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DEFECTIVE REGULATION OF THE RYANODINE RECEPTOR INDUCES HYPERTROPHY IN CARDIOMYOCYTES

Tomoyo Hamada^{*}, Jaya P. Gangopadhyay^{*}, Adel Mandl^{*}, Peter Erhardt^{*}, and Noriaki Ikemoto^{*,#,†}

^{*}Boston Biomedical Research Institute, Watertown, Massachusetts 02472

[#]Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Abstract

Recent studies on cardiac hypertrophy animal model suggest that inter-domain interactions within the ryanodine receptor (RyR2) become defective concomitant with the development of hypertrophy (e.g. destabilization of the interaction between N-terminal and central domains of RyR2; Circulation 111, 3400–3410, 2005). To determine if destabilization of the inter-domain interaction in fact causes hypertrophy, we introduced DPc10 (a peptide corresponding to the G²⁴⁶⁰-P²⁴⁹⁵ region of RyR2, which is known to destabilize the N-terminal/central domain interaction) into rat neonatal cardiomyocytes by mediation of peptide carrier BioPORTER. After incubation for 24 hrs the peptide induced hypertrophy, as evidenced by significant increase in cell size and [³H]leucine uptake. K201 or dantrolene, the reagents known to correct the de-stabilized inter-domain interaction to a normal mode, prevented the DPc10-induced hypertrophy. These results suggest that disruption of the normal N-terminal/central inter-domain interaction within the RyR2 is a causative mechanism of cardiomyocyte hypertrophy.

Keywords

ryanodine receptor; cardiomyocyte; hypertrophy; domain peptide; dantrolene; endothelium

Introduction

The cardiac ryanodine receptor (RyR2) not only plays a central role in excitation-contraction (E-C) coupling, but also its defective operation causes some cardiac diseases, such as sudden cardiac death (SCD), such as catecholaminergic polymorphic ventricular tachycardia (CPVT). As widely recognized, the sites of CPVT mutations reported in the literature are not randomly distributed, but they are localized to the four well definable regions: I, residues 77–466 (N-terminal domain); II, 2246–2534 (central domain); III, 3778–4201; IV, 4497–4959 [1]. According to the hypothesis deduced from our recent studies [2–4], the N-terminal domain and the central domain of RyR2 form a pair of interacting domains, which serves as the implicit on/off switch for channel opening/closing (designated as a Domain Switch). In diastole (in a

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[†]Address for correspondence: Noriaki Ikemoto, Ph.D., Boston Biomedical Research Institute, 64 Grove St., Watertown MA 02472, Tel: 617-658-7774, Fax: 617-972-1761, ikemoto@bbri.org.

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resting state), the interaction of the two domains is tight, or the Domain Switch is in a 'zipped' configuration, to stabilize the closed state of Ca^{2+} channel. In systole (upon activation), these critical inter-domain contacts are weakened, producing 'unzipped' configuration, which is recognized by the channel as an activation signal. CPVT-linked mutation in either N-terminal domain or central domain weakens the inter-domain interaction (defective domain unzipping), causing channel activation in an otherwise resting state. This is manifested in 'diastolic SR Ca^{2+} leak' widely observed in diseased RyR2 channels. Defective domain unzipping is also produced in other types of cardiac myopathy (e.g. hypertrophy and oxidative stress), which is not caused by RyR2 mutation but rather, by a still unknown allosteric mechanism.

As shown in recent studies of ventricular pacing-induced dog cardiac hypertrophy [5,6], the aforementioned defective domain unzipping takes place concomitant with the development of hypertrophy, suggesting that defective domain unzipping may be a causative mechanism of hypertrophy. However, it is not clear whether this defect is in fact the cause of, or the side effect of, hypertrophy, because the ventricular pacing protocol most likely affects many proteins. In order to test the causative role of defective domain unzipping, therefore, we have to examine whether defective inter-domain interaction produced by cell-based manipulation of RyR2 in fact develops hypertrophy. The aim of the present study is to test this possibility. Here we demonstrate that introduction of a central domain peptide DPc10, which is known to produce defective domain unzipping by interfering with the inter-domain interaction between the N-terminal domain and the central domain [7], into neonatal cardiomyocytes in fact develops hypertrophy.

Materials and Methods

Reagents

K201 was provided by Aetas Co, Ltd. (Tokyo Japan) and dantrolene was from Sigma.

Isolation of primary cardiomyocytes

Neonatal cardiac myocytes were prepared using a Percoll gradient method as described in Ref. 8. Myocytes from 1–2-day-old Sprague-Dawley rats were cultured in a serum-containing medium (Dulbecco's modified Eagles's medium, 10% horse serum, 5% fetal bovine serum, 1 units/ml penicillin, 0.1 mg/ml streptomycin, 0.25 $\mu\text{g}/\text{ml}$ Amphotericin B, 0.1 mM Brdu and 2 mM L-glutamine).

Domain peptide

DPc10, corresponding to amino acids Gly²⁴⁶⁰-Pro²⁴⁹⁵ of RyR2, was synthesized on an Applied Biosystems model 431A synthesizer employing Fmoc as the α -amino protecting group. The peptide was cleaved and de-protected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography. The mutation residue corresponds to human CPVT mutation (Arg²⁴⁷⁴ to Ser) [7]

Induction of hypertrophy by DPc10 or ET-1

After isolation, cardiomyocytes were cultured in a serum-free Dulbecco's modified Eagle's medium containing 0.5% Nutridoma (Roche Applied Science) for 24 hrs. At this time point, we introduced 50 μM RyR2-directed peptide DPc10 (or DPc10-mut) into the living cell using the BioPORTER reagent (Gene Therapy Systems, Inc.) for 4 hrs, which can deliver bioactive molecules across the cell membrane into the cell. As a reference experiment, the cells were treated with 0.1 μM ET-1 instead of DPc10 in the absence of added BioPORTER. The myocytes were incubated with 10 μM dantrolene or 0.3 μM K201 for 15 min, and were further incubated with 50 μM peptides in the presence of BioPORTER. We followed the development

of hypertrophy in the cardiomyocytes at 24 hrs incubation with domain peptide or ET-1 and various inhibitors as indicated (see Figure legends). This general protocol was based on the most appropriate protocol deduced from our pilot experimental data showing that (i) BioPORTER allowed direct translocation of various membrane impermeable peptides and antibodies into the living cell, (ii) this induced hypertrophy in the myocytes within 24–48 hrs after the incorporation of the peptides, and (iii) incubation of myocytes with BioPORTER without added effectors produced no appreciable effect.

Criteria to evaluate the development of hypertrophy

(i) Increase in [³H]leucine uptake—At 24 hrs incubation with domain peptide or ET-1, the control and treated cells were incubated with 1 μ Ci/ml [³H]leucine (PerkinElmer Life Sciences). The cells were harvested by detaching from the culture plate using a minimum volume of cell harvesting solution. A portion of the solution containing the harvested myocytes was treated with trichloroacetic acid (Sigma) to precipitate the cellular proteins for radioactivity assay by liquid scintillation counting, and a portion of the solution was subjected to the hemacytometer counting of the number of cells. The ratio (the determined radioactivity in a unit volume of the harvest solution)/(the number of the cells in the corresponding unit volume of the harvest solution) was the amount of [³H]leucine uptake per cell.

(ii) Increase in cell size—After incubation of the treated and control cells for 24 hrs, the control and treated cells were fixed with 3.7% formaldehyde. To facilitate the measurements of cell size, the fixed cells were stained with anti-sarcomeric α -actinin Ab (Sigma) (for clear recognition of the cytoplasmic area) and TO-PRO-3 (Invitrogen) (for staining of nucleus). We then determined the cytoplasmic area of individual cells, in the confocal fluorescence microscopy (BioRad) image, using ImageJ software (NIH).

(iii) Expression of fetal and embryonic genes—RNA was isolated from cardiomyocytes using TRIzol reagent (Invitrogen) and processed according to the manufacturer's instructions. Reverse transcription for first-strand cDNA synthesis was performed with the SuperScript III system (Invitrogen). Semi-quantitative PCR amplifications employed 30 cycles with steps at 94.5 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1.5 min with Taq DNA polymerase (New England Biolabs) using selected primers.

Statistical Analysis

Values are presented as mean $\pm$ S.E.M. Data analysis was performed with unpaired Student's *t*-test, with Prism software (GraphPad Software, Inc.). We accepted a *p* value less than 0.05 as statistically significant.

Results and Discussion

DPc10 (a domain-unzipping peptide) as well as Endothelin-1 (ET-1: a trigger of pharmacological hypertrophy) induced hypertrophy in the neonatal cardiomyocytes

DPc10 (a peptide corresponding to the G²⁴⁶⁰-P²⁴⁹⁵ region of the central domain) binds to the 1–450 residue region of the N-terminal domain in competition with its corresponding native domain [4]. Thus, the peptide interferes with the N-terminal/central domain interaction, and causes defective domain unzipping, as determined by site-specific fluorescence labeling and spectroscopic monitoring of local protein conformational change [5,7]. According to our hypothesis, CPVT mutation in the G²⁴⁶⁰-P²⁴⁹⁵ region (e.g. R2474S) abolishes the ability of this region to interact with the N-terminal domain. Therefore, a single CPVT mutation made in DPc10 (DPc10-mut: R2474S) abolishes the ability of the peptide to interfere with the inter-domain interaction, providing us with an excellent negative control [5,7].

We introduced DPc10 into cultured neonatal cardiomyocytes with the aid of peptide/protein carrier BioPORTER (DPc10, Fig. 1). As a control, the cells were treated with BioPORTER, without added peptides (BP). To compare the effect of DPc10 with that of neurohormonal trigger of hypertrophy (well-characterized hypertrophy model of neonatal cardiomyocytes), the cells were treated with ET-1 (ET-1), or without treatment (control, Ctrl). After incubation for 24 hrs these cells were fixed and immuno-stained. As shown in Fig. 1A, the size of the cells treated with either DPc10 or ET-1 is considerably larger than the size of the corresponding control cells. We then determined the area of *n* number of cells to plot the cell size distribution for five groups of cells (Ctrl, ET-1, BP, DPc10, and DPc10-mut) (Fig. 1B). In agreement with previous report [8], ET-1 produced a significant increase in cell size (hypertrophic growth). Incubation with the peptide carrier BioPORTER without DPc10 (BP) produced no appreciable effect on the cell size. However, incubation with DPc10 and BioPORTER produced a significant increase of cell size: the effect comparable with that of ET-1. Introduction of DPc10-mut into the cell via BioPORTER produced no appreciable effect on the cell size (DPc10-mut, Fig. 1B).

A considerable number of reports are available in the literature concerning the hypertrophy model of neonatal cardiomyocyte induced by neurohormonal triggers, such as ET-1 and phenylephrine (PE). In these studies two more parameters, beside the increase in the cell size, have been used as criteria to assess the development of hypertrophy: namely, increase in the uptake of amino acids from the culture medium [8,9], and expression of fetal and embryonic genes such as ANP and BNP [9–11]. To confirm the effect of DPc10 on cardiomyocyte hypertrophy, we measured [³H]leucine uptake and up-regulation of fetal gene expression, ANP and BNP, after treatment of cardiomyocytes with ET-1, BP, DPc10 and DPc10-mut. DPc10 and ET-1 approximately doubled the [³H]leucine uptake, but DPc10-mut showed virtually no effect (Fig. 2A). DPc10 and ET-1 increased also the amount of both ANP and BNP mRNAs, but DPc10-mut produced little or no effect on ANP and BNP expression (Fig.2B).

As determined from these criteria of hypertrophy, these results suggest that the RyR2-directed peptide DPc10, which produces defective inter-domain interaction within the Domain Switch of RyR2, induces hypertrophy in neonatal cardiomyocytes, working equally well as ET-1 (the trigger of pharmacological hypertrophy).

Both DPc10-induced and ET-1-induced hypertrophies are prevented by RyR2-directed agents that correct defective inter-domain interaction

K201, the 1,4-benzothiazepine derivative, binds to the 2114–2149 region of RyR2, corrects the defective inter-domain interaction and prevents the development of cardiac hypertrophy as shown in recent reports [12]. Dantrolene is a muscle relaxant by action on the ryanodine receptor, and it is the only specific and effective treatment for malignant hyperthermia. As shown in our recent reports [13,14], dantrolene binds to the L⁵⁹⁰-C⁶⁰⁹ sequence of RyR1 (skeletal muscle-type RyR) and corrects defective unzipped configuration of Domain Switch to a normal mode. Dantrolene has widely been thought that it would be a specific inhibitor of RyR1. However, the drug binding sequence is exactly identical between RyR1 (L⁵⁹⁰-C⁶⁰⁹) [13,14] and RyR2 (L⁶⁰¹-C⁶²⁰) [15]. In fact, the drug does become accessible to, and works as an inhibitor of, the diseased RyR2 by correcting defective inter-domain interaction as in the case of RyR1 (unpublished data).

We carried out the same ET-1 and DPc10-induced hypertrophy experiments as shown in Fig. 1 in the presence of dantrolene or K201. As shown in Fig. 3, dantrolene as well as K201 prevented the DPc10-induced hypertrophic growth, as evidenced by the fact that the cell size distribution in the presence of these reagents is basically identical with that of control (cf. BP, Fig. 1) even after the treatment with DPc10. These results confirm the concept that defective

inter-domain interaction within the RyR2 is the causative mechanism for the development of hypertrophy in the neonatal cardiomyocytes.

An exciting finding in this experiment (Fig. 3) is that dantrolene and K201, the RyR2-channel stabilizing reagents, not only prevented DPc10-induced hypertrophic growth, but also prevented ET-1-induced hypertrophic growth. These results suggest that defective inter-domain interaction within the RyR2 is involved in some steps of the pathway of pharmacological hypertrophy.

In conclusion, defective inter-domain interaction between the N-terminal and central domains of RyR2, produced by domain peptide DPc10 that interferes with a normal inter-domain interaction, induced hypertrophy in the neonatal cardiomyocytes. RyR2-directed agents that correct defective inter-domain interaction (K201 and dantrolene) prevented the DPc10-induced hypertrophy. These results indicate that the defective inter-domain interaction is the causative mechanism for the development of hypertrophy in these cells. The present results also suggest that RyR2 is involved in some steps of the pathogenic process of pharmacological hypertrophy, although the question as to how the neurohormonal stimulation of the surface receptor causes defective inter-domain interaction within the RyR2 has remained open for future studies.

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A

Figure 1. RyR2-directed peptide DPc10 as well as ET-1 induce hypertrophic growth of neonatal rat cardiomyocytes

We prepared five groups of cells classified by the type of treatment: no treatment (Ctrl), 0.1 μ M endothelin-1 (ET-1), BioPORTER (peptide/protein carrier across the cell membrane) alone (BP), or together with either 50 μ M DPc10 (DPc10) or DPc10-mut (DPc10-mut). **A.** After incubation cell culture for 24 hrs, the cells were stained with anti- α -actinin antibody (to visualize cell area: red) and TO-PRO-3 (to visualize nucleus: blue). Bar shows a 50 μ m scale. **B.** The area of individual cells was determined from the confocal images using the NIH Image-J program. Mean (line) of each graph was shown on the histogram. One pixel corresponds to 0.15 μ m².

B

Figure 2. DPc10 as well as ET-1 increases [^3H]leucine uptake, and fetal gene expressions in neonatal cardiomyocytes

A. At 24 hrs of incubation of the cells with DPc10 or ET-1, the cells were incubated with 1 $\mu\text{Ci/ml}$ [^3H]leucine and the total amount of [^3H]leucine taken up by the group of cells was measured by a liquid scintillation counter. The amount of [^3H]leucine per cell was calculated by dividing the total count of [^3H] by cell number, which was counted with a hemacytometer. ** Ctrl vs ET-1, $p < 0.01$; * BP vs DPc10, $p < 0.05$; BP vs DPc10-mut, not significant. **B.** ANP and BNP expression levels were calculated by RT-PCR. Relative amount of mRNA in the presence of ET-1, or DPc10 and DPc10-mut was compared with the amount in the absence of reagents (Ctrl) or in the presence of BioPORTER (BP) alone.

Figure 3. Dantrolene and K201 prevent hypertrophic growth of neonatal cardiomyocytes induced by DPc10 or ET-1

Cardiomyocytes were incubated with either 10 μ M dantrolene (Dan) or 0.3 μ M K201 (K201) during the 24 hrs treatment of DPc10 or ET-1. Histogram of ET-1+dantrolene or +K201 and that of DPc10+dantrolene or +K201 are comparable with that of ET-1 control (ET-1, Fig. 1B) and that of DPc10 control (DPc10, Fig. 1B), respectively. Mean (line) of each graph was shown on the histogram. One pixel corresponds to 0.15 μ m².