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INTRACELLULAR CA^{2+} STORE IN EMBRYONIC CARDIAC MYOCYTES

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1. ABSTRACT

In mature cardiac myocytes, Ca^{2+} influx through the L-type Ca^{2+} channel activates the ryanodine receptor and triggers Ca^{2+} release from the sarcoplasmic reticulum (SR). This Ca^{2+} signal amplification, termed Ca^{2+}-induced Ca^{2+} release (CICR), occurs within the junctional membrane complex between the plasma membrane and the SR, and is essential for cardiac excitation-contraction (E-C) coupling. On the other hand, Ca^{2+} available during E-C coupling is predominantly derived from Ca^{2+} influx in embryonic cardiac myocytes. To examine the role of the intracellular Ca^{2+} store in immature cardiac myocytes, we have generated knockout mice lacking the cardiac type of the ryanodine receptor (RyR-2), or junctophilin (JP-2) contributing to formation of the junctional membrane complex. Both RyR-2- and JP-2-knockout mice show lethality at early embryonic stages immediately after beginning of heart beating. The loss of RyR-2 produced abnormal SR elements exhibiting vacuolated structures and Ca^{2+}-overloading in embryonic cardiac myocytes. In JP-2-deficient cardiac myocytes, formation of junctional membrane complexes, called pheripheral couplings, was disturbed, and abnormal Ca^{2+} transients without spatial and temporal synchronization were observed. Therefore, the knockout mice have demonstrated that RyR-2-mediated Ca^{2+} release at the junctional membrane complex is essential for cellular Ca^{2+} homeostasis in immature cardiac myocytes.

Table 1. Features of ryanodine receptor family members

RyR subtype	Locus	Tissue distribution	Knockout phenotype
Mammalian			
• RyR-1	Mouse 7A2-B3 Human 19q13.1	Skeletal Muscle, Brain	Neonatal Lethality, Respiratory Failure
• RyR-2	Mouse 13 Human 1q42-43	Cardiac & Smooth Muscles, Brain	Embryonic Lethality, Heart Failure
• RyR-3	Mouse 2E5-F3 Human 15q14-15	Skeletal & Smooth Muscles, Brain	Impaired Learning/Memory, Hyperlocomotion
Invertebrate			
• Nematode RyR	Chromosome V K11C4.5	Muscle Cells	Hypolocomotion
• Fruit Fly RyR	Chromosome II Position 44F	Muscle Cells	Embryonic &Larval Lethality, Muscle Dysfunction

2. INTRODUCTION

The ryanodine receptor (RyR) constitutes a major class of intracellular Ca^{2+} release channels that mediate Ca^{2+}-induced Ca^{2+} release (CICR), a mechanism that enhances cytoplasmic Ca^{2+} concentrations during excitation-contraction (E-C) coupling (1). The RyR purified from skeletal muscle has been shown to form a homotetramer with the characteristic "foot" structure which spans the gap between the membranes of the sarcoplasmic reticulum (SR) and transverse tubule (2). The monomeric RyR is composed of ~5000 amino acid residues with the carboxyl-terminal channel region containing transmembrane segments and the remaining large cytoplasmic portion constituting the foot structure (3,4). A single gene encoding RyR is found in invertebrates, but mammalian genomes contain three genes for RyR subtypes, namely RyR-1, RyR-2 and RyR-3. The RyR subtypes are different not only in tissue distribution but also in physiological properties (for example,5,6). During E-C coupling in skeletal muscle, opening of RyR-1 is directly controlled by the voltage-gated Ca^{2+} channel/dihydropyridine receptor (DHPR), while in other cell types RyRs are thought to contribute to Ca^{2+} signal amplification by the CICR mechanism.

The junctional membrane complex between the plasma membrane and the endoplasmic reticulum (ER) is common among excitable cells and is thought to provide a structural foundation for crosstalk between ionic channels (7,8). In skeletal muscle, the transverse (T-) tubule and the SR form a junctional complex, designated as the "triad junction" (9), where a proposed direct coupling between DHPR and RyR-1 converts the depolarization signal to Ca^{2+} release from the SR (10-12). Previous studies have demonstrated that the triad junctions are formed in mutant skeletal muscle cells lacking either DHPR or RyR (13,14), suggesting that the physiological coupling between DHPR and RyR requires the junctional membrane complex to be formed by as-yet-unidentified molecules. In our current experiments, junctophilin (JP) subtypes have been identified as major transmembrane proteins at junctional membrane complexes in excitable cells. JP subtypes are most likely to contribute to the stabilization of the junctional membrane complexes by anchoring the ER/SR and interacting with the plasma membrane to provide a structural framework for physical coupling between cell-surface and intracellular channels (15).

Heart muscle cells contain two types of junctional membrane complexes where DHPR (the L-type Ca^{2+} channel) and RyR-2 (the channel responsible for CICR) are functionally coupled. The "diad" is formed by the T-tubular and SR membranes in mature myocytes, and the "peripheral coupling" is composed of the normal cell-surface membrane and SR in immature myocytes. During E-C coupling in mature cardiac myocytes, Ca^{2+} flowing through DHPR binds to RyR and triggers a larger Ca^{2+} release from the SR, generating a Ca^{2+} signal that is essential for contraction (16,17). On the other hand, Ca^{2+} available for E-C coupling in fetal cardiac myocytes is predominantly derived from Ca^{2+} influx through DHPR (18), and therefore the function of the intracellular store in immature myocytes is not yet clear. In this review, we focus on the physiological role of Ca^{2+} release from the developing SR in embryonic cardiac myocytes, with insights given by the observation of heart failure in mutant mice (15,19).

3. RESULTS AND DISCUSSION

3.1. RyR-mutant animals

Cloning studies so far demonstrate that invertebrate and vertebrate genomes contain a single RyR gene and three RyR subtype genes, respectively, and that there is no RyR gene in yeast. In nematode and fruit fly, the RyR genes are predominantly activated in muscle cells. Voltage-dependent Ca^{2+} influx through the DHPR homologue seems to be essential in E-C coupling in invertebrate muscle cells (20). RyRs probably contribute to Ca^{2+} signal amplification as SR Ca^{2+} release channels in invertebrates, because the RyR-knockout nematode exhibits hypolocomotion due to weakened body-wall muscle cells (21), and the RyR-knockout fruit fly shows lethality at larval stages likely due to dysfunction of body-wall muscle (22).

Some general features of RyR subtypes are listed in Table 1. RyR-1 is expressed predominantly in skeletal muscle and weakly in the brain. RyR-1-knockout mice die

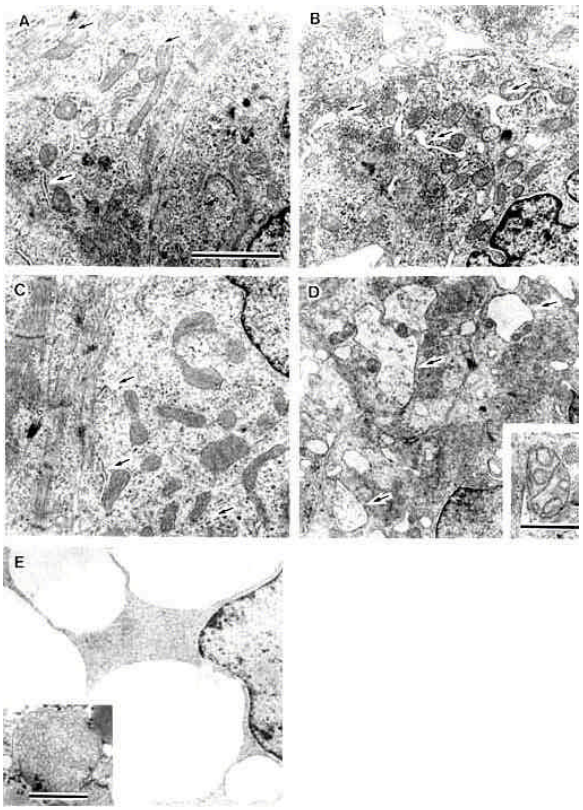

Figure 1. Ultrastructural abnormalities in cardiac myocytes from the RyR-2-knockout embryonic mice. Electron micrographs were obtained from cardiac myocytes in (**A**) E8.5 wild-type, (**B**) E8.5 mutant, (**C**) E9.5 wild-type, (**D**) E9.5 mutant and (**E**) E10.5 mutant embryos. Abnormal vacuoles were detected in the mutant myocytes, and the growth of the vacuoles in size was observed during embryonic development. The normal rER (or developing SR) in wild-type myocytes and the rER carrying swelling parts and abnormal vacuoles in the mutant myocytes are indicated by arrows. The majority of mitochondria contained abnormal tubular cristae in the E9.5 mutant myocytes, and were further swelling in the E10.5 mutant myocytes (insets in **D** and **E**). Scale bars; 5 μm in **A-E**, 1 μm in insets of **D** and **E**. It has been demonstrated that the abnormal vacuoles of the mutant myocytes contain higher Ca^{2+} than the developing SR of wild-type myocytes in other experiments. The data suggest that Ca^{2+} overloading results in the formation of the vacuoles from the SR in the mutant myocytes.

immediately after birth because E-C coupling is abolished in the mutant skeletal muscle (11,23). RyR-2 is predominantly expressed in cardiac muscle and is also distributed in smooth muscle and neurons. RyR-2-knockout mice exhibit embryonic lethality as described below. RyR-3 is detected in skeletal and smooth muscles, brain and certain non-excitable cells at low levels. RyR-3-knockout mice do not show lethality or obvious anatomical abnormalities (24), but bear impaired muscle and brain functions including weakened muscle contraction (25), hyperlocomotion (24) and insufficient learning and memory (26-28).

In human genetic diseases, several mutations have been determined in the RyR subtype genes. Genomic point mutations and resulting amino acid substitutions in the RyR-1 primary structure are responsible for malignant hyperthermia and central core disease, and both diseases are caused by abnormalities in SR Ca^{2+} release in skeletal muscle (29). A recent study found that mutations in the RyR-2 gene underlie catecholaminergic polymorphic ventricular tachycardia (30).

3.2. Cardiac failure in RyR-2 knockout mice

To prepare RyR-2-knockout mice, a deletion mutation was introduced at the first exon of the RyR-2 gene in embryonic stem cells. The resulting knockout mice showed cardiac arrest and lethality at about embryonic day (E) 10.5. Histological analysis demonstrated that cardiac myocytes were irregularly arranged in the hearts (cardiac tubes) from the E9.5 RyR-2-knockout embryos. Ultrastructural analysis using an electron microscopy showed that the ER/SR elements were partly swollen in E8.5 mutant cardiac myocytes, and were further vacuolated at E9.5 and E10.5 (Figure 1). In the analysis with calcium oxalate precipitates, Ca^{2+}-overloading was suggested in the vacuolated ER/SR from the RyR-2-knockout myocytes (data not shown). Moreover, mitochondria exhibited tubular cristae and were swollen in the mutant cardiac myocytes. The abnormal morphological features prior to cardiac arrest suggested that the RyR-2 deficiency primarily damages embryonic cardiac myocytes.

The hearts of both RyR-2-knockout and control embryos show beating at E9.5, and the cardiac myocytes exhibited spontaneous Ca^{2+} oscillations that can be monitored with fluorometric Ca^{2+} indicators (Figure 2). Application of caffeine, an activator of RyR subtypes, evoked Ca^{2+} transients in wild-type cardiac myocytes, but not in the RyR-2-knockout myocytes. Therefore, RyR-2 appears to be solely expressed in embryonic cardiac myocytes, although skeletal and smooth muscle cells contain at least two RyR subtypes. To determine the possible contribution of Ca^{2+} release via RyR-2 to E-C coupling in embryonic myocytes, control myocytes were examined under store-depleting conditions using caffeine and ryanodine (Figure 3). Spontaneous contractions and Ca^{2+} oscillations were still retained after ryanodine treatment, even though depletion of stores was confirmed by the lack of response to subsequent application of caffeine. Therefore, the loss of CICR mediated by RyR-2 does not abolish E-C coupling in the hearts at the early embryonic stages.

On the basis of the above observations, it seems reasonable to conclude that RyR-2 does not play a significant role in Ca^{2+} signaling during E-C coupling in embryonic hearts; instead, it appears that RyR-2-mediated Ca^{2+} release maintains the normal range of luminal Ca^{2+} levels in the developing SR (Figure 7). Our proposal is that in the RyR-2-knockout cardiac myocytes, cytoplasmic Ca^{2+} derived from the extracellular fluid during E-C coupling may gradually accumulate in the developing SR; the cytoplasmic Ca^{2+} that cannot be sequestered by the

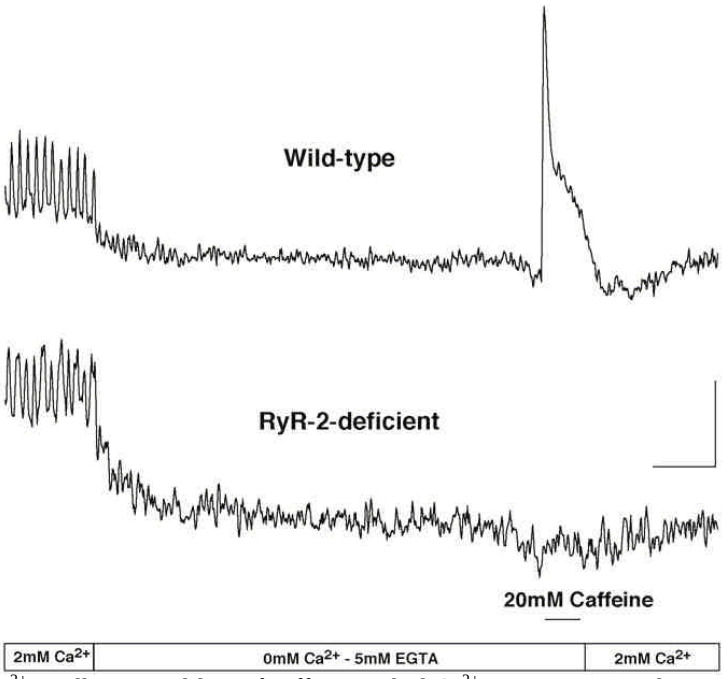

Figure 2. Spontaneous Ca^{2+} oscillations and loss of caffeine-evoked Ca^{2+} transients in cardiac myocytes from E9.5 RyR-2-knockout mice. Intracellular Ca^{2+} concentrations of myocytes from wild-type (upper trace) and RyR-2-knockout (lower trace) embryos were measured with Fluo-3, and the time course of change in fluorescence intensity is shown. Ca^{2+} oscillations in both genotypes were abolished in a Ca^{2+}-free solution containing 5 mM EGTA. The application of 20 mM caffeine in the Ca^{2+}-free solution induced Ca^{2+} transients in wild-type myocytes, but not in the mutant myocytes. The horizontal scale indicates 20 s, and the vertical scale shows 10% change relative to the diastolic level in fluorescence intensity in upper trace and 5% in lower trace.

overloaded SR may then flow into mitochondria, causing defective organelles and/or the abnormal Ca^{2+} homeostasis that leads to cellular dysfunction. Therefore, it is likely that RyR-2 can function as a safety valve for the intracellular Ca^{2+} store in embryonic cardiac myocytes. This conclusion is supported by the result that the vacuolated SR is shared by mutant skeletal muscle from double-knockout mice lacking both RyR-1 and RyR-3 (14). Skeletal muscle contains RyR-1 and RyR-3 as the major and minor components, respectively, but mutant muscle cells lacking either RyR-1 or RyR-3 do not exhibit such severe ultrastructural defects (11,24). In striated muscle cells, the complete loss of Ca^{2+} release channels may produce such abnormalities in SR structure. The molecular mechanism for the vacuolated SR is unclear, because Ca^{2+} is not a major ion in comparison with K^+, Na^+ or Cl^-, and Ca^{2+}-overloading alone can not produce obvious osmotic changes between the SR lumen and the cytoplasm. One possibility is that luminal Ca^{2+}-sensitive K^+ or Cl^- channels may be involved in formation of the vacuolated SR. The predicted channel might be probably shared by skeletal and cardiac myocytes and could have an important physiological role in SR Ca^{2+} handling.

3.3. Identification of JP as component of junctional membrane complex

To search for proteins supporting the structure of the triad junction, we prepared monoclonal antibody (mAb) libraries from mice immunized with membrane vesicles from rabbit skeletal muscle (31). Of the antibodies screened, mAb2510 labeled intracellular rows oriented transversely in the longitudinal skeletal muscle cryosections, and the location of the rows was assigned to interfaces between the A and I-bands, where triad junctions are localized. In the ultrastructural analysis of sections labeled with the immunocolloidal gold, specific labeling was detected near the triad junction and frequently located in the junctional gap between the T-tubule and SR. We named the antigen protein of ~72 kilodaltons (kda) mitsugumin72 or junctophilin type 1 (JP-1). cDNA cloning demonstrated that rabbit JP-1 is composed of 662 amino acid residues, and contains a single transmembrane segment at its carboxyl-terminal end but no amino-terminal signal sequence. Therefore, the bulk of JP-1 is located in the cytoplasmic region of the junctional gap between the SR and T-tubular membranes. In the cytoplasmic region of JP-1, motif sequences of 14 residues, called "MORN motif" sequences, were found repeated eight times (Figure 4A). The putative consensus sequence for this motif is "Tyr-Gln/Glu-Gly-Glu/Gln-Trp-x-Asn-Gly-Lys-x-His-Gly-Tyr-Gly".

To examine functional aspects, JP-1 mRNA was generated in vitro and was injected into amphibian embryos (15). Immunofluorescence observation indicated that expressed JP-1 was localized on the plasma membrane in the embryonic cells (Figure 4B). Electron microscopy revealed junctional complexes between the ER and plasma membrane in the JP-1-expressing cells (Figure 4C); these ultrastructures could not be detected in control cells. Thus,

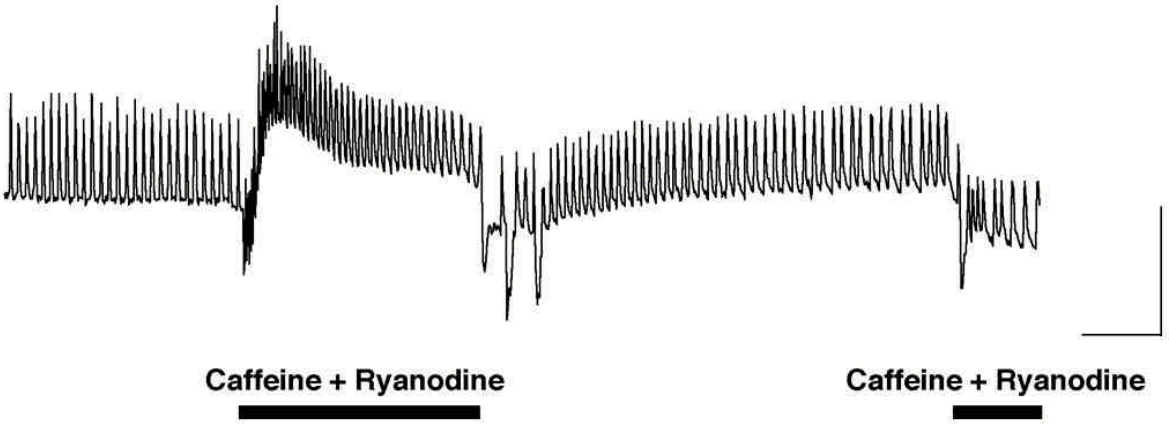

Figure 3. Effects of ryanodine on spontaneous Ca^{2+} oscillations in embryonic cardiac myocytes. The time course of change in fluorescence intensity of Fluo-3 is shown, and representative responses are shown from experiments using E9.5, E10.5 and E11.5 wild-type cardiac myocytes. Ryanodine (100 μM) was applied with caffeine (20 mM) to deplete intracellular Ca^{2+} stores (the binding of ryanodine to RyR is enhanced when the RyR channel is opened by caffeine). Depletion of the stores was confirmed by no response to the secondary application of caffeine. The horizontal scale indicates 20 s, and the vertical scale represents 10% change in fluorescence intensity relative to the diastolic level. Downward deflections were due to movement artifacts of the specimen by solution exchanges.

it is likely that the junctional membrane complexes correspond to the immunoreactive plasma membrane observed histochemically. Furthermore, when the soluble form of JP-1 lacking the carboxyl-terminal transmembrane segment was expressed, immunolabeling was detected specifically on the plasma membrane, but no junctional membrane complexes were formed (data not shown). The subcellular distribution of the truncated JP-1 demonstrates the specific binding affinity of the cytoplasmic domain for the plasma membrane, and the membrane-spanning segment is essential for the generation of the junctional membrane structures. Successive expression experiments of deletion-bearing JP proteins indicated that MORN motifs contribute to specific binding with the plasma membrane in the cytoplasmic region (data not shown). Therefore, it is proposed that JP-1 is involved in the formation of the skeletal muscle triad junction by interacting with the T-tubule and spanning the junctional SR membrane as shown in Figure 4D.

In an attempt to search for JP family members (15,32), we used the cross-hybridization technique and isolated cDNAs encoding JP-2 and JP-3 from libraries derived from heart and brain, respectively. The defined mouse JP subtypes, composed of 660-744 amino acid residues, show homology in sequence (overall ~40% identity among them) and share structural features with rabbit JP-1. The regions containing the MORN motif sequences (~80% identity) and carboxyl-terminal hydrophobic segment spanning the ER/SR membrane (~50% identity) are conserved well among the JP subtypes, whereas the regions of 211-286 residues immediately preceding the transmembrane segment are highly divergent (~6% identity). Based on the regional sequence identities among the subtypes, structural features and genomic organization, six intermolecular domains are proposed in JP subtypes (Figure 4A).

3.4. JP-knockout animals

As in the case of the RyR gene, invertebrate genomes contain a single JP gene, mammalian genomes carry three JP subtype genes, and no JP gene is found in yeast. The nematode JP gene is predominantly activated in muscle cells. Nematodes, in which expression of JP was inhibited by RNA-mediated interference (RNAi), showed hypolocomotion. Taking into account data in the RyR-knockout nematodes (see section 3.1), the hypolocomotion is likely due to the deficiency of junctional membrane structures and the resulting reduction of Ca^{2+} signaling during E-C coupling in muscle cells (33). Mutant phenotype on the JP gene has not yet been reported in fruit fly.

Some general features of JP subtypes are listed in Table 2. Northern and Western blot experiments in mouse and human tissues indicated that JP-1 is predominantly expressed in skeletal muscle, JP-2 is detected in skeletal, cardiac and smooth muscles, and JP-3 is abundantly expressed in the brain (15,32). Therefore, the JP subtypes seem to be distributed throughout excitable tissues and may take part in junctional membrane complexes including the subsurface cisternae. JP-1-knockout mice die within a day after birth and show no milk suckling. In the mutant skeletal muscle containing JP-2 at normal levels, formation of triad junctions is impaired and contraction responses to low-frequency stimuli are reduced. These results suggest that JP-1 and JP-2 are functionally different in skeletal muscle, and that JP-1-mediated formation of triad junctions is required for efficient E-C coupling in newborn mice (34). JP-2-knockout mice exhibit embryonic lethality as described below. JP-3-knockout mice do not show lethality or obvious morphological abnormalities, but bear impaired motor coordination in different tasks (35). The brain-specific expression of JP-3 suggests that irregular functions of certain neurons induce motor uncoordination

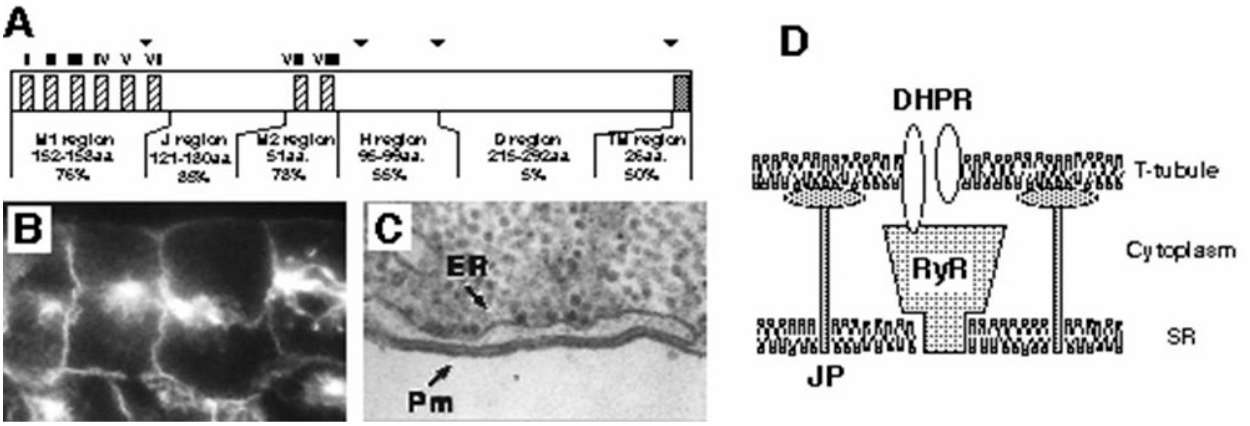

Figure 4. Structure and proposed function of JP. Structural features of the JP subtypes (**A**). Several observations predict six intramolecular domains in JP; M1 region, MORN motif region 1; J region, joining region; M2, MORN motif region 2; H region, putative α-helical region; D region, divergent region; TM region, membrane-spanning region (for details see ref. 32). Amino acid residue number and sequence identity among the JP subtypes are shown in each proposed domain. The locations of introns interrupting the JP subtype-coding sequences in human genome are indicated by arrows; the genomic organization is conserved among the JP subtypes. Analysis of amphibian embryonic cells expressing JP-1 (**B**,**C**). An immunofluorescence image of the cells expressing full-length JP-1 is shown in **B**. Specific staining with monoclonal antibody against JP-1 was observed on the plasma membrane of the embryonic cells. An electron micrograph of JP-1-expressing cells is shown in **C**. Junctional complexes between the plasma membrane (Pm) and the endoplasmic reticulum (ER) were formed in cells injected with JP-1 mRNA. No such ultrastructures were detected in control cells. Proposed role of JP at the triad junction in skeletal muscle (**D**). The subcellular localization and biochemical features suggest that JP contributes to formation of the triad junction, where proposed direct coupling between DHPR and RyR converts the depolarization signal into the cytoplasmic Ca^{2+} signal. As described in the text, skeletal muscle triad junctions contain both JP-1 and JP-2, and they may have different physiological roles.

Table 2. Features of junctophilin family members

JP subtype	Locus	Tissue distribution	Knockout phenotype
Mammalian			
• JP-1	Mouse 1A2-5 Human 8q21	Skeletal Muscle	Perinatal Lethality, Suckling Failure
• JP-2	Mouse 2H1-3 Human 20q12	Skeletal, Cardiac, Smooth Muscle	Embryonic Lethality, Heart Failure
• JP-3	Mouse 8E Human 16q23-24	Brain	Motor Discoordination
Invertebrate			
• JP Subtype	Chromosome I T22C1.7	Muscle Cells	Hypolocomotion
• Fruit Fly JP	Chromosome II Region 30B	?	?

in the mutant mice. It was currently reported that triplet repeat expansions in the JP-3 gene are associated with a human disorder very similar to Hungtington's disease, termed HDL-2, Hungtington's-Disease-Like-2 (36).

3.5. Cardiac failure in JP-2 knockout mice

In the adult mouse heart, an antibody against JP-2 mainly recognized cytoplasmic rows along the Z-lines within myocytes. In fetal mice on E9.5, the looped cardiac tube of the immature heart was immunofluorescence-positive, and the labeled region was in the periphery of cardiac myocytes. These observations suggest that JP-2 is localized on the junctional SR in diads and peripheral couplings. The targeted disruption of the mouse JP-2 gene induced embryonic lethality in the homozygous state. In the E9.5 knockout embryos the hearts showed spontaneous contractions, but the heartbeats were often weak and irregular. More than half of the E10.5 mutants exhibited cardiac arrest and congested peripheral tissues, and the E11.5 mutants showed autolysis after death. Although JP-2 expression is observed throughout muscle cell types in adult mice, neither skeletal nor smooth muscle cells appear to be functionally developed in the early embryonic stages. Therefore, the loss of JP-2 in the mutant embryos affects

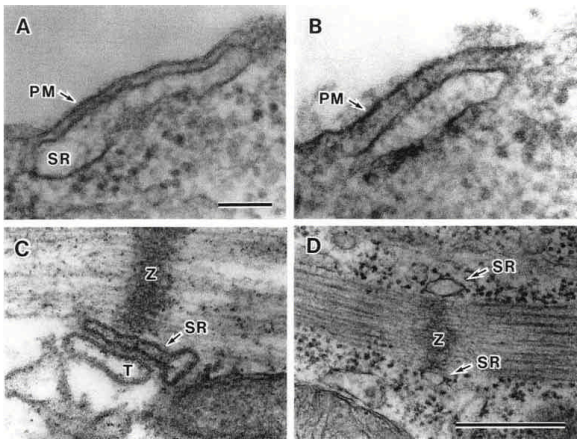

Figure 5. Junctional membrane structures in embryonic cardiac myocytes. Cardiac myocytes from wild-type E9.5 embryos contained two types of junctional complexes between the cell-surface membrane and the SR with gap sizes of ~12 (**A**) and ~30 nm (**B**). In the diad observed in mature cardiac myocytes from adult mice, the gap size between the T-tubular and SR membranes was ~12 nm (**C**). Therefore, the junctional membrane complex bearing the 12 nm gap is likely to correspond to the functional peripheral coupling as the structural foundation for the physiological crosstalk between the DHPR and RyR in the embryonic myocytes. The connection between the SR and Z-line is common to striated muscles, and the structures are also found in immature cardiac myocytes from E9.5 embryos (**D**). PM, plasma membrane; SR, sarcoplasmic reticulum; T, transverse tubule; Z, Z-line. Scale bars, 0.1 μm in **A**-**C** and 0.5 μm in **D**.

mainly cardiac myocytes.

Cardiac myocytes from wild-type E9.5 embryos contained two types of junctional membrane complexes with gap sizes of ~12 and ~30 nm (Figure 5A and B). According to previous reports, the gap size in the diad from mature cardiac myocytes is predominantly ~12-nm (Figure 5C), suggesting that the 12-nm junctions correspond to functional peripheral couplings in embryonic myocytes. Statistical analysis demonstrated that in the JP-2-knockout myocytes the appearance of the 12-nm junction was reduced to only ~10% of the control value; numbers of the junctions per 100 μm plasma membrane in wild-type and mutant myocytes are 12.4±0.2 and 1.5±0.7, respectively. Furthermore, the average length of the 12-nm junctional membrane complex in the mutant myocytes was significantly shorter than that in controls; 0.17±0.06 μm in mutant myocytes and 0.37±0.16 μm in controls. Also, there were no differences between the genotypes in control SR structures, the 30-nm junction and the close association between the SR and Z-line, namely "Z tubule" (37). The deficiency of the peripheral coupling, demonstrated in the mutant myocytes prior to cardiac arrest, clearly supports the hypothesis that JP subtypes contribute to the formation of the junctional membrane complexes in various cell types.

Functional abnormalities of the JP-2-knockout hearts from the E9.5 mutants were examined in Ca^{2+}-imaging analysis. Because cardiac E-C coupling requires Ca^{2+} influx via DHPR, heart beats are abolished under Ca^{2+}-free conditions. In wild-type hearts, all myocytes showed synchronized Ca^{2+} transients, and the transients disappeared in a Ca^{2+}-free bathing solution (Figure 6). However, in mutant hearts from the JP-2-knockout embryos, a large number of myocytes showed irregular Ca^{2+} transients that were not synchronized with the heartbeats and occurred randomly in space. Although the random transients were observed in all mutant hearts examined, the frequency of myocytes showing the abnormal transients was higher in the mutant hearts with infirm heart beatings. Moreover, the random transients were retained in the Ca^{2+}-free bathing solution, albeit the frequency was significantly reduced. Of the RyR subtypes only RyR-2 is expressed in the embryonic cardiac myocytes as described in the above section. The random transients in the JP-2-knockout myocytes were abolished by combined application of caffeine and ryanodine, and intracellular Ca^{2+} waves were observed during the random Ca^{2+} transients (data not shown). These results indicate that the abnormal transients in the JP-2-knockout hearts are evoked by Ca^{2+} release from the SR through RyR-2.

RyR-2 expressed in embryonic cardiac myocytes is prone to regenerative Ca^{2+} release responses even under resting Ca^{2+} levels because of its high Ca^{2+} sensitivity for channel activation. Thus, RyR-2 produces Ca^{2+} waves and oscillations independent of Ca^{2+} influx when expressed in cultured skeletal myocytes that have high SR Ca^{2+} contents (5). Moreover, abnormal intracellular Ca^{2+} waves have been observed in cardiac myocytes that have high SR Ca^{2+} loading and have been implicated in arrhythmia (38,39). As described above, the data from the RyR-2-knockout hearts suggest that SR Ca^{2+} levels can be increased by the reduction of RyR-2-mediated Ca^{2+} release in embryonic cardiac myocytes. It may be reasonable then that the loss of JP-2 disconnects the physiological coupling between cell-surface DHPR and SR RyR-2, because the deficiency of the functional peripheral coupling likely prevents close association of the channel molecules and interferes with effective activation of RyR-2 by DHPR-mediated Ca^{2+} influx. The resulting reduction of SR Ca^{2+} release may temporally produce the Ca^{2+}-overloaded SR in the mutant myocytes. The overloaded Ca^{2+} may be randomly released through RyR-2, and the generated Ca^{2+} rise in a microdomain may be expanded to neighboring SR regions by CICR to produce the intracellular Ca^{2+} waves (Figure 7). The random Ca^{2+} transients in the mutant heart probably produce local extrasystoles and infirm heartbeats.

Mutant cardiac myocytes from both RyR-2-kockout and JP-2-knockout embryos contained mitochondria that have irregular internal structures. The abnormal Ca^{2+} homeostasis may produce mitochondria dysfunction and further toxic effects on the mutant myocytes. Stress and damage in mitochondria provokes permeability transition and triggers the cytochrome release as the cell-death signal (40). Cardiac failure in the mutant embryos likely underlies the cell death triggered by damaged mitochondria. Furthermore, possible crosstalk in Ca^{2+} handling between the SR and mitochondria might be

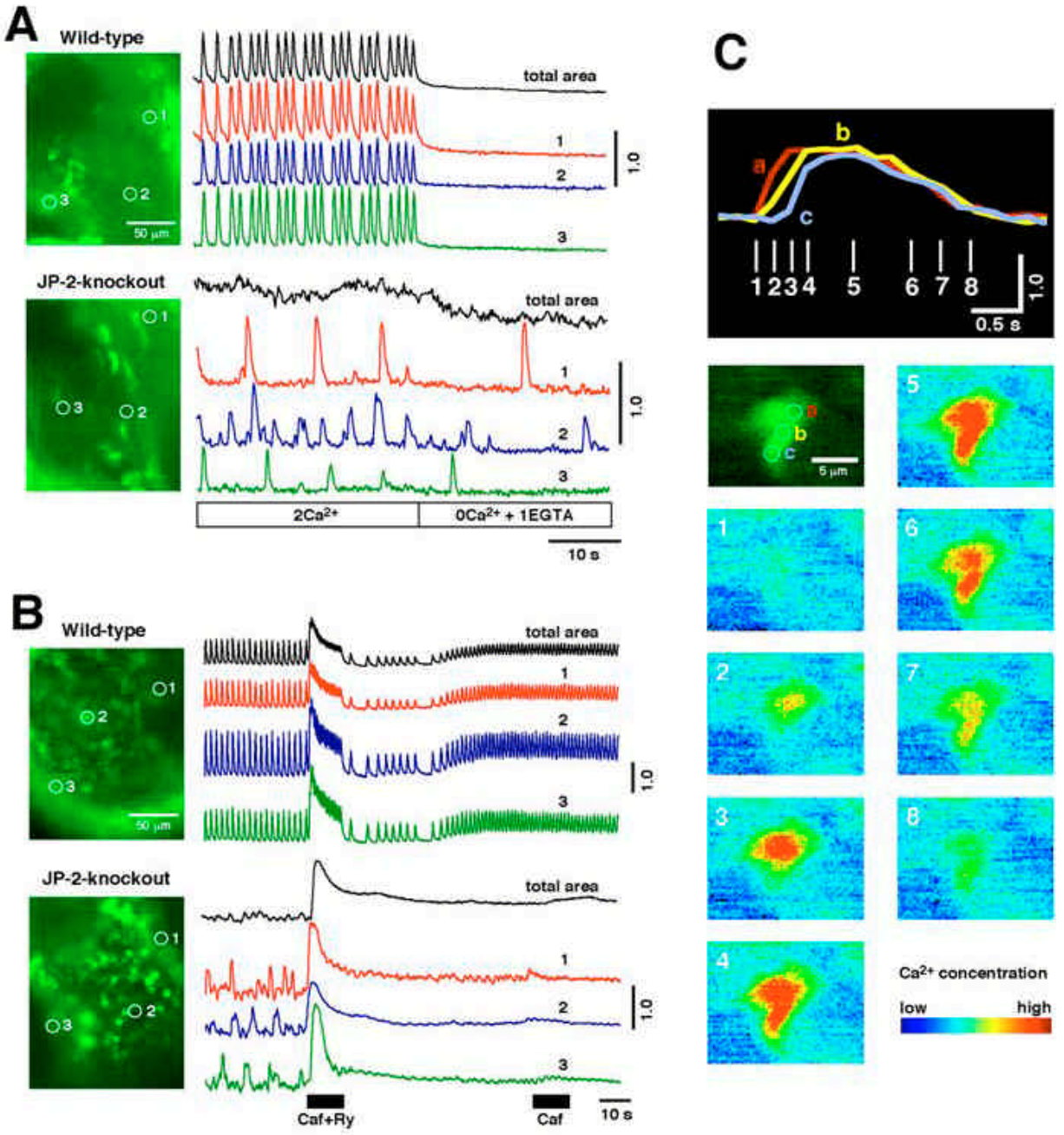

Figure 6. Abnormal Ca^{2+} transients in cardiac myocytes from the E9.5 JP-2-knockout embryos. Intracellular Ca^{2+} changes during spontaneous oscillations in wild-type (upper traces) and JP-2-knockout (lower traces) cardiac myocytes from the cardiac tubes were measured in the normal bathing solution and the Ca^{2+}-free solution containing 1 mM EGTA (**A**). The myocytes, examined for traces (1-3), are indicated by white circles in the fluorescence image of the embryonic heart. Abolishment of the random Ca^{2+} transients in the mutant hearts under Ca^{2+}-store-depleted conditions (**B**). The effects of the application of 20 mM caffeine (Caf) and 100 μM ryanodine (Ry) on the Ca^{2+} transients were examined in the normal bathing solution. In wild-type hearts, rhythm disturbances of heart beating were often observed after the caffeine applications with or without ryanodine. Spatial and temporal patterns of the random Ca^{2+} transient in a single JP-2-knockout cardiac myocyte (**C**). The upper traces show the time-courses of changes in fluorescence intensity during a Ca^{2+} transient; the intracellular regions examined are indicated in the fluorescence image of the myocyte (a-c). Intracellular Ca^{2+} concentrations during the Ca^{2+} transient are shown in pseudo-color images at the frames indicated in the upper panel (1-8). The vertical scales are indicated in ΔF/F0.

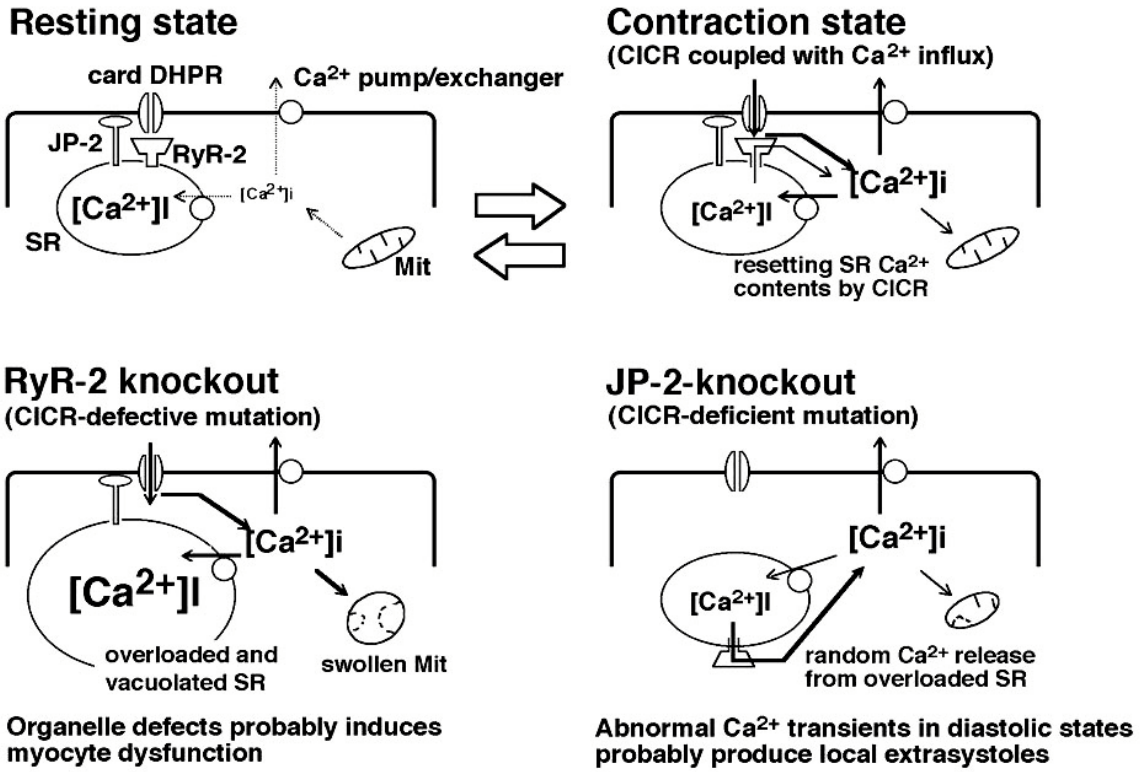

Figure 7. Proposed roles of JP-2 and RyR-2 on the SR in embryonic cardiac myocytes. Based on the results of gene targeting studies, major intracellular Ca^{2+} flows in embryonic cardiac myocytes are illustrated. Even though contributing slightly to Ca^{2+} transients during E-C coupling, RyR-2-mediated Ca^{2+} release coupled with Ca^{2+} influx is essential for regulating Ca^{2+} contents of the SR to maintain the cellular functions in embryonic cardiac myocytes. The loss of RyR-2 seems to induce Ca^{2+}-overloading in the developing SR and produce SR vacuoles. JP-2-mediated formation of peripheral coupling appears to be essential for functional crosstalk between DHPR and RyR. Structural abnormalities of mitochondria are thought to be induced by irregular Ca^{2+} homeostasis in RyR-2 and JP-2 knockout myocytes.

important to understand pathological defects in the failing hearts.

4. PERSPECTIVE

In the embryonic heart, Ca^{2+} release from the SR has no important role in Ca^{2+} signaling during E-C coupling. However, our mutant mice lacking RyR-2 or JP-2 demonstrated that SR Ca^{2+} release coupled with Ca^{2+} influx is essential for cellular Ca^{2+} homeostasis in embryonic cardiac myocytes, and further imply that reduction of Ca^{2+} release and abnormalities in membrane structures may cause cardiac failure and heart arrest. Therefore, it is important to examine micro-structures at cellular and organelle levels for understanding RyR-mediated Ca^{2+} release from intracellular stores. Current progress in the E-C coupling research field requires the combination of physiology with molecular biology. Further unification of molecular physiology with morphology and cell biology would be needed in future studies in this field.

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