Sarcoplasmic Reticulum Calcium Overloading in Junctin Deficiency Enhances Cardiac Contractility but Increases Ventricular Automaticity

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Background—Abnormal sarcoplasmic reticulum calcium (Ca) cycling is increasingly recognized as an important mechanism for increased ventricular automaticity that leads to lethal ventricular arrhythmias. Previous studies have linked lethal familial arrhythmogenic disorders to mutations in the ryanodine receptor and calsequestrin genes, which interact with junctin and triadin to form a macromolecular Ca-signaling complex. The essential physiological effects of junctin and its potential regulatory roles in sarcoplasmic reticulum Ca cycling and Ca-dependent cardiac functions, such as myocyte contractility and automaticity, are unknown.

Methods and Results—The junctin gene was targeted in embryonic stem cells, and a junctin-deficient mouse was generated. Ablation of junctin was associated with enhanced cardiac function in vivo, and junctin-deficient cardiomyocytes exhibited increased contractile and Ca-cycling parameters. Short-term isoproterenol stimulation elicited arrhythmias, including premature ventricular contractions, atrioventricular heart block, and ventricular tachycardia. Long-term isoproterenol infusion also induced premature ventricular contractions and atrioventricular heart block in junctin-null mice. Further examination of the electrical activity revealed a significant increase in the occurrence of delayed afterdepolarizations. Consistently, 25% of the junctin-null mice died by 3 months of age with structurally normal hearts.

Conclusions—Junctin is an essential regulator of sarcoplasmic reticulum Ca release and contractility in normal hearts. Ablation of junctin is associated with aberrant Ca homeostasis, which leads to fatal arrhythmias. Thus, normal intracellular Ca cycling relies on maintenance of junctin levels and an intricate balance among the components in the sarcoplasmic reticulum quaternary Ca-signaling complex. (Circulation. 2007;115:300-309.)

Key Words: proteins ■ arrhythmia ■ calcium ■ sarcoplasmic reticulum

One of the most prevalent causes for premature death in Western countries is lethal ventricular arrhythmias. The proximate cause of death in approximately half of all heart failure patients is ventricular tachycardia or fibrillation,1 and polymorphic ventricular tachycardia is responsible for unexpected exercise-induced sudden death in children and adolescents with no overt evidence of heart disease.2 It has been shown that abnormal automaticity plays a great role in the induction and maintenance of different types of ventricular arrhythmias in patients with either structurally normal hearts or severe structural diseases.3-5 One type of the abnormal automaticity, known as “triggered activity,” induces ventricular arrhythmias through activation of delayed afterdepolarizations (DADs),6 the spontaneously occurring secondary depolarizations sometimes observed in abnormal cardiomyocytes after complete repolarization.7 Although the precise

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biochemical mechanisms that lead to propagation of arrhythmias by DADs are not entirely known, altered ryanodine receptor (RyR)–mediated calcium release has been implicated as a potentially important pathological mediator.8

Ca release by RyRs occurs subsequent to their activation by the L-type Ca channel–mediated inward Ca current. It has been proposed that RyRs are not only activated by cytosolic Ca during excitation-contraction coupling but also by increases in sarcoplasmic reticulum (SR) luminal Ca,9 which is itself dependent on proteins, including calsequestrin (CSQ), triadin, and junctin,10 that together with RyR constitute the macromolecular SR Ca-regulating complex.11 Within this complex, CSQ can act as a Ca sensor for RyRs as follows: Decreased SR luminal [Ca] (and therefore Ca-bound CSQ) itself dependent on proteins, including calsequestrin (CSQ), triadin, and junctin, appear to be critical for normal regulation of RyR-mediated Ca release. Consistent with this notion, mutations in CSQ are associated with the development of DAD-triggered ventricular tachycardia and sudden death, and forced expression of triadin in cardiomyocytes decreases the threshold for DAD-related arrhythmias.12,13

Less is known about junctin, another component of the RyR protein complex. Junctin and triadin exhibit 60% to 70% amino acid homology in their transmembrane domains, including repeated KEKE motifs important for macromolecular protein–protein interactions within their SR luminal tails.14 The effects on cardiac function of both junctin and triadin have been examined with transgenic overexpression strategies in mouse hearts, which exhibit varying phenotypes ranging from mild SR structural alterations, prolongation of Ca transient decay, impaired relaxation, and cardiac hyper trophy and/or heart failure.15–18 Although demonstrating that these components of the RyR complex can modify SR Ca cycling and Ca-dependent cardiac function, the potential for promiscuous or nonspecific activity with protein overexpression precludes a clear assessment of the physiological/pathophysiological role of junctin in normal SR Ca cycling. Therefore, the present study generated junctin-deficient mice to elucidate its essential functional roles in the in vivo heart. We find that junctin gene ablation enhances SR Ca cycling and contractility but is associated with DAD-induced arrhythmias and premature mortality under conditions of physiological stress.

Methods

Generation of Targeted Mice

The murine junctin gene was isolated from the PAC (P1-derived Artificial Chromosome) genomic library by screening it with mouse junctin cDNA. The targeting strategy was designed to replace exons 1 and 2 with a neomycin (neo)-resistance gene. The generation of the targeting construct, targeting of embryonic stem cells, and handling and maintained according to protocols by the ethics committee of the University of Cincinnati. The investigation conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

Quantitative Immunoblotting Analysis of Cardiac Ca-Handling Protein Levels

Mice were anesthetized, and hearts were excised, washed with ice-cold PBS, and then quickly frozen in liquid nitrogen before homogenization. To assess the levels of Ca-cycling proteins in junctin-knockout (KO) hearts, quantitative immunoblotting was performed,20,21 The antibody to histidine-rich Ca-binding protein was a generous gift from Dr Woo Jin Park (Gwangju Institute of Science and Technology, Gwangju, Korea); the junctin antibody was homemade and specific to the last 15 C-terminal residues of mouse junctin; the polyclonal antibody to SR Ca-ATPase was homemade; the polyclonal antibody to phosphorylated RyR at serine 2809 was a generous gift from Dr Andrew R. Marks (Columbia University, New York, NY); and the polyclonal antibodies to CaMKII and CaMKII-δ were generous gifts from Dr Harold A. Singer (Albany Medical College, Albany, NY). All the other antibodies were purchased from Affinity Bioreagents (Golden, Colo). For immunoblotting of triadin, a specific polyclonal antibody to residues 146 to 160 of mouse triadin 1 was used, and immunoblotting was performed as described previously.19 Protein levels were determined with AlphaEaseFC software (Alpha Innotech, San Leandro, Calif).

Isolated Myocyte Mechanics and Ca Kinetics

Isolation of mouse ventricular myocytes and measurements of mechanics and Ca kinetics were performed in the absence or presence of isoproterenol 100 nmol/L at 0.5 Hz.20

Electrophysiology of Isolated Left Ventricular Myocytes

The L-type Ca current and the Na-Ca exchanger (NCX) current were recorded from isolated ventricular myocytes with the whole-cell patch-clamp technique with an Axopatch-200B amplifier (Axon Instruments, Foster City, Calif), as described previously.20,22

SR Ca Uptake

SR Ca uptake in whole-heart homogenates was determined with the Millipore (Billerica, Mass) filtration technique.19

Induction of Aftercontractions and DADs in Isolated Cardiomyocytes

Rod-shaped ventricular myocytes, which exhibited no spontaneous activity at rest, were paced at 5 Hz in the presence of 1 μmol/L isoproterenol in 1.8 mmol/L Ca-Tyrode’s solution at room temperature. After 2 to 3 trains of stimulations, pacing was stopped to allow the recording of spontaneous aftercontractions within 2 to 5 seconds. Ca transients were examined with the same protocol. Ryanodine (10−7 or 10−6 mol/L) was applied to myocytes once aftercontractions were observed. Action potentials were recorded under current-clamp mode and triggered by 2-ms just-threshold current steps at a frequency of 5 Hz in the presence of isoproterenol 1 μmol/L at 32°C. Cells were then rested to determine the presence of DADs.

Measurement of Ca Sparks

To assess the effects of junctin ablation on RyR activity, Ca sparks were measured in permeabilized cardiomyocytes as described previously,23,24 except that the free Ca was 50 nmol/L.

Isoproterenol-Induced Arrhythmias

Surface ECG Recordings After Injection of Isoproterenol

Mice were anesthetized with pentobarbital 90 mg/kg, and the ECG was monitored on an ECG recording system (PowerLab, ADInstruments, Colorado Springs, Colo) after the mice were injected with isoproterenol 0.25 μg/g IP (Sigma, St. Louis, Mo).
Telemetry Recordings After Implantation of Isoproterenol Minipumps

A telemetry transmitter (EA-F20, Data Science International, St Paul, Minn) and an Alzet 2002 pump (Braintree Scientific, Inc, Braintree, Mass) that can release isoproterenol at a rate of 15 mg · kg⁻¹ · d⁻¹ for 2 weeks were implanted. Subsequently, ECG recordings were monitored for 2 weeks. Values from ECG (R-Rint, P-wave duration, PRint, QRS, and QT int) were analyzed with Dataquest A.R.T. software (EA-F20, Data Science International).

Assessment of In Vivo Cardiac Function With Echocardiography

Echocardiography was performed to examine cardiac contractile function in a noninvasive manner.²⁰

Statistical Analysis

All data are expressed as mean±SEM. Comparison between groups was evaluated with the Student t test. Protein levels in wild-type (WT) and KO hearts were also compared with 1-way ANOVA. For the studies that used isolated myocytes, 5 to 10 cells per heart were studied; n indicated the number of hearts. Aftercontractions and DADs were analyzed with χ² test. Probability values of <0.05 were considered significant.

The authors had full access to the data and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Ablation of Junctin In Vivo by Gene Targeting

The physiological role of junctin in vivo was investigated with gene targeting to ablate its expression in the intact mouse (Figure 1A, 1B, and 1C). First, the PAC genomic library was screened with mouse junctin cDNA. One of 5 positive clones, which contained all 5 exons of the junctin gene, was sequenced, characterized (Figure 1A), and used to generate the targeting construct, which was designed to delete exons 1 and 2 of the junctin gene (Figure 1B and 1C). Heterozygous mice were interbred to produce the homozygous deficient mice, which were screened by polymerase chain reaction (online-only Data Supplement Figure IB and IC). Offspring exhibited the expected mendelian ratios, and the homozygous junctin-KO mice were viable, without any overt abnormalities. Quantitative

Figure 1. Targeted disruption of the mouse junctin gene in embryonic stem cells and Ca cycling protein expression levels in junctin-null hearts. A, Restriction map of the WT junctin genomic locus. Bold bars represent exons of the junctin gene. B, Targeting construct, consisting of a junctin HindIII–SalI genomic fragment with exons 1 and 2 replaced by a neo gene in this 7.1-kb fragment. A copy of the TK gene with its own promoter and polyadenylation signal was attached to the 5′ end of the targeting construct. C, Predicted structure of the disrupted junctin allele after homologous recombination. D, Representative blots of SR Ca-cycling protein levels in WT and junctin-KO hearts. E, Quantititation of protein levels in WT and KO hearts. p-RyR indicates phosphorylated RyR at serine 2809; FKBP12.6, FK506 binding protein 12.6; Cal, calreticulin; PLN, phospholamban; LTCC, L-type Ca channel; and HRC, histidine-rich Ca-binding protein. Values are mean±SEM; n=7, except that n=12 for SERCA, CSQ, and HRC. P<0.05 vs WTs.
immunoblotting revealed that there was no junctin protein detected in homozygous hearts (Figure 1D).

**Ca-Cycling Proteins**

Because junctin interacts with CSQ, triadin, and the RyR, ablation of junctin may result in compensation from 1 or more of its partners in the SR Ca-cycling complex. Quantitative immunoblotting, however, did not reveal any significant changes in the levels of RyR, phosphorylation of RyR at serine 2809, triadin, or calsequestrin in junctin-KO hearts. Likewise, there were no alterations in the histidine-rich Ca-binding protein, FKBP12.6, SR Ca-ATPase, or phospholamban levels (Figure 1D and 1E). Because junctin ablation may alter SR Ca homeostasis, we also examined SR Ca-transport activity in KO and WT hearts. There were no significant changes in $V_{\text{max}}$ (58.6±5.6 versus 60.6±8.7 nmol Ca·mg$^{-1}$·min$^{-1}$ in WT versus junctin-KO hearts; $n=6$) or $EC_{50}$ (279±15 versus 274±12 nmol/L in WT versus KO hearts, respectively; $n=6$) of the SR Ca transport for Ca (online Data Supplement, Figure II). Further assessment of the major sarcolemmal Ca-cycling proteins indicated no alterations in L-type Ca-channel protein level on junctin ablation (Figure 1D and 1E). Moreover, the L-type Ca currents were recorded in isolated WT and junctin-KO ventricular myocytes by whole-cell voltage clamp. There were no differences in membrane capacitance (WT: 174.4±11.2 pF, $n=23$; KO: 174±7.5 pF, $n=27$ myocytes) or average peak current density ($-9.4±0.6$ and $-9.2±0.4$ pA/pF; WT versus KO; Figure 2A and 2B). There were no differences in the average current-voltage relationships of the L-type Ca currents (Figure 2B) and kinetic properties of the Ca current (data not shown) between WT and KO cells; however, the current levels of NCX in junctin-KO myocytes were significantly enhanced. The average NCX density at 80 mV was increased by 67% in junctin-KO cells (1.06±0.09 pA/pF) compared with WT cells (0.64±0.07 pA/pF; Figure 2E). Consistent with these findings, there was a 70% increase in NCX protein levels in junctin-KO hearts (Figure 2F and 2G). Thus, ablation of junctin was not associated with alterations in any of the major Ca-cycling proteins except the NCX.

**Increased Contractility in Junctin-Deficient Mice**

Because we proposed that junctin is important for the regulation of SR Ca cycling, it was important to determine whether junctin deficiency had any effects on overall cardiac contractility. Cardiac echocardiography of intact mice indicated that ejection time was significantly abbreviated...
1.91 versus 40.4/10006 0.62 seconds, P/10021 0.05), whereas the fractional shortening (35.7/10006 1.93 versus 42.5/10006 2.54, P/10021 0.05) and the rate-corrected velocity of circumferential fiber shortening (8.1/10006 0.59 versus 10.5/10006 0.58 circumference/s, P/10021 0.05) were markedly increased by 19% and 30%, respectively, in junctin-KO mice compared with WTs (mean/10006 SEM; WT, n = 9; KO, n = 11). Consistent with these findings, assessment of the mechanical parameters and Ca transients in isolated cardiomyocytes, which represent a mechanically unloaded preparation, revealed that junctin ablation resulted in significant increases in the fractional shortening (51%), dL/dt (66%), and −dL/dt (89%; Figure 3A, 3B, 3C, and 3D). Analysis of the Ca transients showed that there were no significant changes in diastolic Ca concentration; however, the Ca peak was increased by 54% in junctin-KO myocytes (Figure 3E and 3F), and the time constant of Ca transient decay (τ) was significantly shorter than in WTs (Figure 3G). The amplitude of caffeine-induced Ca release was also increased by 30% (Figure 3H and 3I), which indicates a higher SR Ca content in junctin-deficient cells. Furthermore, the τ of caffeine-induced Ca release was significantly abbreviated in junctin-KO cells compared with WTs (Figure 3J), consistent with the increased NCX activity in these cardiomyocytes. The present data are in agreement with those obtained from transgenic mice overexpressing junctin in cardiac muscle, which exhibited opposite phenotypic alterations: depressed contractile function, Ca kinetics, and SR Ca content.16,25

Figure 3. Mechanics and Ca kinetics of junctin knockout myocytes and their response to isoproterenol. A, Representative cell shortening tracings of WT and KO cells before isoproterenol stimulation. B, +dL/dt in the absence and presence of isoproterenol 100 nmol/L. C, −dL/dt in the absence and presence of isoproterenol 100 nmol/L. D, The fractional shortening in the absence and presence of isoproterenol. E, Representative tracings of Ca transients in WT and KO cells before isoproterenol stimulation. F, Amplitude of Ca transients in the absence and presence of isoproterenol 100 nmol/L. G, Time constant of twitch Ca decay (τ) in WT and KO cardiomyocytes with or without isoproterenol 100 nmol/L. H, Representative tracings of caffeine-induced Ca transients in WT and KO cells. I, Amplitude of caffeine-induced Ca transients without isoproterenol. J, τ of caffeine-induced Ca transients without isoproterenol. Data are mean±SEM. In the absence of isoproterenol, for mechanics, n = 36 to 42 cells from 5 hearts; for twitch Ca transients, n = 52 to 64 cells from 6 hearts; for caffeine-induced Ca transients, n = 30 to 49 cells from 6 hearts. In the presence of isoproterenol, mechanics were measured in 30 to 40 cells from 4 hearts; Ca transients were measured in 40 to 52 cells from 3 hearts. Values represent mean±SEM. *P<0.05 vs WT at basal condition. §P<0.05 vs KO before isoproterenol stimulation. Iso indicates isoproterenol.

(47.2±1.91 versus 40.4±0.62 seconds, P<0.05), whereas the fractional shortening (35.7±1.93 versus 42.5±2.54, P<0.05) and the rate-corrected velocity of circumferential fiber shortening (8.1±0.59 versus 10.5±0.58 circumference/s, P<0.05) were markedly increased by 19% and 30%, respectively, in junctin-KO mice compared with WTs (mean±SEM; WT, n = 9; KO, n = 11). Consistent with these findings, assessment of the mechanical parameters and Ca transients in isolated cardiomyocytes, which represent a mechanically unloaded preparation, revealed that junctin ablation resulted in significant increases in the fractional shortening (51%), +dL/dt (66%), and −dL/dt (89%; Figure 3A, 3B, 3C, and 3D). Analysis of the Ca transients showed that there were no significant changes in diastolic Ca concentration; however, the Ca peak was increased by 54% in junctin-KO myocytes (Figure 3E and 3F), and the time constant of Ca transient decay (τ) was significantly shorter than in WTs (Figure 3G). The amplitude of caffeine-induced Ca release was also increased by 30% (Figure 3H and 3I), which indicates a higher SR Ca content in junctin-deficient cells. Furthermore, the τ of caffeine-induced Ca release was significantly abbreviated in junctin-KO cells compared with WTs (Figure 3J), consistent with the increased NCX activity in these cardiomyocytes. The present data are in agreement with those obtained from transgenic mice overexpressing junctin in cardiac muscle, which exhibited opposite phenotypic alterations: depressed contractile function, Ca kinetics, and SR Ca content.16,25

To examine the effects of β-adrenergic agonists, isolated cardiomyocytes were subjected to maximal isoproterenol (100 nmol/L) stimulation, and the contractile parameters and Ca kinetics were evaluated. Isoproterenol stimulation was associated with significant increases in contractile parameters, including +dL/dt and fractional shortening in both WT and junctin-deficient myocytes. Although the relative increases were smaller in the KO cells, the maximally stimulated parameters were not different between WT and KO cells (Figure 3B, 3C, and 3D). Similarly, Ca transient amplitude and τ were significantly stimulated in both groups, and the maximally stimulated parameters were not different between WT and KO cells (Figure 3F and 3G). The apparently blunted responses of the KO cardiomyocytes to isoproterenol stimulation may be due to their highly elevated basal levels, which allows smaller further increases by β-adrenergic stimulus, similar to the phospholamban KO models.26

Ca Sparks on Ablation of Junctin
The increases in SR Ca load and luminal [Ca] have been shown to stimulate the open probability of the RyR channel, which results in increased spontaneous SR Ca release.27 Ca sparks, the local and temporally restricted fluorescence Ca
signals that represent the coordinated openings of RyRs gating in situ, were examined. Experiments in permeabilized cells, although not entirely physiological, resolve more directly the effect of junctin ablation on RyR gating. In the absence of external Ca fluxes (no L-type Ca channel or NCX contribution), in permeabilized cells, the excitation-contraction coupling machinery is practically reduced to SR Ca\(^{2+}\)/H\(^{+}\) release and uptake, with the greatest regulation of these events dictated by RyR/luminal proteins and SR Ca\(^{2+}\)/H\(^{+}\) ATPase/phospholamban, respectively. Cell permeabilization also allows for controlled buffering of the internal medium, which dictates the level of SR Ca\(^{2+}\) load. Because, as we show in the present study, WT and junctin-KO cardiomyocytes exhibit dissimilar NCX activity (Figure 2E) but similar Ca uptake rates (online Data Supplement, Figure II), experiments in permeabilized cells resolve directly the effect of junctin ablation on RyR gating without the compound effects of unequal Ca removal processes. Thus, we examined Ca sparks in saponin-permeabilized myocytes. Representative line-scan images of Ca sparks acquired at a constant cytosolic [Ca] of \(50\) nmol/L and their surface plots, obtained by averaging multiple individual events, are illustrated in Figure 4A and 4B for WT and junctin-KO cells. In WT myocytes, spontaneous Ca sparks occurred with an average frequency of \(0.187\pm0.018\) sparks/\(\mu\)m \(\cdot\)s. In myocytes from junctin-null hearts, spark frequency was increased to \(0.266\pm0.010\) sparks/\(\mu\)m \(\cdot\)s (Figure 4C). Furthermore, the event amplitude was significantly increased in junctin-KO cells with respect to WTs (2.19±0.01 and 1.76±0.01; Figure 4D), but duration was decreased (35.452±0.543 and 33.433±0.304 ms; Figure 4E). Ca spark width was unchanged (Figure 4F). The decreased duration of Ca sparks in junctin-KO cells likely indicates faster termination of the Ca release event once [Ca] underneath the active RyRs drops to subthreshold levels. The increased spark frequency and amplitude strongly suggest that SR Ca load is increased in junctin-KO cells, in agreement with our findings in cardiomyocytes (Figure 3H and 3I).

Premature Death in Junctin-KO Mice

It was unexpected that \(\approx25\%\) of the junctin-KO mice died by 3 months of age, and \(>50\%\) of them died before 12 months of age, which is the mid point of the average mouse life span (24 to 30 months; Figure 5); there were no deaths among WT control mice up to 12 months of age, however. Histological examination of cardiac muscle did not reveal any cardiac morphological alterations or structural abnormalities in junctin-null hearts at 3 months of age (online Data Supplement, Figure IV). Furthermore, the heart weight/body weight ratio was not different between WT and KO mice (online Data Supplement, Table II), and there were no increases in hypertrophic signaling cascades, including p38, ERK, calcineurin, CaMKII, and Akt (data not shown) in the KO hearts.

Figure 4. Properties of Ca sparks in control and junctin-null cardiomyocytes. A and B, Representative confocal line-scan images and 3D Ca sparks recorded in saponin-permeabilized WT and junctin-KO cardiomyocytes, respectively. C through F, Bar graphs depicting Ca spark frequency, measured as the number of events per unit time and length; the maximal Ca spark amplitude (peak F/F\(_0\)); the average of full-duration-at-half maximal amplitude (FDHM) of Ca sparks; and the average of full-width-at-half maximal amplitude (FWHM) of Ca sparks in WT and KO saponin-permeabilized cells, respectively. Fluo-4 salt was used as the Ca indicator for all experiments. Data are presented as mean±SEM of 2279 sparks from WT cells and 4292 sparks from junctin-KO cells. *P<0.05, WT vs junctin-KO.

Figure 5. Premature death in junctin-KO mice. Survival curve of the junctin-KO and WT mice (n=21 KOs and 18 WTs). Data were analyzed by logistic regression with exact method. *P<0.05, WT vs KO.
In Vivo Cardiac Arrhythmias Induced by Isoproterenol Stimulation

The sporadic premature death without notable cardiac pathology suggested that junctin-deficient mice may be predisposed to arrhythmias. Thus, catecholaminergic-induced stress, such as isoproterenol stimulation, would be expected to trigger arrhythmias in junctin-KO mice. To test this hypothesis, the surface ECG was monitored on short-term intraperitoneal injection of isoproterenol or after implantation of an isoproterenol minipump with a telemetry system. Short-term isoproterenol administration (0.25 μg/g IP) was associated with ventricular tachycardia in junctin-null mice (Figure 6A). Frequent premature ventricular contractions and atrioventricular heart block were also observed (data not shown). However, WT mice showed only benign and expected arrhythmias, such as sinus tachycardia and infrequent atrial premature contractions. Similarly, telemetry studies under long-term isoproterenol stimulation revealed cardiac arrhythmias that included premature ventricular contractions (Figure 6B), sinus asystole, and atrioventricular heart block (online Data Supplement, Figure V). The predominant arrhythmias were premature ventricular contractions, which occurred in isolation and as couplets over 2 weeks.

Stress-Induced Aftercontractions and DADs in Junctin-Null Cardiomyocytes

We postulated that in vivo catecholaminergic-elicited arrhythmias might be the consequence of increased RyR spontaneous Ca release in the junctin-null mice. If this was the case, arrhythmias could also be observed in isolated myocytes under stress, and inhibition or stabilization of the RyR activity could block these events. To test our hypothesis, junctin-null and WT cardiomyocytes were subjected to 2 to 3 trains of 5-Hz field stimulation in the absence or presence of isoproterenol. Increased stimulation did not cause any disturbance in contractile cycles in WT cardiac myocytes, whereas there were aftercontractions in ≈16±6% of junctin-deficient cells (Figure 7B). Inclusion of isoproterenol resulted in aftercontractions in 54±13% of the junctin-null cells, compared with 8±3% of WT cells (Figure 7A and 7B). Importantly, incubation of WT cells with 10⁻⁷ mol/L ryanodine or junctin-KO cells with 10⁻⁶ mol/L ryanodine completely blocked the aftercontractions within 9 to 15 minutes, which suggests that the aftercontractions were associated with aberrant discharge of SR Ca through the RyR channel. Consistent with the increase in aftercontractions, further investigation of the Ca transients at 5 Hz in the presence of isoproterenol 1 μmol/L showed that 60±14% of the junctin-KO cells exhibited Ca aftertransients, whereas only 9±3% of the WT cells developed abnormal Ca transients after the electrical stimulation was stopped (Figure 7C).

Previous studies indicated that aberrant RyR Ca release may induce arrhythmias by activation of DADs. To determine whether the mechanism for the arrhythmias elicited by junctin ablation is related to DADs, electrical activity was examined under increased frequency of stimulation (5 Hz) in the absence or presence of isoproterenol, as well as at 2 to 5 seconds after termination of the stimulation in separate experiments. In the absence of isoproterenol, 32±7% of the junctin-KO cells exhibited DADs compared with 6±4% of the WT cells (Figure 7E). In the presence of isoproterenol, DADs were observed in 90±5% of the junctin-deficient cells, and among those, 72% were suprathreshold. However, only 21±2% of WT cells developed DADs (Figure 7D and 7E), and 43% of them were suprathreshold, which suggests that the increased frequency of DADs may underlie the mechanisms for the elicited arrhythmias in the junctin-null hearts.

Discussion

Herein, we used in vivo genetic techniques to establish that junctin is essential for normal cardiomyocyte SR Ca cycling,
for Ca-dependent cardiac inotropy, and to suppress DAD-triggered arrhythmias that can lead to sudden death. Ablation of junctin was associated with enhanced SR Ca cycling in cardiomyocytes and increased cardiac function in vivo, which is expected with maneuvers that increase SR Ca load. Unexpectedly, however, when the KO mice were subjected to forms of physiological cardiac stress, malignant ventricular arrhythmias developed that were associated with DAD-related aftercontractions in junctin-deficient cardiac myocytes. These results not only demonstrate a necessary role for junctin in cardiomyocyte SR Ca homeostasis but also reveal a novel aspect of the previously postulated relationship between RyR function and electrical stability of cardiomyocytes, the critical regulatory role of junctin.  

By what mechanisms did ablation of junctin result in increased SR Ca load? It is known that CSQ-binding capacity, SR Ca uptake, and NCX activity are the key determinants of SR Ca load.19,28–30 We did not find significant changes, however, in the SR Ca-ATPase Ca uptake activity or its affinity for Ca, and the CSQ protein levels were not altered. By contrast, the expression level and the activity of the NCX were enhanced, which would be expected to result in diminished SR Ca load. Thus, we hypothesize that the increased SR Ca load in the absence of junctin reflected increases in the CSQ Ca-binding capacity. It is well known that the repeated aspartate-rich region at the C-terminus of CSQ is the Ca-binding domain and acts as a Ca reservoir.31 Importantly, this is also the domain that interacts with the KEKE motifs of junctin/triadin.31 Intuitively then, ablation of junctin could elicit availability of additional Ca-binding sites in the aspartate-rich region of CSQ, leading to an increase in the CSQ Ca-binding capacity.

The increase in SR Ca load was likely the primary factor responsible for the augmented Ca transient and hypercontractility in junctin-deficient cardiomyocytes. In this regard, the increase in contractile function was consistent with the enhanced Ca transients and kinetics. The gain of function of NCX appeared to also be an important compensatory mechanism along these lines, because the forward mode of the NCX favors Ca extrusion to maintain a normal diastolic [Ca] in the face of increased SR Ca load.32

Importantly, ablation of junctin was associated with DAD-induced arrhythmias under stress. Indeed, genetic defects of 2 other proteins in the SR Ca release channel complex, RyR and CSQ, have been also linked to catecholaminergic polymorphic ventricular tachycardia in human patients through activation of DADs.2 The cellular basis for DADs appeared to also be an important compensatory mechanism along these lines, because the forward mode of the NCX favors Ca extrusion to maintain a normal diastolic [Ca] in the face of increased SR Ca load.32

Figure 7. Aftercontractions and DADs in junctin-KO cardiomyocytes. A, Representative traces of aftercontractions (Acs) in WT and junctin-KO myocytes at 5 Hz and 1 μmol/L isoproterenol stimulation. B, Percentage of the WT and junctin-null cardiomyocytes that developed Acs at 5 Hz without or with isoproterenol (ISO; n=49 to 51 cells from 6 mice; values are percent of cells with Acs). C, The occurrence of Ca aftertransients in junctin WT and junctin-KO myocytes at 5 Hz in the presence of isoproterenol 1 μmol/L (n=21 to 23 cells from 3 hearts; values represent the percentage of cells with Ca-after transients). D, Representative traces of action potential in WT and junctin-KO myocytes at 5 Hz and isoproterenol stimulation. DAD is marked with arrows. E, Bar graph showing the occurrence of DADs in WT and junctin-KO cells at 5 Hz without or with isoproterenol (n=19 to 32 cells from 5 hearts; values are percent of cells with DADs). *P<0.05 vs WT.
esize that junctin ablation may act in a similar manner on RyR activity, leading to lethal cardiac arrhythmias.

What are the mechanisms underlying the increased likelihood of DAD-associated arrhythmias in junctin-deficient mice? Junctin ablation may be associated with defective regulation of RyR activity by SR luminal Ca. Similar to the scheme in catecholaminergic polymorphic ventricular tachycardia–related CSQ mutations, junctin ablation may directly prevent the interaction between CSQ and the RyR complex, impairing the sensitivity of RyR to SR Ca, which may lead to aberrant RyR openings. Potentially, ablation of junctin may directly enhance RyR activity. It was shown that Ca negatively regulates the interaction between CSQ and junctin/triadin.11 Thus, when SR [Ca] increases during diastole, junctin and triadin may be free to interact and modulate RyR activity. On consequent decrease in [Ca] during Ca-induced Ca release, the interaction between CSQ and junctin/triadin may increase, and the effects of junctin/triadin on RyRs may be weakened by CSQ. Alternatively, the effects of junctin/triadin on regulation of RyR activity may be dominant during diastole. Indeed, the present Ca spark data from permeabilized myocytes clearly showed that spontaneous RyR Ca release was increased, which constituted an important molecular mechanism for the induction of DAD-associated arrhythmias, which suggests that junctin may represent a brake that inhibits RyR activity. Although it has been widely accepted that junctin and triadin mediate the interaction between RyR and CSQ,10,11,15,31,35 studies in skeletal muscle have indicated a direct interaction between RyR and CSQ, with CSQ enhancing RyR activity.35,36 Because it was suggested that junctin/triadin may also prevent this direct interaction,35,37 deletion of junctin might favor the positive regulation of CSQ on RyR activity and contribute to the increase in RyR spontaneous Ca release. In addition, it is known that calsequestrins can induce spontaneous SR Ca release by increasing SR Ca load.38 Thus, isoproterenol stimulation most likely exacerbated the high SR Ca load of junctin-null cells. The suprathreshold levels of SR Ca load coupled with decreased FKBP12.6 binding to RyR enhanced the spontaneous RyR openings. The larger amount of Ca released during each RyR opening increased the likelihood of DAD-associated arrhythmias. However, DADs and aftercontractions were also observed in the absence of isoproterenol, which indicates that dissociation of FKBP12.6 may not be required for the generation of arrhythmias in this model. Finally, because DAD is activated by an inward current via the NCX,39 the enhanced NCX activity in junctin-deficient cardiomyocytes could be an additional contributor to the generation of DADs, which further induce arrhythmias.

In conclusion, the present findings show that cardiomyocyte SR Ca content and contractile function are inversely related to the level of cardiac junctin expression under normal conditions, which establishes that junctin can be an important modulator of SR Ca cycling. Surprisingly, junctin deficiency also decreased the threshold for malignant ventricular arrhythmias by increasing the incidence of DADs, which may have been related to our observations of sudden death in junctin-KO mice. Thus, maintenance of junctin expression levels appears necessary for proper SR Ca handling, cardiac function, and resistance to abnormal ventricular automaticity.

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**Disclosures**

None.

**References**

CLINICAL PERSPECTIVE

Sudden cardiac death, largely attributable to malignant ventricular arrhythmia, is responsible for 300 000 to 400 000 annual deaths in the United States. Although sudden cardiac death is a frequent end point for patients with organic heart diseases, such as chronic or acute ischemia and dilated or hypertrophic cardiomyopathies, it often occurs in individuals with no detectable cardiac pathology, as in catecholaminergic polymorphic ventricular tachycardia. Recently, an abnormal sarcoplasmic reticulum Ca leak has been correlated with increased ventricular arrhythmias in patients with heart failure or catecholaminergic polymorphic ventricular tachycardia, caused by aberrant ryanodine receptor (RyR) Ca release. Although the underlying mechanisms for this leak have not been completely elucidated, human mutations in the RyR and calsequestrin genes, which alter RyR gating and calcium release, have been associated with lethal cardiac arrhythmias. Junctin is another member of the sarcoplasmic reticulum Ca-handling apparatus, and it complexes with RyR to affect cardiac performance in vivo but also increased Ca leak, which further triggered arrhythmias and predisposed to sudden cardiac death. Thus, it may be valuable to identify polymorphic variants in the human junctin gene and determine their association with cardiac arrhythmias in subjects with and without known cardiac disease. Identification of such variants may suggest more aggressive targeted use of primary preventative measures for sudden cardiac death.
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