as secretory organ, mechanical stretch or sheer stress sensor, its differential cholinergic and purinergic innervation, and its role in regulation of blood pressure makes it a unique myocardial tissue with multiple functions. Consistent with multiplicity of atrial function, these myocytes express significantly higher levels of IP_3Rs (both type 1 and type 2) as compared to ventricular cells [123, 124]. The parallel IP_3 Ca^{2+} signaling pathway may have evolved to regulate the signaling pathways associated to other functions of atria, as well as expression of transcription factors related to development and growth of the myocytes [125].

II. Ca^{2+} signaling in hiPSC-CMs

As discussed in the first section of this review, cellular evaluation of Ca^{2+} signaling requires critical measurements of amount of charge carried by I_{Ca}, its inactivation kinetics, the magnitude of Ca^{2+} released from the SR by caffeine, the gain of CICR, and the fractional release of SR Ca^{2+} by I_{Ca}.[126] The gain of CICR. measured as the degree to which I_{Ca} triggers Ca^{2+} release normalized to the Ca^{2+} content of the SR store and the charge carried by I_{Ca} [4, 127], shows unexpected voltage-dependence in adult cardiomyocytes, as discussed above.

To determine whether hiPSC-CMs represent a good model of adult human cardiomyocytes or merely replicate the physiology of immature cardiomyocytes, it is critical to compare and quantitate the structural and functional differences of the two tissues, as they pertain to the different cellular physiological signaling pathways. On Structural level, hiPSC-CMs have

flat rounded or polygonal/spindle shapes with large surface to volume ratios [13, 129], low levels of myofibrils, and disorganized z-lines and sarcomeres [12, 130, 131]. They also lack t-tubular network [132–134] and have non-uniform distribution of calcium release units [12, 130]. Functionally, hiPSC-CMs beat spontaneously in culture, unlike adult cardiomyocytes [13, 128], express variable levels of $I_{f,}$ lower levels of I_{K1}, and T-type Ca^{2+}channels [13, 128, 135]. In addition, hiPSC-CMs show mixture of atrial, ventricular, and nodal action potentials, possibly related to their varied ancestral origins. These signaling parameter may result in the observed lower rate of rise and decay of cellular Ca^{2+} transients [10, 129], and lower fractional Ca^{2+} release [4] as compared to adult cardiomyocytes. Nevertheless, the fundamental Ca^{2+} signaling parameters of hiPSC-CMs are almost identical to neonatal rat cardiomyocytes and similar to adult rat cardiomyocytes [13]. For instance, their Ca^{2+} release is gated by L-type Ca^{2+} channel, the voltage dependence of I_{Ca} and Ca-transients are similar and bell-shaped and indistinguishable from those of neonatal or adult rat cardiomyocytes, Fig.2. [4]. HiPSC-CMs also express large SR Ca^{2+} pools that are effectively released by 5–10mM caffeine generating significant inward NCX1 currents in voltage-clamped myocytes [13, 129]. The magnitude of I_{NCX} was significantly larger in hiPSC-CMs as compared to rat neonatal and adult cardiomyocytes (2–3 vs 1–1.5 pA/pF)[13], suggestive of either larger levels of expression of NCX1 protein in hiPSC-CMs, or more effective rise of intracellular Ca^{2+} in hiPSC-CMs that have small cell volumes but large surface areas [13, 129]. As to the proteins of Ca^{2+} signaling pathway, hiPSC-CMs express significant levels of RyR2, calcium-ATPase (SERCA2a), phospholamban, PLB, Junctin (Jun), Triadin (TRDN), calsequestrin (CSQ2) and inositol tris phosphate receptor (IP_3R) [130, 136–139]. Even though the expression levels of these proteins vary greatly in reports from different labs, most likely related to their developmental stage or their in vitro culture conditions, it is clear that hiPSC-CMs express all the molecular components of cardiac Ca^{2+} signaling pathway.

2.1. Functional components of Ca^{2+} signaling: Ca^{2+} current, NCX1 current, Ca^{2+} sparks, and SR Ca^{2+} store

2.1a. Calcium channels.—In adult ventricular myocytes L-type calcium channels are highly expressed both on the sarcolemma and t-tubular network. Despite the paucity of t-tubular network in hiPSC-CMs, the density of I_{Ca} in these cells appears to be comparable to those of adult human cardiomyocytes averaging around 8 pA/pF [4] and significantly higher, 10–12pA/pF, in monolayers of engineered heart tissue [140]. The pharmacology and voltage-dependence/kinetics of I_{Ca} in hiPSC-CMs suggest that the current is carried by the L-type Ca^{2+} channels (CaV1.2), where I_{Ca} activates at −30mVs, reaches its peak values around zero, reverses at potentials positive to 80mVs, and dihydropyridine blockers and divalent cations like Cd^{2+} and Ni^{2+} block, while BayK 8644 effectively enhances the current [13, 140]. Direct measurements of Ca^{2+} channel (CaV1.2) gene and proteins, or α−1C subunit of L-type Ca^{2+} channels, also confirm similar expression levels of calcium channel protein in hiPSC-CMs and adult human heart [130, 135, 141]. These findings suggest that irrespective of their originating lab and differentiation procedures used to create them, hiPSC derived cardiomyocytes express robust and similar levels of Ca^{2+} channels, comparable to those of adult human cardiomyocytes, compare Fig.1 of 2.

Calcium dependent inactivation (CDI), a biophysical property specific to the dihydropyridine sensitive CaV1.2 channel, allows the more Ca^{2+} permeating through the channel to regulate the time course of the current. It has been consistently shown, for instance, that intracellular Ca^{2+} buffers like EGTA or BAPTA or replacement of Ca^{2+} by Ba^{2+} as the permeating cation, markedly slows the kinetics of inactivation of only CaV1.2 channel (L-type Ca^{2+} current). Although the kinetics of inactivation of I_{Ca} seem to vary greatly in different hiPSC-CM cell populations, intracellular Ca^{2+} buffers or extracellular replacement of Ca^{2+} by Ba^{2+} have the same effects on the kinetics of channel inactivation as those reported for those expressed in the adult or neonatal cardiomyocytes. A more recent report suggests that hiPSC-CMs may express two types of nifedipine-sensitive Ca^{2+} channel cells, based on the kinetics of inactivation of I_{Ca} [142]. The rapidly inactivating I_{Ca} cells (tau<40ms) appeared to be dominant in 3–5 days post-dissociation hiPSC-CM cultures, while the slowly inactivating I_{Ca} cells (tau≥ 40ms) were more prevalent in 6–8 days post-dissociation cultures. The authors suggest that differences in kinetics of inactivation of I_{Ca} were in part determined by the proximity of the L-type channels to dyadic calcium release structures, such that in younger cells where dyadic Ca^{2+} stores are in close proximity of sarcolemmal calcium channels, the cells show rapidly inactivating I_{Ca}. In older and much larger cells, on the other hand, the dyadic Ca^{2+} pools distributed in much larger cellular volumes maybe at a distance from the sarcolemma, thus decreasing the effectiveness of released Ca^{2+} and CDI in regulating the inactivation kinetics of I_{Ca} [142]. These studies also showed that slowly inactivating I_{Ca} cells had longer *ventricular-like* action potentials, while the rapidly inactivating I_{Ca} cells had shorter *atrial-like* action potentials, a finding also confirmed in rat neonatal cardiomyocytes, see fig 3. If these findings are consistently confirmed in hiPSC-CMs created in different labs, it might resolve the issue of whether diversity in the shapes and durations of action potentials in hiPSC-CM cultures represents different ancestral origins of hiPSC-CMs, or whether they are a consequence of proximity-dependence of dyadic Ca^{2+} release pools to sarcolemmal Ca^{2+} channels caused by cell growth in culture. Some researchers, but not all, have reported the expression of T-type Ca^{2+} currents in hiPSC-CMs [135, 140, 143].

2.1b. β-Adrenergic regulation of I_{Ca}—Cardiac myocytes respond to β-adrenergic agonists by enhancing their I_{Ca}, global Ca-transients, the rate of development of contraction and relaxation. Similarly, hiPSC-CMs when exposed to β-adrenergic agonists also show both positive chronotropic and inotropic effects in a manner similar to adult or neonatal cardiomyocytes [128, 135, 137, 141, 144, 145]. The expression of β-adrenergic receptors (ARs) and their functional regulation of I_{Ca} in hiPSC-CMs, however, appears to be cell maturation dependent [146]. In younger cell cultures of hiPSC-CMs, (<~30 days), β2-ARs are the primary source of cAMP/PKA signaling, while in older cultures (30–60 days) β1-AR signaling appears to increase markedly, reaching cAMP generating values of 56.8 ± 6.6%. PKA signaling also shows a similar pattern of expression, increasing from day 30 to 90 [146]. Consistent with this idea, the positive inotropic and lusitropic effects of both isoproterenol and forskolin (%) appear to be more prevalent in older 90-day cultures of hiPSC-CMs [147]. HiPSC-CMs or micro-tissues derived from them show robust β-adrenergic positive chronotropic but not the positive inotropic response [148]. In such studies PLB expression by recombinant adenoviruses appears to restore the positive

inotropic response of β-adrenergic stimulation [148], suggesting that the positive inotropic effect is mostly dependent on the SERCA2a/PLB activation.

2.1c. Caffeine-induced Ca^{2+} release, Na/Ca-exchanger, and Fractional Ca^{2+} release.—Sarcoplasmic reticulum is the major intracellular Ca^{2+} pool of the mammalian hearts that is generally gated by RyR2 channel, and is activated by rise of intracellular calcium exceeding 10^{-7} M or by caffeine which sensitizes RyR2 to Ca^{2+} such that resting cytoplasmic concentrations (~10^{-7}M) of Ca^{2+} triggers the release of Ca^{2+} from the SR [41, 149]. Caffeine-releasable Ca^{2+} stores of hiPSC-CMs were reported to be comparable to those of adult rabbit and mouse cardiomyocytes [129]. Our studies show that hiPSC-CMs have rapid and robust releases of Ca^{2+} from the SR on rapid application of caffeine that in turn also activate the sarcolemmal Na/Ca exchanger. The caffeine induced I_{NCX} current density was significantly larger in hiPSC-CMs than in rat neonatal cardiomyocytes (~2.5pA/pF vs. ~1.0pA/pF) [13]. Hwang et al also compared the caffeine induced Ca^{2+} release of hiPSC-CMs with those of adult ventricular myocytes, but found significantly lower I_{NCX} values of 0.6 pA/pF in hiPSC-CM as compared to 2.5 pA/pF in our hiPSC-CMs [129]. The discrepancy between the two reported values is not readily apparent, but it might be related to developmental stage of hiPSC-CMs used or concentrations of intracellular Ca^{2+} buffers introduced via the patch pipette in the two studies.

Fractional Ca^{2+} release (ratio of I_{Ca} to caffeine triggered Ca^{2+} release), appears to vary significantly among hiPSC-CMs, ranging from 0.3–0.6, depending in part on the age of cells in culture and the procedures used to differentiate them. The smaller values of fractional release in hiPSC-CMs as compared to adult cardiomyocytes (0.8–0.9) may reflect the development of subcellular couplons of SR and their proximity to calcium channels that gate them, rather than differences in the mode or mechanisms of their activation, as also reflected in the studies comparing calcium channel inactivation kinetics in 3–5 day versus the 6–8day hiPSC-CM cultures [142].

2.1d. Ca^{2+} sparks.—Ca^{2+} release from ryanodine receptor clustered in a dyadic junction has been shown to generate localized rise of intracellular Ca^{2+} called Ca^{2+} sparks. In rat ventricular myocytes, Ca^{2+} sparks represent the release of ~100,000 calcium ions. The morphology of Ca^{2+} sparks in the subsarcolemmal space of rat ventricular cells, imaged with high resolution TIRF microscopy, compares well to sparks recorded with confocal microscopy [150]. Using TIRF imaging with higher spatial resolution (0.1 μm), we found that the majority of spontaneously occurring sparks in hiPSC-CMs were of brief duration, had spatiotemporal properties analogous to those of adult rat cardiomyocytes, ranged between 40–60ms in duration, had occurrence frequency of 10–40 sparks/s in different cells, see fig 4 [4, 151]. In sharp contrast, TIRF-imaging of Ca^{2+} sparks in adult rat ventricular cells showed lower occurrence frequencies (7.1 sparks/cell/s) and shorter durations (~15ms) [152]. Confocal line scan recording of sparks in hiPSC-CMs from different groups show occurrence frequencies ranging from 0.007 sparks/μm^2/s, [138], to ~4sparks/100μm/s [133]. Other groups, have identified stochastic and repetitively occurring spontaneous Ca^{2+} sparks at the same cellular sites with duration at half maximum amplitude of 30.9±0.6ms vs. 26.1±1.7ms in adult rat CMs [11]. Ca^{2+} sparks in hiPSC-CMs appear to be predominately

triggered by activation of L-type Ca^{2+} channels, blocked by nifedipine and enhanced in frequency by elevation of extracellular Ca^{2+} or 50nM ryanodine [11].

2.2. IP_3-gated Ca^{2+} pools

IP_3-gated Ca^{2+} signaling pathway is thought to have an important role in immature developing hearts, playing a critical role in nuclear signaling during growth and development [125, 153]. Although the primary Ca^{2+}-signaling pathway in adult mammalian cardiomyocytes occurs through activation of RyR2-gated store, in some myocytes and at different stages of their development, or humoral regulation, there may be significant contribution from the IP_3-gated calcium stores [125, 153]. IP_3R-gated Ca^{2+} release is expressed both in endoplasmic reticulum and in the nuclear envelop [154]. While functional role of IP_3R in beat-to-beat regulation of cardiomyocyte contractility remains clouded, an early study shows that IP_3R mRNA levels were about 50-fold lower than those of RyR2 in adult cardiac myocytes [155]. Nevertheless, abundant levels of IP_3R protein has been reported in embryonic, neonatal, and adult atrial myocytes [123, 125, 156, 157]. This has led to the suggestion that the spontaneous pacing activity of embryonic myocytes and stem cell-derived cardiomyocyte may depend on IP_3-mediated Ca^{2+} signaling [158, 159]. In this respect, since IP_3-gated Ca^{2+} signaling is reported to trigger both sub-sarcolemmal and perinuclear Ca^{2+} releases in developing cardiomyocytes [125], it is possible that the sub-sarcolemmal Ca^{2+} release would activate the Na^+/Ca^{2+} exchanger causing the depolarization of the surface membrane and setting off pacing, while Ca^{2+} released at the nuclear envelop may serve to replenish the SR stores or have a transcriptional cellular role [160]. Cytoplasmic Immuno-staining studies in newborn mice also show that IP_3Rs are mostly distributed around the nucleus consistent with the findings in hiPSC-CMs and their possible involvement in modulation of Ca^{2+}-signaling [137, 161]. Consistent with this idea, IP_3R antagonist 2-APB or phospholipase C inhibitor U73122 have been reported to slow significantly the kinetics and decrease the amplitude of spontaneously activated Ca^{2+} transients in hiPSC-CMs [136]. We have failed to confirm these findings in hiPSC-CMs using U73122 at 5μM concentrations, which only slightly affected the beating frequency of hiPSC-CM, suggesting that IP_3R Ca^{2+} signaling pathway does not play a major role in generation of Ca^{2+} transients and regulation of pacing. In sharp contrast, our studies show that interactions between Ca^{2+} release from the SR and mitochondria contribute significantly to the mechanism of spontaneous pacing in hiPSC-CMs and rat neonatal cardiomyocytes [13].

2.3. Mitochondrial Ca^{2+} signaling

The possible interaction between mitochondrial and SR Ca^{2+} signaling in cardiac myocytes has been a topic of great interest recently. We have examined the contribution of mitochondrial Ca^{2+} signaling to spontaneous beating activity of hiPSC-CM using genetically encoded probes targeted to mitochondrial subunit VIII of cytochrome C. These studies showed both release and uptake of Ca^{2+} by different mitochondrial populations in hiPSC-CM and rat neonatal cardiomyocytes either during spontaneous beating or on rapid application of caffeine [13]. Generally, perinuclear mitochondrial population released Ca^{2+}, though not always, while the peripheral mitochondria took-up Ca^{2+}. FCCP, a mitochondrial uncoupler, at 50nM abolished the spontaneous beating of hiPSC-CM and prolonged the

relaxation time of caffeine-induced Ca^{2+} transients from the SR, suggesting a direct role for mitochondria in the cycling of cytosolic Ca^{2+} uptake and regulation of pacing [13, 162]. These findings support the idea that in developing myocytes (rat neonatal and hiPSC-CMs) Ca^{2+} released from mitochondria may contribute to generation and regulation of spontaneous pacing activity. Consist with our finding, another study reports that spontaneously triggered cytoplasmic Ca^{2+} transients were abolished by 300nM FCCP and 1 μM oligomycin and mitochondrial NCX blocker, CGP-37157, suggesting that mitochondrial Ca^{2+} flux contributes to the spontaneous beating in hiPSC-derived ventricular-like CMs [163]. Irrespective of possible role of mitochondria in spontaneous pacing of hiPSC-CMs, the targeted mitochondrial Ca^{2+} probes when used in combination with cytosolic Ca^{2+} dyes, suggests that mitochondria may play a critical role in uptake of Ca^{2+} under cellular Ca^{2+} overload conditions caused either by caffeine-triggered Ca^{2+} release or sodium withdrawal mediated Ca^{2+} influx on NCX1. Fig.5 shows simultaneous recording of rise and fall of Ca^{2+} in a mitochondrion and in the cytosol of a patch-clamped hiPSC-CM. Note that rapid puffs of caffeine or withdrawal of Na^{+} activate the exchanger current in response to rapid rise and fall of global cytosolic Ca^{2+} followed by rise of mitochondrial Ca^{2+} as recorded by GCamP3 probe targeted to mitochondrial matrix. The mitochondrial Ca^{2+} uptake, though activating with a delay and slower kinetics, outlasts the rise and fall of cytosolic Ca^{2+} back to baseline levels. Given the differences in Ca^{2+} uptake functions of mitochondria based on their proximity to Ca^{2+} release dyadic structures or plasma membrane Ca^{2+} transporters, it is likely that there will be wide distribution in activation kinetics of Ca^{2+} uptake or its release in different populations of mitochondria that cannot be easily detected by imaging techniques that fail to differentiate their ultrastructural cellular locations. Thus, the detailed role of mitochondria in cardiac Ca^{2+} signaling must await the availability of higher resolution imaging technics that use targeted probes to various proteins of Ca^{2+} signaling pathway.

2.4. Maturity of Ca^{2+} signaling in hiPSC-CMs

2.4.a. Spontaneous beating and intracellular rhythmic Calcium Oscillations

—The spontaneously pacing properties of hiPSC-CMs is similar to that observed in rat neonatal cardiomyocytes and is considered to reflect the immature state of the hiPSC-CMs. Supporting this idea spontaneous rhythmic beating is absent or rare in freshly isolated adult ventricular myocytes. Not generally considered, however, are the findings that adult ventricular myocytes when maintained in culture conditions for a week or longer, lose their brick-like shapes, become flat, develop protruding cellular processes, generate repetitive calcium oscillations, and beat rhythmically. It is therefore reasonable to ask whether rhythmic beating is a consequence of myocyte shape changes or whether it represents regression of myocyte to an immature state? We propose the former possibility because Ca^{2+} signaling, as discussed in previous sections of this review, does not show significant differences in its biophysical or pharmacological parameters from those of adult cardiomyocytes to suggest possible regression of myocytes into an immature state. It also remains unrecognized that the spontaneous rhythmic beating, in rat neonatal cardiomyocytes and hiPSC-CMs, is initiated by regular and rhythmic rises and falls of cytosolic Ca^{2+} that persists even when the myocyte membrane potentials are voltage-clamped at a constant holding potentials of-50 or −70mVs, and that the rhythmic Ca^{2+} oscillations are insensitive

to applications of nifedipine (1.0uM) or Cd^{2+}(1.0mM), that prevent activation of any plasma-membrane ionic channels [13]. Since these spontaneous Ca^{2+} oscillations in hiPSC-CMs or neonatal rat cardiomyocytes were blocked by very low concentrations (50nM) of mitochondrial uncoupler, FCCP [13], we considered the possibility that they may originate directly from mitochondria or indirectly through mitochondrial modulation of SR stores [13]. What indeed triggers this rhythmic Ca^{2+} oscillation remains somewhat unclear. Perhaps the mechanisms responsible for cyclic generation ATP initiate directly or indirectly the release of Ca^{2+} from the mitochondria. Considering the large surface area of hiPSC-CMs compared to their small cell volumes, and their robust Ca^{2+} release mechanism, it is likely that the Na/Ca exchanger is effectively activated to depolarize the membrane to trigger spontaneous trains of action potentials. The robust expression of Na/Ca exchanger in hiPSC-CMs, and the paucity of t-tubules in them would further enhance the effectiveness of released calcium to depolarize the membrane and initiate spontaneous rhythmic beating. We propose therefore that spontaneous beating of hiPSC-CMs or neonatal rat cardiomyocytes are caused by changes in myocyte shape that creates large surface areas with smaller cellular volumes, allowing the robust cytosolic rise of Ca^{2+} to depolarize the cell and set off rhythmic spontaneous beating, rather than a genetic consequence of regression to an immature cellular state. It is intriguing to consider whether a similar mechanism may also be active in cardiac pacemaker cells of the mammalian SA-node, where the myocytes are small with surface to volume ratios exceeding unity and have robust Ca^{2+} releases [162].

2.4.b. Cellular maturity and ASIC family of Channels—HiPSC-CMs unexpectedly express acid sensitive ion channels (ASIC), which are absent in adult rat ventricular or neonatal rat cardiomyocytes. ASIC currents in hiPS-CMs show the same biophysical and pharmacological properties as those reported for ASIC currents in neuronal tissues [164, 165]. Significant expressions of proton-gated ASIC currents, ASIC1 mRNA, and ASIC1 protein was reported in 15–60 days old hiPSC-CMs, but only small or undetectable levels in hiPSC-CM cultures older than 100-days post differentiation, suggesting that younger myocytes may have remnants of neuronal signaling pathways [166]. The presence of ASIC family of proteins in hiPSC-CM lines may be a consequence of ancestral dermal-fibroblasts origins of these cells, but only low or undetectable levels of ASIC mRNA or proton-gated currents was found in dermal fibroblast [166]. Measurements of ASIC currents in cardiomyocytes derived from hematopoietic cells may be helpful in probing this possibility, but remain to be carried out.

2.4.c Hormonal and metabolic procedures to promote maturity—To promote maturation of hiPSC-derived cardiomyocytes, multiple approaches have been attempted that include long term maintenance of cells in culture, hormone treatments, 3D culture conditions, mechanical stretching, electrical stimulation, and addition of fatty acids to the culture media [133, 167–170]. It appears that maintaining hiPSC-CMs in culture for prolonged periods (80–120days) can promote some degree of maturation in cellular ultrastructure and Ca^{2+} handling properties, such as enhanced rate of Ca^{2+} release and uptake, but with no significant effects on the amplitude of Ca^{2+} transients [131]. In another study, maturation in culture was accompanied by tight packing of myofibrils forming parallel arrays of sarcomeres, accompanied by the appearance of mature Z-, A-, H-, and I-

bands, but not M-bands in 180-days hiPSC-CMs cultures, where M-bands became detectable in 360 day [169]. In sharp contrast, Hwang et al report that although 15 day hiPSC-CMs exhibit lower twitch tensions, Ca^{2+} transients and SR Ca^{2+} stores compared to 21 day old hiPSC-CMs, the Ca^{2+} handling properties did not continue to mature further in 21 to 30 day myocytes [129].

Tri-iodo-l-thyronine (T3) is reported to increase the hiPSC-CM myocyte size, sarcomere length, mitochondrial respiratory capacity, where the enhancement of contractile force is accompanied by increases in rates of calcium release and reuptake and SR-ATPase expression [168]. Similarly, fatty acid-treatment of hiPSC-CMs appears to also improve contractile force and kinetics of calcium transients as well as action potential upstroke velocity [167]. Combined use of thyroid hormones and dexamethasone appear also to induce t-tubular development, enhance the CICR, and EC-coupling of hiPSC-CMs grown on matrigel [133]. A combination of biochemical factors, thyroid hormone, dexamethasone, and insulin-like growth factor-1 (TDI) are also reported to enhance hiPSC-CMs maturation in 3D cardiac micro-tissues. In such cultures, the levels of t-tubule-associated genes junctophilin2 and RyR2, increased significantly when cardiomyocytes were treated with TDI. Similarly, the mRNA and protein levels of sarcoplasmic reticular Ca^{2+} ATPase and the Na/Ca exchanger were also significantly increased in the treated myocytes [171]. Thus, it appears that manipulating hiPSC-CM culture environment can lead to more robust EC-coupling parameters, but it is unclear whether these procedures alter the fundamental or qualitative nature of gating of Ca^{2+} release by I_{Ca}, the sensitivity of RyR2 to calcium, or pharmacology of EC-coupling. Although great emphasis has been placed on development of t-tubular network as an indicator of cardiomyocyte maturity, it must be noted that the mammalian atrial myocytes, with their paucity of t-tubules, have only minor Ca^{2+} signaling differences as compared to ventricular cells that have fully developed t-tubular network. It is likely that Ca^{2+} signaling and its regulation evolves and matures early in myocyte development, long before the myocyte attains its adult structural form. Nevertheless, maturation studies are likely to provide procedures to create metabolically and structurally more robust myocytes that could better survive the challenging micro-environments of infarcted tissue when transplanted into the diseased heart.

III. Conclusion and future trends and directions

In this review, we have attempted to compare Ca^{2+} signaling parameters of hiPSC-CMs with those of native mammalian cardiomyocytes. The comparative studies suggest that qualitatively hiPSC-CMs have similar Ca^{2+} signaling properties as those of adult cardiomyocytes, but quantitative differences do exist in the magnitude of calcium transients and their kinetics and the ability of I_{Ca} to release fully the RyR2 gated stores of SR. These differences may be, in part, related to subcellular geometry dyadic couplons and their proximity to sarcolemmal calcium channels and absence of t-tubular network in developing hiPSC-CMs, and not to a fundamental difference in CICR mechanism.

The flat and often spindle-shaped hiPSC-CMs with disorganized sarcomeric structures beat spontaneously unlike the adult cardiomyocytes, a property, we believe, arising from their large surface to volume ratios, where the robust calcium release effectively depolarizes the

cells leading to spontaneous rhythmic pacing, quite similar to neonatal rat cardiomyocytes. The finding that NCX currents were two to 3 folds larger in hiPSC-CMs would further enhance their spontaneous beating rates. Despite these *quantitative* differences in the Ca^{2+} signaling parameters, the *qualitative* aspects of Ca^{2+} signaling are extremely similar in hiPSC-CMs and adult cardiomyocytes, suggesting that these cells may be used as reliable models for studies of human heart EC-coupling.

It is critical to recognize, that maturity of cardiac myocytes is a multi-dimensional time- and metabolic-dependent process. It is quite likely that some cellular mechanism mature early while others might be slower to reach maturation. In using hiPSC-CMs as a model of cardiac function or pathology, the structural and biophysical maturity of the signaling pathways to be studied must be taken into account. Clearly, cell morphology and development of sub-cellular architecture must be considered in structural or functional studies dependent on the structural development. Nevertheless, on the central issue, *Cardiac Ca^{2+} signaling of hiPSC-CMs* of this review, once the Ca^{2+} signaling processes of these cells are critically evaluated and compared with those of adult cardiomyocytes, and have been found to be similar, conclusions may be drawn as to the physiology or pathophysiology of the signaling pathway, irrespective of structural difference of the two human cell types. Although quantitative differences should be taken into consideration, it is not sufficient to dismiss hiPSC-CM calcium signaling and pathologies imposed on them by the experimenter, simply based on sweeping statements of cells immaturity. There is, for instance, little difference in Ca^{2+} signaling between atrium and ventricles even though atrium expresses low levels or lacks t-tubular network, as compared to ventricular cells. Structural immaturity does not necessarily indicate functional immaturity of some of the fundamental signaling pathways.

The field of Ca^{2+} signaling in adult cardiomyocytes still remains an active area of research as state-of-the-art technologies ranging from molecular, genetic, electrophysiological and imaging approaches are making it possible to explore in greater details the molecular mechanism regulating cardiac EC-coupling. Even though it is generally accepted that I_{Ca}-gated Ca^{2+} release is the dominant pathway for activation of contraction in the heart, other pathways that include transport of Ca^{2+} across the membrane or Ca^{2+} released from ER and mitochondria are being evaluated for their role in modulation of cardiac Ca^{2+} signaling under pathophysiological or developmental conditions. There still remain questions as to the microarchitecture of dyadic coupling, the couplons, with other ion transporting proteins, the mechanism of voltage-dependence of CICR gain, and the extent to which development or pathology alters these parameters. Transgenic animals either overexpressing or deleted of specific proteins of Ca^{2+} signaling, though have provided some clarity to EC-coupling regulation, have generated more questions as to, for instance why deletion of NCX1, or CSQ2 in adult heart fails to create significant changes in Ca^{2+} release mechanism or induce cardiac pathology?

With the emergence of hiPSC-CMs technology, human cells may become a primary source for basic investigation of human cardiac Ca^{2+} signaling and its regulation, but the issues related to maturity and developmental stages of the cells must be addressed and resolved. Since these cells lend themselves well to genetic engineering, the combined use of hiPSC lines and CRSPR/Cas9 gene editing techniques, that allows introduction of missense

mutations causing cardiac pathology, are making it possible to study the pathophysiology of cardiac disease and its pharmacotherapy systematically in the lab before attempting it in patients afflicted with the disease. Perhaps more compelling is the possibility of applying gene editing technology to probe the sites that bind Ca^{2+} on RyR2 and CSQ2 or are phosphorylated by PKA and CaMKII. We feel that such molecular and genetic engineering techniques will open unlimited vistas in advancing cardiovascular research.

Since the human heart cells are of limited availability, generation of stem-cells derived cardiomyocytes provides a unique source, not only to probe the fundamental processes that underlie Ca^{2+} signaling in human heart, but also address pathophysiological alterations associated with cardiac disease in *in vitro* conditions. Possible use of hiPSC-CMs for cardiac regeneration and transplantation therapy also provides an intriguing possibility, but before using such cells clinically, it is paramount that their physiology is fully explored and understood. It is critical not to use cells with improper Ca^{2+}-handling and electrophysiology that would introduce aberrant sources of Ca^{2+} release and Ca^{2+}-mediated after-depolarizations and/or automaticity resulting in various forms of potentially lethal arrhythmias.

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Highlights

- Functional analysis of Ca^{2+} signaling parameters in human pluripotent stem cell derived cardiomyocytes as compared to adult mammalian cardiomyocytes
- Discussion of maturity level of hiPSC-CMs and whether hiPSC-CMs can serve as reliable models for adult human cardiac myocytes
- Evaluation of contributions of I_{Ca}-gated Ca^{2+} release, Na/Ca exchanger, and mitochondria to hiPSC-CM Ca^{2+} signaling
- Calcium signaling and pathophysiological consequences of genetic deletion or overexpression of proteins of Ca^{2+} signaling pathway in mice
- Qualitatively, hiPSC-CMs serve as a good model of adult mammalian cardiac Ca^{2+} signaling despite the *quantitative* differences between the two cell types

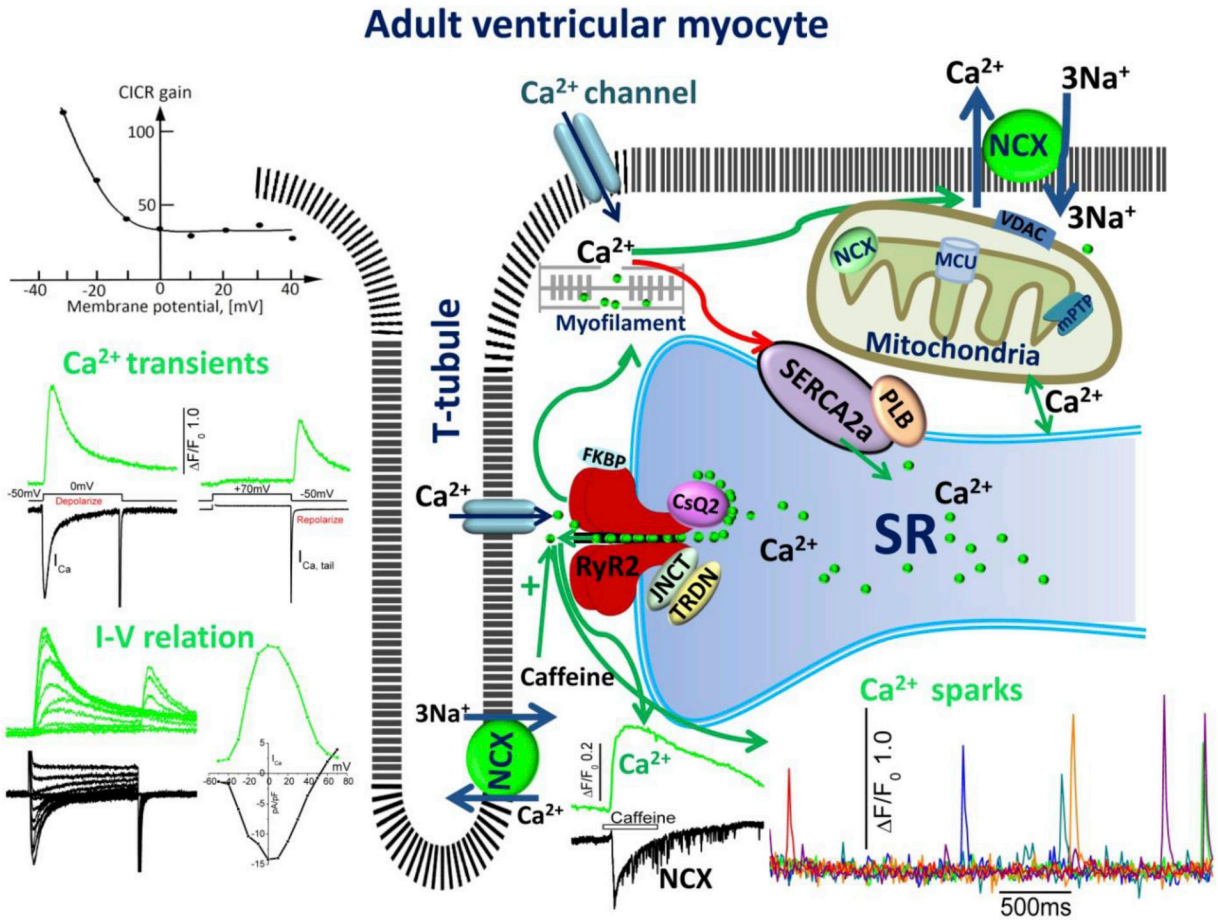

Fig.1.

Excitation-contraction and Ca^{2+} cycling in adult ventricular myocytes. The schematic shows cell surface membrane and t-tubular expression of L-type Ca^{2+} channel, Na/Ca exchanger (NCX), and sarcoplasmic reticulum (SR) expression of Ryanodine receptors (RyR2) in dyadic couplon, as well as SERCA2a/PLB Ca^{2+} uptake, and calsequestrin the storage proteins of the SR. The green arrows depict the influx of Ca^{2+} via the Ca^{2+} channels, activating RyR2, the release of SR Ca^{2+} into the cytoplasm and its binding to myofilaments. Relaxation is initiated by reuptake of Ca^{2+} into the SR via the Ca^{2+} pump (red arrow) and extrusion by NCX across the membrane and possible sequestration by mitochondria. The figure also shows representative traces of I_{Ca} activated by depolarization to zero and by repolarization from +70mVs (I_{Ca} tail current), and the accompanying Ca^{2+} transients, the bell shaped I-V relation of I_{Ca} and Ca^{2+} transients, and voltage-dependence of CICR gain. Also shown are the time courses of Ca^{2+} sparks, activation of Ca^{2+} transients by caffeine and the accompanying I_{NCX}. Also depicted are: TRDN, triadine, JNCT, Junctin, associated with RyR2, and mitochondrial NCX, MCU, Ca^{2+} uniporter, and anion channel VDAC.

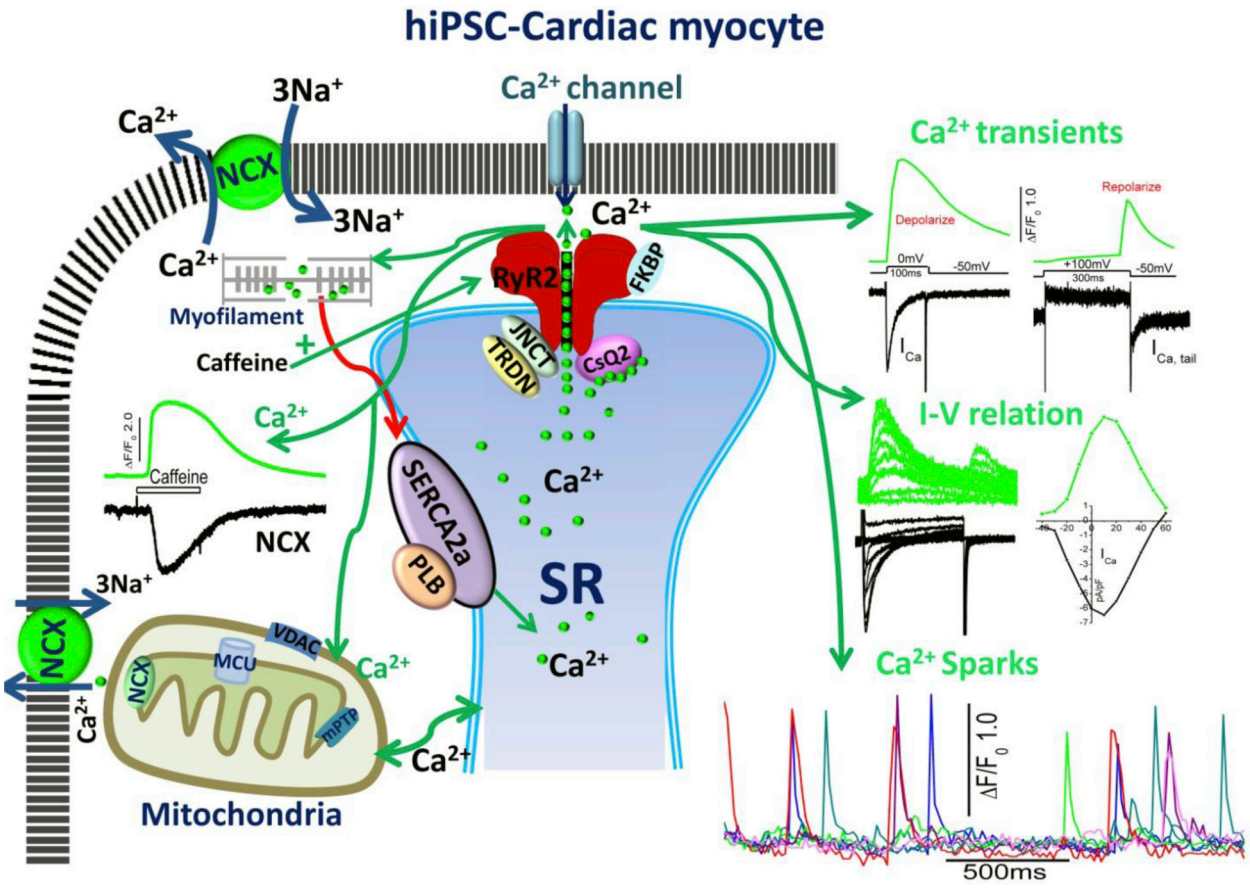

Fig.2.

Excitation-contraction and Ca^{2+} cycling in hiPSC-CMs. The schematic shows cell surface membrane L-type Ca^{2+} channel, Na/Ca exchanger (NCX), and sarcoplasmic reticulum (SR) expression of Ryanodine receptors (RyR2) in dyadic couplon, as well as SERCA2a/PLB Ca^{2+} uptake, and calsequestrin the storage proteins of the SR. The green arrows depict the influx of Ca^{2+} via the Ca^{2+} channels, activating RyR2, the release of SR Ca^{2+} into the cytoplasm and its binding to myofilaments. Relaxation is initiated by reuptake of Ca^{2+} into the SR via the Ca^{2+} pump and extrusion (red arrow) by NCX across the membrane and possible uptake by mitochondria. The figure also shows representative traces of I_{Ca} activated by depolarization to zero and by repolarization from +100mVs (I_{Ca} tail current), and the accompanying Ca^{2+} transients, and the bell shaped I-V relation of I_{Ca} and Ca^{2+} transients. Also shown are the time courses of Ca^{2+} sparks, activation of Ca^{2+} transients by caffeine and the accompanying I_{NCX}. Also depicted are: TRDN, triadine, JNCT, Junctin, associated with RyR2, and mitochondrial NCX, MCU, Ca^{2+} uniporter, and anion channel VDAC. The major difference between hiPSC-CM and adult ventricular myocytes is the absence of t-tubules.

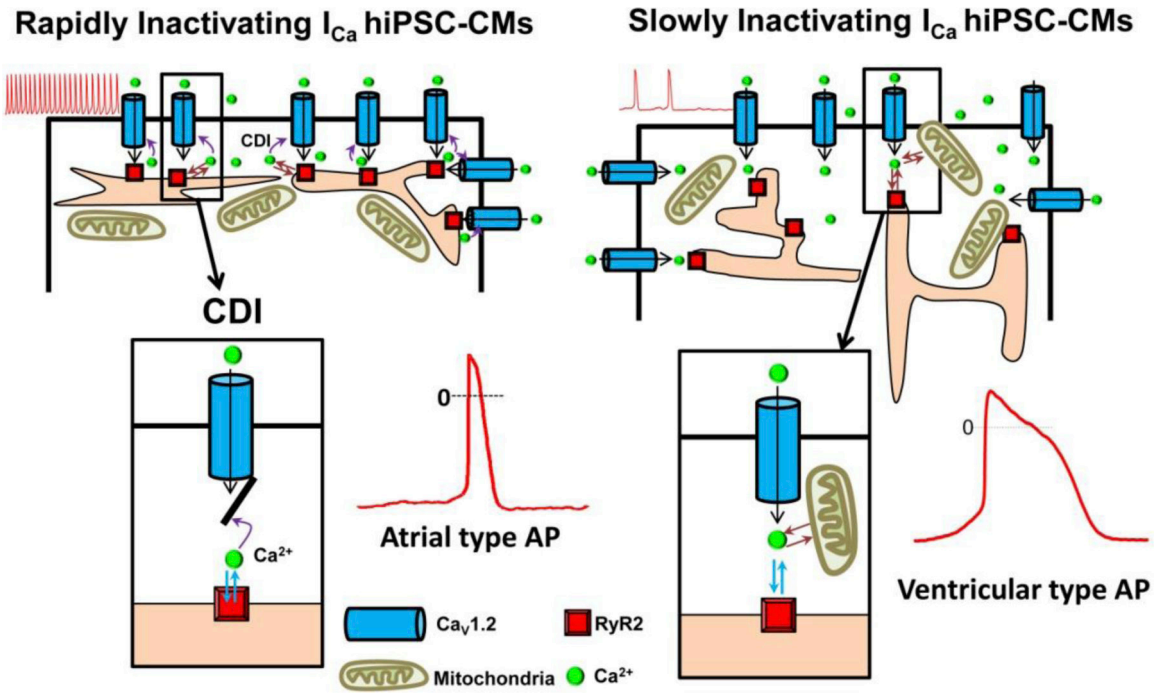

Fig.3.

Two cell-type populations of hiPSC-CMs were identified based on the kinetics of I_{Ca} inactivation. The 3–4 day post-dissociation smaller hiPSC-CMs, expressed predominantly rapidly inactivating I_{Ca} (left panel), shorter action potentials (AP), "*atrial-type"* and rapid rates of spontaneous activity (red traces) whereas the 6–8 day post-dissociation hiPSC-CMs were larger, expressed mostly slowly inactivating I_{Ca} (right panel), longer action potential durations ("*Ventricular-type*"), and slower rates of spontaneous activity. Enlarged cartoon of calcium dependent inactivation, CDI, responsible for the differential rates of I_{Ca} inactivation and durations of APs, showing close proximity of Ca^{2+} channel with SR release machinery in atrial type APs (left box), and at larger distances in ventricular APs, (right box).

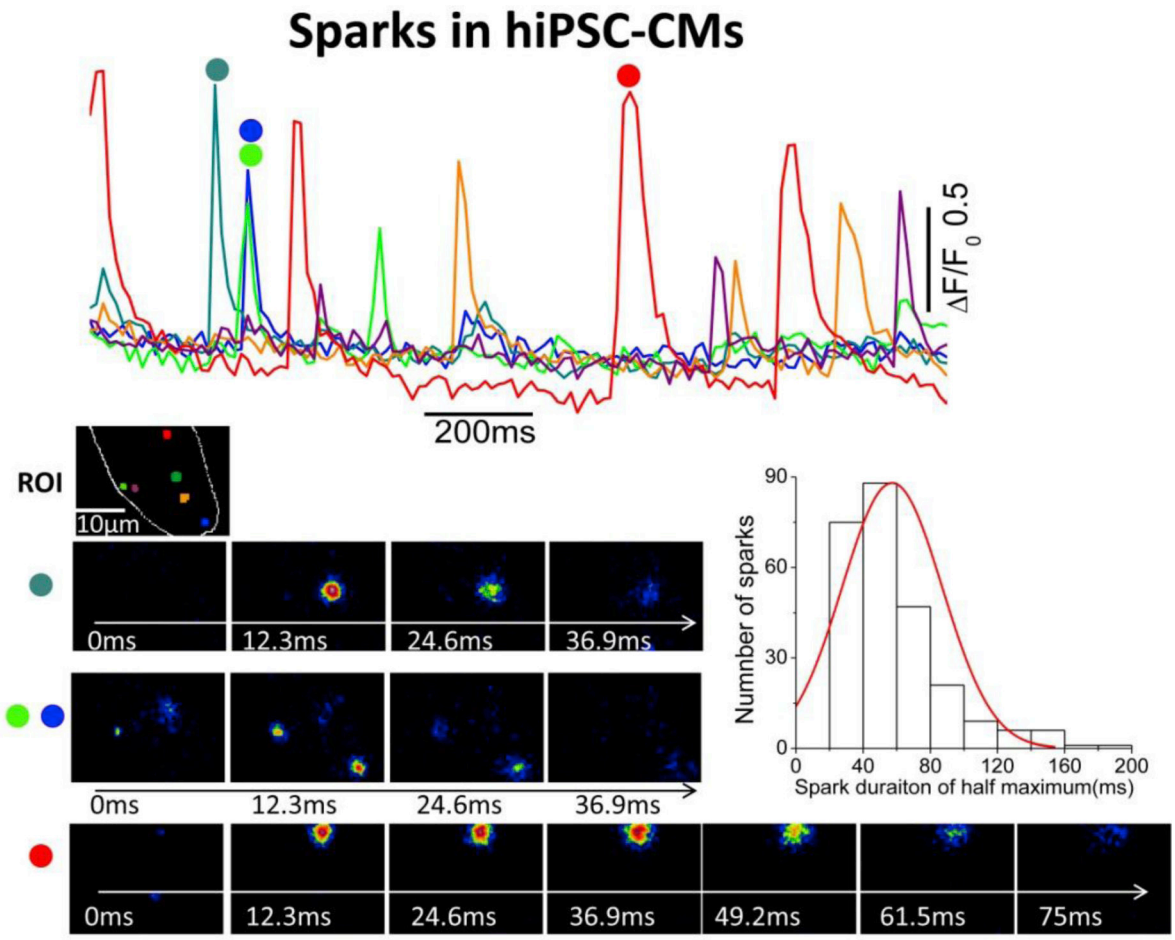

Fig.4.

Ca^{+} Sparks in hiPSC-CMs recorded with TIRF microscopy. The color traces show the time course of normalized Ca^{2+}-dependent fluorescence changes at the selected color-coded regions (ROI) in a single hiPSC-CM. The images show the example of evolution of sparks at different color-coded regions. The sparks were recorded at 12.3ms/frame, ~80Hz. The teal, green and blue sparks are developed and decayed much faster as compared to the red sparks. The histogram shows the distribution of spark durations of half maximum.

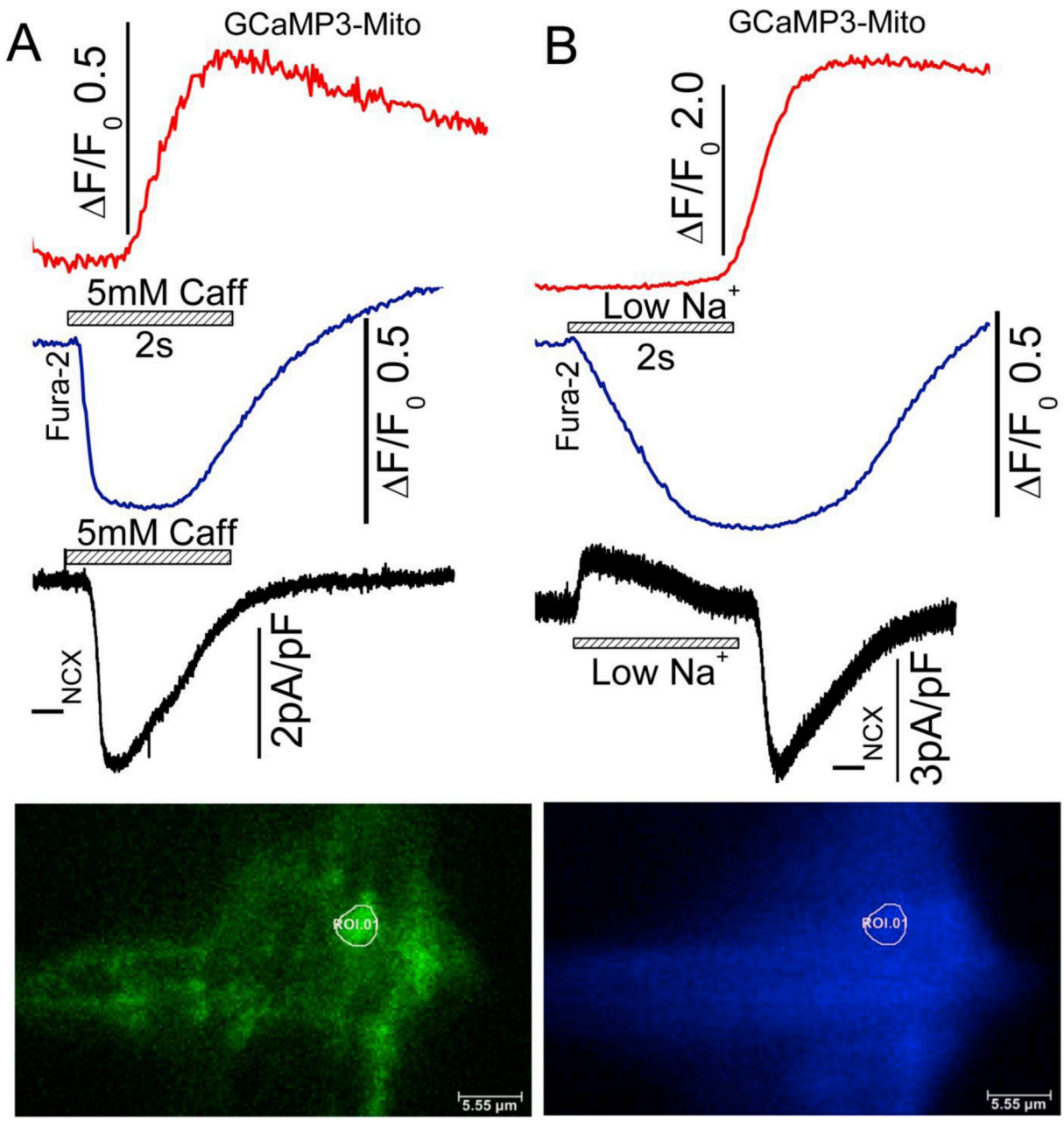

Fig.5.

Simultaneously recorded TIRF images of mitochondrial and cytosolic Ca^{2+} signals in a single patch-clamped hiPSC-CM, in response to Na^+ withdrawal (Na^+ replaced with TEA) and 5mM caffeine applications. hiPSC-CMs were infected with GCaMP3-mito probe for 72 hours and dialyzed with 0.1mM Fura-2 salt through patch pipet during the experiment. Withdrawal of Na^+ activates an outward I_{NCX} and its replacement generates a large slowly decaying inward current, that accompanies the rise and fall of cytosolic calcium. The mitochondrial Ca^{2+} signals recorded from a single mitochondrion (white circle ROI.01 show significant delays when compared to the activation of I_{NCX} or rise of cytosolic Ca^{2+}. The blue traces represent cytosolic Fura-2 Ca^{2+} signal from the same cellular locations. Note that

the rise of mitochondrial Ca^{2+} lags significantly behind the rise in mitochondrial Ca^{2+}. The delays in mitochondrial Ca^{2+} uptake is shorter on release of SR Ca^{2+} compared to withdrawal of Na^{+}. The black traces represent the I_{NCX} currents activated by Na^{+} withdraw and caffeine application. GCaMP3-mito probe was excited at 488nm, where increasing fluorescence signal indicates increases in mitochondrial Ca^{2+}. Fura2 was emission signals measured at 405 nm (decreasing fluorescence indicates increase in cytosolic Ca^{2+}). Temperature 35 degrees C.