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Intracellular translocation of calmodulin and Ca^{2+}/calmodulin-dependent protein kinase II during the development of hypertrophy in neonatal cardiomyocytes

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ABSTRACT

We have recently shown that stimulation of cultured neonatal cardiomyocytes with endothelin-1 (ET-1) first produces conformational disorder within the ryanodine receptor (RyR2) and diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR), then develops hypertrophy (HT) in the cardiomyocytes (Hamada et al., 2009 [3]). The present paper addresses the following question. By what mechanism does crosstalk between defective operation of RyR2 and activation of the HT gene program occur? Here we show that the immuno-stain of calmodulin (CaM) is localized chiefly in the cytoplasmic area in the control cells; whereas, in the ET-1-treated/hypertrophied cells, major immuno-staining is localized in the nuclear region. In addition, fluorescently labeled CaM that has been introduced into the cardiomyocytes using the BioPORTER system moves from the cytoplasm to the nucleus with the development of HT. The immuno-confocal imaging of Ca^{2+}/CaM-dependent protein kinase II (CaMKII) also shows cytoplasm-to-nucleus shift of the immuno-staining pattern in the hypertrophied cells. In an early phase of hypertrophic growth, the frequency of spontaneous Ca^{2+} transients increases, which accompanies with cytoplasm-to-nucleus translocation of CaM. In a later phase of hypertrophic growth, further increase in the frequency of spontaneous Ca^{2+} transients results in the appearance of trains of Ca^{2+} spikes, which accompanies with nuclear translocation of CaMKII. The cardio-protective reagent dantrolene (the reagent that corrects the de-stabilized inter-domain interaction within the RyR2 to a normal mode) ameliorates aberrant intracellular Ca^{2+} events and prevents nuclear translocation of both CaM and CaMKII, then prevents the development of HT. These results suggest that translocation of CaM and CaMKII from the cytoplasm to the nucleus serves as messengers to transmit the pathogenic signal elicited in the surface membrane and in the RyR2 to the nuclear transcriptional sites to activate HT program.

1. Introduction

Since initial demonstration of Paul Simpson that neurohormonal stimulation of cultured neonatal cardiomyocytes causes hypertrophy (HT), this system has been used extensively as a cellular model of cardiac HT to investigate characteristic changes in gene expression and protein kinase signaling [1,2]. The strong advantage of this cellular disease model is that upon stimulation of the cell surface receptor (e.g. the Gαq-mediated receptor) by endothelin-1 (ET-1), the cells develop HT within a day, making it possible to investigate the process of development of HT in a relatively short time. Using this neonatal cell culture model, we have recently shown that the cell developed HT not only by stimulation of the cell with ET-1, but also by DPc10, a domain peptide that destabilizes normal inter-domain interaction within the cardiac ryanodine receptor (RyR2) and causes diastolic Ca^{2+} leak [3]. Furthermore, dantrolene or K201, the reagent that corrects the destabilized inter-domain interaction to a normal mode [4–9], prevented the development of HT that would have been induced by ET-1 or DPc10 [3]. These findings suggest that stimulation of cultured neonatal cardiomyocytes with ET-1 first produces conformational disorder within the RyR2 and diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR), then develops HT in the cardiomyocytes [3].

By what mechanism does crosstalk between defective operation of RyR2 and activation of the HT gene program occur? The present paper addresses this question. As is well known, RyR2-bound CaM inhibits RyR2 channels at a physiological concentration of cytoplasmic Ca^{2+} [10–12]. This implies that the RyR2-bound CaM stabilizes the closed state of RyR2 channels in the resting state of

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normal cells. Accordingly, CaM dissociation from RyR2 will likely activate the channel in an otherwise resting condition, causing a diastolic Ca^{2+} leak, which represents the pro-HT conditions. Consistent with this prediction, Meissner and his colleagues have shown that a mouse with 3 amino acid substitutions in the CaM binding domain of RyR2, which make the RyR2 unable to bind CaM, developed HT and early death [13]. Accumulated evidence also suggests that increased CaMKII-dependent phosphorylation of RyR2 leads to increased SR Ca^{2+} leak, causing elevated cytosolic Ca^{2+} levels, thereby providing a potential arrhythmogenic substrate that triggers cardiac disorder, such as heart failure and atrial fibrillation [14–17]. On the other hands, intensive investigations of *in vitro* and *in vivo* models of HT have revealed crosstalk among multiple parallel pro-hypertorphic signaling pathways, many of which are regulated by CaM and CaMKII. For instance, Ca^{2+}/CaM-dependent activation of calcineurin dephosphorylates NFAT-P to NFAT; the de-phosphorylated NFAT is translocated into the nucleus to activate HT gene program [18]. It is also well established that CaM/CaMKII∂-mediated phosphorylation of histone deacetylase (HDAC) exports the phosphorylated HDAC from the nucleus, and activates HT gene program as a result of removal of the transcriptional suppressor HDAC [19–21]. Thus, it seems that CaM and CaMKII are involved in the upstream pathway leading to the arrhythmogenic diastolic Ca^{2+} leak through RyR2 as well as in the downstream pathway leading to the activation of pro-HT gene program. The above background information suggests the hypothesis that the pathogenic proteins, CaM and CaMKII, also serve as messengers for the crosstalk between the conformational disorder of RyR2 and development of HT. Here we present the data suggesting that translocation of CaM and CaMKII from the cytoplasm to the nucleus serves as messengers to transmit the pathogenic signal elicited in the cytoplasm (i.e. defective inter-domain interaction within the RyR2 and resultant aberrant cytoplasmic Ca^{2+} events) to the nuclear transcriptional sites to activate HT program.

2. Materials and methods

2.1. Reagents

Dantrolene and endothelin-1 were obtained from Sigma.

2.2. Isolation of primary cardiomyocytes

Neonatal cardiac myocytes were prepared using a Percoll gradient method as described in Ref. [22]. Myocytes from 1- to 2-day-old Sprague–Dawley rats were cultured in a serum-containing medium (Dulbecco's modified Eagle's medium, 10% horse serum, 5% fetal bovine serum, 1 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 mg/ml Amphotericin B, 0.1 mM Brdu and 2 mM L-glutamine) for 24 h.

2.3. Induction of hypertrophy by 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 another 24 h. At this time point, the cells were treated with either (i) 0.1 μM ET-1 alone or (ii) 0.1 μM ET-1 supplemented with 10 μM dantrolene or (iii) none (control). Development of HT in the cardiomyocytes was followed at different time points (6–24 h).

2.4. Determination of cell size of the cardiomyocytes

The treated (ET-1 and ET-1 + dantrolene) and control cells were fixed with 3.7% formaldehyde at different times of incubation. For the measurements of cell size, the fixed cells were stained with anti-sarcomeric alpha-actinin antibody (Sigma) and TO-PRO-3 (Invitrogen) (for staining of nucleus). Areas of individual cells in the confocal fluorescence microscopy (BioRad) images were determined using ImageJ software (NIH).

2.5. Immuno-staining of cardiomyocytes

The treated (ET-1 and ET-1 + dantrolene) and control cells fixed with 3.7% formaldehyde at 6, 12 and 24 h of incubation in the course of development of HT were taken for co-immunostaining using anti-CaM monoclonal antibody (Epitomics) and anti CaMKII monoclonal antibodies (Santa Cruz Biotechnology) and imaged in fluorescence confocal microscope.

2.6. CaM-alexa translocation study

For the exogenously introduced CaM relocation study, mutant CaM (with Cysteine at 34 position) was conjugated with alexa546-maleimide. The purified CaM-alexa conjugate was introduced into living cardiomyocytes using the BioPORTER protein-loading reagent (Gene Therapy Systems, Inc.) just before the treatment with ET-1 and incubated for 4 h for the delivery of CaM-alexa across the cell membrane. The CaM-alexa loaded cells were then treated with ET-1 for 24 h for the development of HT, the control cells were incubated without any treatment. Live cells were imaged by following the alexa fluorescence in confocal microscope.

2.7. Studies of calcium transients

At 6, 12 and 24 h time points of ET-1 treatment, the cardiomyocytes were treated with 5 μM fluo 4 AM (cell-permeable calcium indicator, Invitrogen) in an imaging buffer solution (25 mM HEPES, 6 mM glucose, 2 mM $CaCl_2$, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, pH 7.4) and incubated at 37 °C for 30 min. The cells were

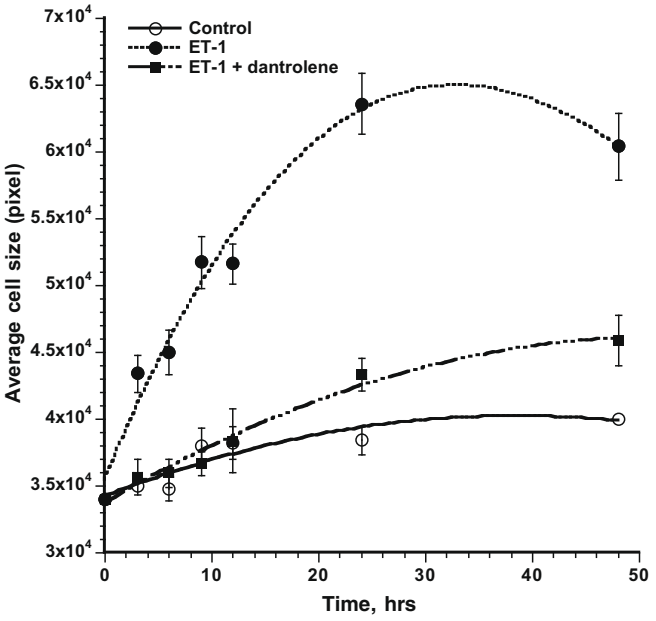

Fig. 1. Effects of ET-1 and dantrolene on the time course of cell growth of neonatal cardiomyocytes. Cardiomyocytes fixed at 3, 6, 9, 12, 24 and 48 h time points of incubation with ET-1 (0.1 μM), ET-1 (0.1 μM) + dantrolene (10 μM) or none (control) were immuno-stained with anti-α-actinin antibody and the areas of individual cells (the cell size) were determined using ImageJ program. The average cell size at each time point was plotted against the time of incubation for all three groups of cardiomyocytes: control (○), ET-1 (●), ET-1 + dantrolene (■).