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Junctin Is a Prominent Regulator of Contractility in Cardiomyocytes

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Abstract

Junctin is one of the components of the ryanodine receptor Ca release channel complex in sarcoplasmic reticulum. To determine the role of acute alteration of junctin protein levels on cardiomyocyte contractility, we used adenoviral-mediated gene transfer techniques in adult rat cardiomyocytes. Acute downregulation of junctin by 40% resulted in significant increases in cell shortening, rate of contraction (+dL/dt) and rate of relaxation (-dL/dt). The alteration of contractile parameters was associated with increased Ca transient peak and accelerated Ca decay. However, all these contractile and Ca kinetic parameters were depressed significantly when junctin levels were upregulated by 60%. Importantly, there were no alterations in other Ca cycling protein levels when junctin levels were either decreased or increased. These findings suggest that junctin plays a prominent role in cardiomyocyte Ca-cycling and contractility.

Keywords

Junctin; Adenoviral; Cardiomyocyte; Calcium; Contractility

Introduction

Junctin (JCN), a 210-amino acid protein, is a sarcoplasmic reticulum (SR) integral component [1]. This 26-kDa protein contains a cytoplasmic NH₂-terminal segment (residues 1-22), a single transmembrane segment (residues 23-44), and a SR intraluminal COOH-terminal segment (residues 45-210), which contains repeated KEKE motifs that are important for protein-protein interaction [1]. Indeed, in cardiac muscle, junctin forms a quaternary protein complex with the ryanodine receptor (RyR), calsequestrin (CSQ), and triadin in the SR lumen [2]. Junctin has been shown to interact with calsequestrin and triadin, and this interaction depends on the SR luminal Ca concentration [1,2]. Junctin also serves as a linker between CSQ and the RyR [1,2, and 3].

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Among the RyR quaternary protein complex, CSQ and triadin have been suggested to regulate RyR Ca release in cardiac myocytes [4,5,6 and 7]; however, the functional role of junctin is not currently clear. Although transgenic studies showed that junctin may be involved in the regulation of SR Ca cycling [8 and 9], adapted mechanisms or even developmental changes associated with chronic expression of high junctin levels may have contributed to the observed phenotype, masking the direct effects of junctin. Intriguingly, in the hearts of mice overexpressing beta-adrenergic receptor 1, junctin was shown to decrease as early as two weeks of age, and preceding the alteration of other Ca cycling protein levels [10]. Notably, the junctin level progressively declined with age and paralleled the development of cardiac hypertrophy and heart failure in this model [10,11, and 12]. Thus, it appears that junctin may be involved in the dysregulation of SR Ca cycling in heart failure. However, it is unknown whether junctin downregulation may be an adaptive mechanism, managing to rescue the deterioration of cardiac function or one of the primary causes for inducing cardiac hypertrophy and heart failure. Therefore, we acutely manipulated the levels of junctin expression, using adenoviral-mediated gene transfer, and examined the effects of downregulation or upregulation of junctin on cardiac myocyte contractile function and Ca cycling. Our findings demonstrate that reduced junctin levels were associated with enhanced cardiac contractility and Ca kinetics, while increases in junctin had the opposite effects, in the absence of alterations in other Ca cycling proteins. Thus, for the first time, we show that contractility may be affected by acute alterations of junctin levels, suggesting that junctin is an important modulator of Ca handling and cardiac function.

Materials and Methods

Recombinant Adenoviral Constructs

Recombinant adenoviruses were developed using the pAdTrack-CMV/pAdEasy-1 system. Briefly, a junctin-adenovirus (named as AdsJCN) was constructed by insertion of junctin cDNA between the cytomegalovirus (CMV) enhancer/promoter and the SV40 polyadenylation signal sequence of the shuttle vector pAdTrack-CMV, which also carries a GFP expression cassette (Figure 1A). Antisense-junctin adenovirus (named as AdasJCN) was generated by cloning the junctin cDNA in reverse orientation relative to the CMV promoter (Figure 1A). The resultant plasmids were linearized and co-transformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinant adenoviral vectors were generated through homologous recombination and selected for kanamycin resistance, and the recombination was confirmed by multiple-restriction endonuclease analyses. Then the linearized recombinant vectors were transfected into HEK 293 cells. AdGFP, which contains GFP cDNAs and LacZ cDNA controlled by a separate CMV promoter, was used as control. The recombinant viruses were harvested after 7-10 days. The recombinant viruses were prepared as high-titer stocks and further purified by CsCl banding. Viral particle titration was performed by using plaque assay. Briefly, multiple dilutions of virus were plated out with proper amount of 293 cells. When there were individual plaques observed and evenly distributed, the individual plaque number was counted and the plaque forming units per mL (pfu) were obtained by multiplying the count by the dilution factor.

Preparation and Infection of Cardiomyocytes

Adult rat ventricular myocytes were obtained from Langendorff-perfused hearts of male Sprague-Dawley rats (~300mg, Harlan Laboratory) at 37°C, as described before [13]. Briefly, rats were anesthetized with sodium pentobarbital (50mg/kg IP) and heparinized (10,000U/kg IP). The hearts were perfused with modified Krebs-Henseleit buffer (KHB) (in mmol/L: NaCl: 118, KCl: 4.8, HEPES: 25, K₂PO₄: 1.25, MgSO₄: 1.25, glucose: 11, taurine: 5 and BDM: 10, pH7.4) for 5 minutes. Hearts were then perfused with an enzyme solution, which contained 0.7mg/mL collagenase type II (263U/mg), 0.2mg/mL hyaluronidase, 0.1% BSA and 25μM Ca²⁺, for 10 minutes. Subsequently, the Ca²⁺ concentration in the perfusion buffer was raised

to 100 μ M, and perfusion continued for 5 additional minutes. Finally, ventricular tissue was excised, minced, pipette-dissociated, and filtered through a 240- μ m screen. Cells were harvested and resuspended in 1.8mM Ca^{2+} -KHB with 1% BSA, centrifuged briefly again, and resuspended in ACCT medium consisting of DMEM containing 2mg/mL BSA, 2mM L-carnitine, 5mM creatine, 5mM taurine, 100IU/mL penicillin, and 100ug/mL streptomycin. Cells were then counted and plated on laminin-coated glass coverslips or dishes. After 1-2 hours, the dishes were infected with adenoviruses in diluted media, at a multiplicity of infection of 500, for 2 hours before addition of suitable volume of culture media. Transfection efficiency, determined by GFP gene expression in the cultured cardiac myocytes under fluorescence microscopy, was consistently >95% by this method (Figure 1B). Myocyte mechanics and Ca^{2+} kinetics were recorded after 24-hour incubation. To obtain intracellular Ca^{2+} signals, cells were incubated with the acetoxymethyl ester form of fura-2 (Fura-2/AM; 2 μ M).

Contractile parameter measurements

Myocytes that adhered to the coverslips were bathed in temperature (37°C)-equilibrated KHB containing 1 mM Ca for 20 min. The myocyte suspension was then placed in a Plexiglas chamber, which was positioned on the stage of an inverted epifluorescence microscope (Nikon Diaphot 200). Myocyte contraction was field stimulated by a Grass S5 stimulator (0.5 Hz, square waves), and contractions were videotaped and digitized on a computer. A video edge motion detector (Crescent Electronics) was used to measure myocyte length and cell shortening, from which the percent fractional shortening (%FS) $\{[(\text{resting cell length} - \text{maximal cell shortening length})/\text{resting cell length}] \times 100\}$ and maximal rates of contraction and relaxation ($\pm \text{dL/dt}$) were calculated. All data were analyzed using software from Felix 1.1 Software (Photon Technology) and Ionwizard.

Intracellular Ca transient measurements

To obtain intracellular Ca signals, myocytes that adhered to coverslips were incubated with the AM form of fura-2 (2 μ M) for 30 min at room temperature and then resuspended in KHB containing 1 mM Ca. After four washes, the coverslip was mounted in a Plexiglas chamber containing 2 ml of fresh KHB (including 1 mM Ca), which was field stimulated to contract at 0.5 Hz between platinum wire electrodes. The fura-2 fluorescence was determined at room temperature using a Delta Scan dual-beam spectrofluorophotometer (Photon Technology) operating at an emission wavelength of 510 nm with excitation wavelengths of 340 and 380 nm. Baseline, amplitude (estimated by the 340-to-380-nm ratio) and the time for 50% decay of the Ca signal were acquired. All data were analyzed using software from Felix 1.1 Software (Photon Technology) and Ionwizard.

Quantitative Immunoblotting of SR Ca Handling Proteins

Cultured myocytes were then harvested and lysed for 20 min at 4°C in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol, 0.4 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.5 mM DTT, and 2 μ l/ml protease inhibitor cocktail]. The supernatants obtained after centrifugation at 8,000 g for 10 min were then used in subsequent experiments. Protein loading (6-30 μ g) varied for each protein; however, a linear range was performed on each immunoblot to ensure that protein loading was within the linear range of detection. Protein samples were electrophoresed on SDS-polyacrylamide gels under reducing conditions and then transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline for 1 h at room temperature and then incubated overnight at 4°C or at room temperature for 2-3 h with the respective primary antibodies: junctin (polyclonal rabbit anti-canine junctin antibody, generous gift from Dr. Larry Jones); SR Ca ATPase (homemade polyclonal rabbit anti-SERCA antibody); L-type Ca channel, Na-Ca exchanger, ryanodine receptor, triadin, phospholamban

and calsequestrin (Affinity Bioreagents, Golden, CO, USA). Blots were then washed in Tris-buffered saline, incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) for 1 h at room temperature, washed again, and subsequently developed using the ECL chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions. All the protein levels were quantitated by using AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

Statistics Analysis

All results are expressed as means $\pm$ SEM. ANOVA was used to compare the parameters among AdGFP control, AdJNC and AdasJNC groups in the isolated myocyte studies. Furthermore, animal-based data (not cell based) was used for statistical comparisons. In all analyses, $p < 0.05$ was considered statistically significant.

Results

Alteration of junctin levels by adenoviral gene transfer in isolated adult rat cardiac myocytes

To investigate the role of junctin in the regulation of cardiac contractile function, the levels of this protein were altered by infection of rat adult cardiac myocytes with adenoviruses containing junctin cDNA in sense or antisense orientation (AdsJCN or AdasJCN, Figure 1A), and AdGFP as a control. Figure 1B shows that over 95% of infectivity was achieved at a MOI of 500 without notable cell death in AdGFP, AdsJNC and AdasJNC groups. After 24 hours of infection, the junctin level was upregulated by 60% in myocytes infected with AdsJCN, whereas it was decreased by 40% by adenoviral transferring of junctin antisense cDNA (Figure 1C).

Effects of alteration of junctin levels on cardiomyocyte contractility

Junctin is one of the components of the RyR protein complex, and overexpression of junctin in mouse hearts was associated with impaired cardiac relaxation [2,8 and 9]. Thus, we hypothesized that alterations in junctin levels may be involved in the regulation of cardiomyocyte contractility. Excitingly, downregulation of junctin by 40% in cardiomyocytes significantly increased the fractional shortening (FS%: by 32%), as well as the maximal rates of contraction (+dL/dt: by 41%) and relaxation (-dL/dt: by 35%) (Figure 2). Accordingly, upregulation of junctin resulted in depressed myocyte contractile parameters (Figure 2).

Effects of junctin levels on Ca kinetics in cardiomyocytes

Since the enhancement of contractility by junctin down-regulation was related to an increase in cytosolic Ca^{2+} cycling, intracellular Ca^{2+} transients were measured by use of the Fura-2 fluorescence indicator. Our results show that down-regulation of junctin increased the amplitude of Ca transients by 19%, whereas the time to 50% decay was shortened by 29% (Figure 3). Thus, the improvement in cellular contractility seems to be associated with parallel acceleration in cytosolic Ca^{2+} cycling by decreased junctin protein levels. Correspondingly, Ca transient amplitude was diminished by 16%, in AdsJCN-infected cardiomyocytes, which is different from the findings in the junctin-overexpressing mice [8]; whereas the time to 50% decay was prolonged, similar to the results from junctin-transgenic model [8].

Effects of overexpression or downregulation of junctin on other Ca cycling protein levels

It has been shown that overexpression of junctin *in vivo* resulted in impaired cardiac relaxation, which was accompanied by reduced expression of the ryanodine receptor and triadin [8,14]. To assess whether acute alteration of junctin levels results in any compensatory effects, immunoblotting analysis was used to examine the Ca cycling protein levels. Our results show that there were no changes in triadin, calsequestrin, ryanodine receptor, phospholamban, SR

Ca-ATPase, Na-Ca exchanger and L-type Ca channel protein levels by either upregulation or downregulation of junctin (Figure. 4 A and B), suggesting alteration of junctin protein levels may directly regulate cardiomyocyte contractility. These data may explain why cell shortening, the rate of contraction and Ca transient peak were not altered in the face of a depressed SR Ca load in junctin-overexpressing mouse models [8,14], which are different findings from our present observations.

Discussion

An exciting new finding of our present study is that acute downregulation of junctin resulted in enhanced contractility and Ca kinetics in the absence of alterations in other Ca handling protein levels. This indicates that downregulation of junctin may be beneficial for improving the depressed SR Ca cycling and contractile function in the failing hearts, suggesting that it may be valuable to investigate whether junctin could serve as a therapeutic target in heart failure.

Previous studies, using transgenic models of cardiac-specific overexpression of junctin, have shown that 10-fold junctin overexpression was associated mild hypertrophy and impaired relaxation [8,9, and 11]; whereas 29-fold junctin overexpression resulted in heart failure and bradycardia [14]. However, both models revealed the adaptive alterations by down-regulating the levels of two other junctional proteins: RyR and triadin. Moreover, 29-fold overexpression of junctin led to increases in L-type Ca channel protein level and its current density [14]. It has also been found that 10-fold overexpression of junctin reduced the NCX expression [9]. Thus, these compensatory effects, associated with chronic overexpression of junctin, prevent the proper assessment of physiological alterations, ascribed directly to junctin. The current studies were performed in cultured adult rat cardiomyocytes, a well-controlled experimental setting, to avoid multiple nonspecific cardiac adaptive alterations using transgenesis. Indeed, acute up-, or down-regulation of junctin in the present study did not induce alterations in other Ca handling protein levels, suggesting that the overexpression of junctin level is responsible for the alterations of Ca-cycling and cardiac function (Figure 4).

Although the underlying mechanisms of altered cardiomyocyte contractility by manipulation of junctin levels are to be determined, we hypothesize that junctin may directly regulate SR Ca cycling, probably by affecting SR Ca load and modulating RyR Ca release. It has been shown that junctin and triadin not only link CSQ to RyR, but also the ratio of junctin/triadin to CSQ strongly affects the disposition of CSQ and the overall shape of the junctional SR cisternae [3]. Thus, it is possible that alteration of junctin levels may directly influence the ratio of junctin to CSQ in the RyR Ca release channel complex, modifying the RyR activity; furthermore, since junctin binds to the Ca-binding region of CSQ [15], alterations of junctin levels may directly alter CSQ-Ca binding capacity. Nevertheless, the present studies points to junctin as a nodal point for cardiomyocyte Ca-cycling and contractility, and paves the way for further studies to elucidate the regulatory role of this protein in cardiac physiology and pathophysiology.

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Figure 1. Generation of recombinant adenovirus vectors: AdsJCN- and AdasJCN, and expression levels of junctin in AdGFP-, AdsJCN- and AdasJCN -infected cardiac myocytes. (A) Diagram of recombinant adenovirus vectors: AdsJCN- and AdasJCN; (B) AdGFP, AdsJCN and AdasJCN infected cardiomyocytes under light microscopy (upper panel) and under fluorescence microscopy (lower panel); (C) Immunoblotting analysis of junctin level in AdGFP, AdsJCN and AdasJCN groups. Protein levels were normalized to that of AdGFP control within the same blot. n=3 hearts in each group. Experiment was repeated three times. Values = Mean $\pm$ S.E.M, *: $p < 0.05$.

Figure 2. **Mechanics in AdGFP, AdsJCN and AdasJCN cardiomyocytes.** After 24-hour incubation with adenoviruses, maximum rates of contraction and relaxation of cardiac myocytes were measured. (A). Representative tracings of cell shortening of AdGFP, AdsJCN and AdasJCN cardiomyocytes. (B). Fractional shortening (FS %) in AdGFP, AdsJCN and AdasJCN cardiomyocytes. (C). Maximum rates of shortening [(+)dL/dt] in AdGFP, AdsJCN and AdasJCN cardiomyocytes (D). Maximum rates of relengthening [(-)dL/dt] in AdGFP, AdsJCN and AdasJCN cardiomyocytes. n = 10-15 cells per experiment; experiment was repeated at least 3 times; values = mean $\pm$ SEM. *: $P < 0.05$.

Figure 3.

Ca kinetics in AdGFP, AdsJCN and AdasJCN cardiomyocytes. After 24-hour incubation with adenoviruses, myocytes were incubated with Fura2-AM for half hour and Ca transients were measured at room temperature. (A). Representative tracings of Ca transients in AdGFP, AdsJCN and AdasJCN myocytes. (B). Ca transient amplitude in AdGFP, AdsJCN and AdasJCN myocytes. (C). Time to 50% of Ca decay in AdGFP, AdsJCN and AdasJCN myocytes. T50: time to 50% decay of Ca transient. n = 10-15 cells per experiment; experiment was repeated at least 3 times; values = mean $\pm$ SEM. *: $P < 0.05$.

Figure 4.

Quantitative immunoblotting analysis of Ca handling protein levels. (A) Representative blots of Ca cycling proteins in AdGFP, AdsJCN and AdasJCN myocytes. SERCA, SR Ca ATPase; PLN, phospholamban; CSQ, calsequestrin; RyR, ryanodine receptor, LTCC, L-type Ca channel; and NCX, Na-Ca exchanger. (B) Quantitative results of Ca cycling protein levels in AdGFP, AdsJCN and AdasJCN myocytes. Protein levels were normalized to that of AdGFP control within the same blot. $n=3$ hearts in each group. Experiment was repeated three times. Values = Mean $\pm$ S.E.M.