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Aberrant interaction of calmodulin with the ryanodine receptor develops hypertrophy in the neonatal cardiomyocyte

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Abstract

We have recently shown that the inter-domain interaction between the two domains of ryanodine receptor (RyR), calmodulin binding domain (CaMBD) and CaM-like domain (CaMLD), activates the Ca²⁺ channel, and this process is called ‘formation of activation-link’ [Gangopadhyay, J. P. and Ikemoto, N. (2008) *Biochem. J.* **411** 415–423]. Thus, CaM that is bound to the CaMBD is expected to interfere the activation-link formation, thereby stabilizing the closed state of channel under normal conditions. Here we report that upon stimulation of neonatal cardiomyocytes with pro-hypertrophy agonist endothelin-1 (ET-1), CaM dissociates from the RyR, which induces a series of intracellular events: increased frequency of Ca²⁺ transients, translocation of the signaling molecules CaM, CaM kinase II (CaMKII) and the transcription factor N-FAT (nuclear factor of activated T-cell) to the nucleus; then these events lead to the development of hypertrophy. Importantly anti-CaMBD antibody that interferes with the activation-link formation prevented all of these intracellular events that would have been triggered by ET-1, and then prevented the development of hypertrophy. These results indicate that the aberrant formation of the activation-link between CaMBD and CaMLD of RyR is a key step in the development of hypertrophy in cultured cardiomyocytes.

Keywords

anti-CaMBD antibody; activation-link; calmodulin; calmodulin binding domain (CaMBD); CaMKII; N-FAT; ryanodine receptor

INTRODUCTION

In the cardiac ryanodine receptor (RyR2), calmodulin (CaM) inhibits channel activity at both low (sub- μ M) and high (μ M) [Ca²⁺] [1,2]. CaM binding seems to take place with residues 3583–3603 of RyR2, designated as CaM binding domain (CaMBD) [3]. Earlier studies on skeletal muscle RyR (RyR1) have revealed another important segment (a.a. 4064–4210 of RyR1) that resembles the structure of CaM and contains putative Ca²⁺ binding sites [4,5]. Homologous segment (a.a. 4020–4166) is present in the RyR2, which we designate as CaM-like domain (CaMLD). Our recent studies on the RyR1, [6] suggest that inter-domain interaction between the CaMBD and the CaMLD activates Ca²⁺ channel. To

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AUTHOR CONTRIBUTION

Jaya P. Gangopadhyay and Noriaki Ikemoto designed the experiments and analyzed results.

Jaya P. Gangopadhyay carried out the experiments and wrote the manuscript.

facilitate discussion, we designate this phenomenon as an 'activation-link' formation. According to this model, the CaM that is bound with the CaMBD is expected to interfere with the activation-link formation, and block channel activation. This mechanism accounts for the aforementioned fact that the bound CaM inhibits channel activity of RyR2, which would help to maintain the closed state of the RyR2 Ca²⁺ channel in a diastolic phase, and facilitate relaxation in a post-systolic phase.

In many different types of cardiac disease, the RyR2 Ca²⁺ channel opens erroneously in an otherwise resting state; a common syndrome known as 'diastolic Ca²⁺ leak' [7,8,9]. Several reports suggest that defects in the CaM interaction with RyR2 become a pathogenic factor in heart disease. For instance, according to Meissner and his colleagues [10], transgenic mice containing RyR2 with a mutated CaMBD unable to bind CaM, developed hypertrophy and early death. Ono *et al.* [11] analyzed the RyR2-CaM interaction in normal and the ventricular pacing-induced canine heart failure model with the fluorescent photo-affinity CaM probe, and have shown that the CaM-affinity in the failing RyR2 is significantly lower than that of normal RyR2. Furthermore, Xu *et al.* [12] have shown that upon beta-adrenergic stimulation of the transgenic mice carrying CPVT R2474S mutation in RyR2, the affinity of CaM binding to the CPVT RyR2 is reduced considerably with no appreciable change in the WT RyR2.

The above facts suggest the hypothesis that in the pathological conditions of cardiac myocytes, abnormally tight activation-link is formed between the CaMBD and the CaMLD, resulting in the weakening of CaM binding and eventual CaM dissociation from RyR2, and in turn induces pathogenic diastolic Ca²⁺ leak. The main aim of the present study is to test this hypothesis using neonatal cardiomyocytes as a cell model of hypertrophy. Neurohormonal stimulation of the neonatal cardiomyocyte culture causes hypertrophic growth within a day, showing characteristic changes in gene expression [13–16] and in proteins signaling [17–19]. This makes this system a versatile cell model to study various intracellular molecular events during the course of development of hypertrophy. In our recent study [20,21], we induced hypertrophy in the neonatal rat cardiomyocytes by endothelin-1 (ET-1) as well as by direct manipulation of inter-domain interaction between the N-terminal domain and the central domain of RyR2 with a domain peptide, DPc10, corresponding to the central domain of RyR2. We then found that during the development of hypertrophy, CaM and CaMKII are translocated to the nucleus. CaM translocation coincides with a moderate increase in the frequency of spontaneous Ca²⁺ transients, while CaMKII translocation coincides with an appearance of the 'trains' of spontaneous Ca²⁺ transients. These findings suggest that neurohormonal stimulation induces conformational disorders in RyR2, which cause aberrant cytoplasmic Ca²⁺ events; the patterns of aberrant Ca²⁺ events are registered in the CaM/CaMKII system; this message is then transmitted to the nucleus as a pathogenic signal to develop hypertrophy.

Here we report that hypertrophic stimulus of neonatal cardiomyocytes with ET-1 produces CaM dissociation from RyR2, which leads to sequential intracellular events including increased frequency of spontaneous Ca²⁺ transients, translocation of CaM, CaMKII, and N-FAT to the nucleus. Importantly, it has been found that an anti-CaMBD antibody, used as a 'molecular wedge' of the CaMBD/CaMLD interaction, prevented all of these ET-1-induced pathological intracellular events, then prevented the development of hypertrophy. This supports the hypothesis that aberrant formation of the channel activation-link between the CaMBD and the CaMLD of RyR2 is an early key event leading to the development of hypertrophy in this cell model.

EXPERIMENTAL

Isolation of primary cardiomyocytes and induction of hypertrophy by ET-1

Neonatal cardiac myocytes were prepared using a Percoll density gradient method as described previously [13]. Myocytes from 1–2 days old Sprague-Dawley rat hearts 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. The cardiomyocytes were cultured for another 24 h in a serum free Dulbecco's modified Eagle's medium containing 0.5 % nutridoma. At this time point the cells were treated with 0.1 μ M ET-1, then incubated for 24 h for the development of hypertrophy. The animals used for the isolation of cardiomyocytes were handled following the animal protocol approved by NIH and the cells were disposed following the biohazard disposal regulations of the Institute.

In vivo cross-linking assay

Neonatal cardiomyocytes were cultured in fibronectin coated 10 mm culture dish with 8–10 million cells per dish. For *in vivo* cross-linking the culture medium was replaced with 2 % formaldehyde in PBS. After 2 min of incubation the cells were washed with PBS and harvested by scraping the cells off the plate, centrifugation at 10,000 g for 10 minutes and the cell pellet was suspended in an extraction solution containing 50 mM Tris, 10 mM EGTA, 2 % SDS, pH 8.0 supplemented with protease inhibitors. Cells were disrupted in a tissuelyser for 1 min at 25 Hz/min frequency. After centrifugation the supernatant was used for Western blot analysis. RyR2 was detected by anti 34C antibody (1 : 200) and the RyR2 associated CaM was detected with anti-CaM antibody (1 : 2000).

Introduction of the anti-CaMBD antibody to the cardiomyocytes

The custom made antibody corresponding to the CaM binding domain (CaMBD) of RyR1 was purified using peptide affinity column [6]. To determine the specificity of the antibody for RyR2, the neonatal cardiomyocytes cell extract was used for Western blot analysis with a 1 : 15,000 dilution of the antibody. The purified antibody was complexed with BioPORTER protein-loading agent (Genlantis) for 5 min for its delivery across the cell membrane, and it was added to the serum free culture media. After 4 h of incubation the cells were washed with PBS and the antibody loaded cells were treated with ET-1 for the development of hypertrophy. The untreated cells were used to check the effect of the antibody alone.

Calcium transient assay

The cardiomyocytes were treated with 5 μ M fluo 4 AM (cell permeable calcium indicator, Invitrogen) in an imaging buffer solution (25 mM HEPES, 6 mM glucose, 2 mM CaCl₂, 150 mM KCl, 1.2 mM MgSO₄, pH 7.4) and incubated for 30 min at 37 °C. The cells were washed with the imaging buffer alone to remove excess fluo 4 AM and incubated another 30 min at 37 °C. Live cells were imaged at 22°C in a confocal microscope (Leica TCS SP5) for the synchronous calcium transients following the green fluorescence of fluo 4 for 2 minutes. [$\lambda_{\text{excitation}} = 494 \text{ nm}$, $\lambda_{\text{emission}} = 516 \text{ nm}$].

Immuno-cytological analysis

The cells fixed with 3.7 % formaldehyde for 15 min were used for immuno-staining. Monoclonal antibodies for anti-CaM (epitomics) and anti-CaMKII (Santa Cruz biotechnology) were used for co-immuno-staining at a dilution of 1 : 200. The anti-N- FAT monoclonal antibody (Cell Signaling) was used at a 1 : 100 dilution. The fluorescence intensity in the cytoplasm and the nucleus were quantified using the ImageJ software (NIH).

Determination of the cell-size

The cells were fixed with 3.7 % formaldehyde for 15 min and immuno-stained with antisarcomeric alpha-actinin antibody (Sigma). The areas of individual cells in confocal microscopy images were determined using imageJ software (NIH).

Statistical analysis

The statistical significance of the data were analyzed by unpaired student's *t*-test using Prism software (GraphPad Software, Inc). A *p* value less than 0.05 was considered to be statistically significant.

RESULTS

Pro-hypertrophy stimulus of neonatal cardiomyocytes dissociates the RyR2-bound CaM

In several animal models of cardiac disease, there is a clear indication of reduced affinity of CaM binding to RyR2 [11,12]. It has also been shown that depletion of the ability to bind with CaM from RyR2 causes cardiac hypertrophy and early cardiac death [10]. These facts suggest that the RyR2-bound CaM plays an important role in regulating normal function of RyR2, and defective interaction of CaM with RyR2 will cause cardiac disorder (cf. Introduction). The first question we addressed in the present study is whether CaM dissociates from the RyR2 during the development of hypertrophy in the neonatal cardiomyocytes. In the experiment shown in Figure 1, we fixed the cardiomyocytes treated with ET-1 and matching control cells at 24 h with 2% formaldehyde for *in vivo* protein/protein cross-linking, then subjected to Western blot analysis of electrophoretically separated protein bands by immuno-staining with anti-RyR2 Ab and anti-CaM Ab. The expression level of RyR2 estimated by the immuno-staining intensity of RyR2 was about identical between the control and the ET-1 treated cells. However, the immuno-staining intensity of the RyR2-bound CaM of the ET-1 treated cell, expressed as the density ratio of CaM/RyR2, was about 30% of that of the control cells. This suggests that neurohormonal stimulation of the cardiomyocytes produced some structural changes (i.e. according to the hypothesis, abnormally tight CaMBD/CaMLD interaction) in the RyR2, which decreased the affinity of CaM binding to the RyR2 and partial dissociation of the bound CaM.

Pathological changes in the RyR2 induce a series of aberrant intracellular events that lead to the development of hypertrophy

As shown by many investigators, diastolic Ca^{2+} leak and intracellular Ca^{2+} overload represent pathological states of cardiomyocytes, which are usually recognized as increased frequency of spontaneous Ca^{2+} sparks [22–24]. In our recent report [21], we have shown that ET-1 stimulation of the neonatal rat cardiomyocytes causes increased frequency of spontaneous Ca^{2+} transients in an early phase and eventually it develops to a train of Ca^{2+} spikes during the course of development of hypertrophy. We have also shown that the cytoplasmic CaM is translocated into the nucleus in an early phase of hypertrophy development, and the cytoplasmic CaMKII is translocated into the nucleus in a later phase of hypertrophy development. Importantly, the CaM translocation coincides with a moderate increase in the frequency of Ca^{2+} transients, and the CaMKII translocation coincides with the appearance of trains of Ca^{2+} spikes. These findings suggested the new concept that the patterns of aberrant intracellular Ca^{2+} transients are registered in the CaM/CaMKII system as pathogenic signals, which are transduced to the nuclear transcriptional sites to activate hypertrophy program as CaM and CaMKII move into the nucleus.

In the present study, we investigated several key events presumably involved in the pathogenic pathway using the neonatal cardiomyocytes that have been treated with ET-1 for 24 h with reference to the untreated matching control cells. They are: (i) increased frequency

of spontaneous Ca^{2+} transients, (ii) translocation of cytoplasmic CaM and CaMKII into the nucleus, (iii) nuclear import of N-FAT, one of the most extensively investigated mechanisms in the literature involved in the cytoplasm-to-nucleus transduction of the transcriptional signal to activate the hypertrophy gene program [25–27,18,19], and (iv) cell size increase as an indicator of the development of hypertrophy. In agreement with our previous report [21], at 24 h incubation the ET-1-treated cells show trains of spontaneous Ca^{2+} spikes, that are often synchronized among the neighboring cells, whereas there are virtually no spontaneous Ca^{2+} transients in the untreated control cells (Figure 2).

Figure 3 depicts confocal images of the immuno-staining patterns of the control and the ET-1-treated cells that are double-stained with anti-CaM Ab and anti-CaMKII Ab, showing CaM staining (left column, CaM), CaMKII staining (middle column, CaMKII). Importantly, in the control cells the staining intensity of both CaM and CaMKII is localized chiefly in the cytoplasm, indicating their less abundance in the nucleus than in the cytoplasm. In the ET-1 treated cells, however, the distribution of the staining density between the cytoplasm and the nucleus is reversed in both cases of CaM and CaMKII, indicating that a larger population of both cytoplasmic CaM and CaMKII has moved into the nucleus in the ET-1-treated cells.

Of various pathogenic mechanisms of cardiac hypertrophy, the mechanism mediated by nuclear import of N-FAT is well established in the literature [25–27,18,19]. Namely, Ca^{2+} /CaM-mediated activation of calcineurin de-phosphorylates the transcriptional factor N-FAT, and the de-phosphorylated N-FAT is translocated into the nucleus to activate the hypertrophy program. In the present study, we performed immuno-staining of N-FAT of the control cells and the ET-1-treated cells using anti-N-FAT Ab (Figure 4). As shown, the staining intensity is chiefly localized in the cytoplasm in the untreated cells (N-FAT-control). In the ET-1-treated and hypertrophied cells (N-FAT-ET1), however, major intensity has been shifted from the cytoplasm to the nucleus, indicating that coupled with the aforementioned aberrant intracellular events, N-FAT has moved from the cytoplasm to the nucleus. This suggests that the aberrant intracellular events described here led to the activation of the hypertrophy gene program via the N-FAT-mediated transcription mechanism.

Figure 5 shows morphological evidence that ET-1 treatment and the induced intracellular events described above have developed hypertrophy in the neonatal cardiomyocytes: (A) the histograms of cell size distribution of the control and the ET-1 treated and hypertrophied cells at 24 h, and (B) mean values of the size of these cells. As shown, ET-1 treatment has produced a considerable increase in both average cell size and variation in the cell size distribution compared with those of the untreated cells.

Anti-CaMBD antibody inhibits these pathological intracellular events and prevents the development of hypertrophy in the neonatal cardiomyocytes

According to our hypothesis, partial dissociation of CaM from the RyR2 (an event in the upstream of the series of intracellular events described here) is produced by abnormally tight interaction between the CaMBD and the CaMLD, which would have been suppressed by the CaMBD-bound CaM (owing to its molecular wedging effect) in a normal diastolic state. A possible way to test this hypothesis might be to introduce a sufficiently large amount of CaM into the cell and to examine whether the exogenously introduced CaM prevents the aberrant intracellular events and development of hypertrophy. However, this is not a feasible approach, because it is known that over-expression of CaM induces hypertrophy by activating the pro-hypertrophy pathogenic pathways [28,29], and the exogenously introduced CaM is expected to affect a variety of cell functions because of its ubiquitous multiple functions [30–32]. In the present study, we introduced anti-CaMBD Ab, which is raised against the CaMBD using CaMBP (a synthetic peptide corresponding to the CaMBD

of RyR1) as an antigen. RyR1 and RyR2 are highly homologous in their primary structure [33,34], and as expected the anti-CaMBD Ab reacted with RyR2 of neonatal cardiomyocytes as shown in Figure 2 (inset). Our strategy is that the binding of this antibody to the CaMBD will block the excessive and abnormal interaction between the CaMBD and CaMLD, as the CaMBD-bound CaM would do in normal conditions.

We introduced the anti-CaMBD Ab into the control and ET-1-treated neonatal cardiomyocytes by mediation of the protein carrier, BioPORTER [20,21], and examined its effect on each of the pathogenic intracellular events and hypertrophic growth of the cell described above. As shown in Figures 2 through 5, the introduced antibody inhibited all of these events – increased frequency of the spontaneous Ca^{2+} transients (Figure 2), nuclear translocation of CaM and CaMKII (Figure 3), nuclear import of N-FAT (Figure 4) – and prevented the development of hypertrophic growth (Figure 5). The antibody produced no effect on the untreated control cells (Figures 2–5), and non-immune serum had no effect on the ET-1 treated cells (data, not shown). These results provide a strong support to the notion that the abnormal activation-link formation between the CaMBD and the CaMLD within RyR2, which must be prevented in a normal channel regulation at a resting/diastolic state, is a causative mechanism for the development of hypertrophy in the neonatal cardiomyocytes.

DISCUSSION

According to several reports [4,5,35], CaMBP (a peptide corresponding to the CaMBD) activates the skeletal muscle RyR (RyR1) by binding to the CaMLD (residues 4064–4210), and on this basis it was suggested that CaMBD serves as an intrinsic regulator of the RyR1 Ca^{2+} channel [6]. According to the work by Hamilton and co-workers [4], the expressed peptide corresponding to the CaMLD (i.e. CaMLP) has several CaM-like properties, and importantly it binds with CaMBP. This suggests that the *in vivo* counterparts of these peptides, namely CaMLD and CaMBD, can interact with each other. In our recent studies of RyR1 we have shown that the fluorescent probe attached to the CaMLD became inaccessible to fluorescence quenching by a large molecular weight fluorescence quencher with an increase of $[\text{Ca}^{2+}]$ in a range of 0.03–2.0 μM , or in the presence of the RyR1 agonist 4-CmC, indicating that there is in fact the inter-domain interaction between the CaMBD and the CaMLD [6]. Also we have shown that at physiological concentration of Mg^{2+} (mM) the Ca^{2+} -dependent CaMBD/CaMLD interaction is inhibited [6]. Importantly, the Ca^{2+} -dependent activation and Mg^{2+} -dependent inhibition of the CaMBD/CaMLD interaction show a close parallel to the Ca^{2+} dependent activation and Mg^{2+} -dependent inhibition of the RyR1 channel activity, respectively. These results suggest that the mode of interaction between the CaMBD and the CaMLD serves as a key mechanism in the conformation-dependent regulation of the Ca^{2+} channel function. Since the RyR2 and the RyR1 share homologous primary structure [36–38] and common Ca^{2+} -dependent channel activation and Mg^{2+} -dependent channel inhibition mechanism, it seems that the same CaMBD/CaMLD interaction-based mechanism operates in the regulation of RyR2 Ca^{2+} channels as discussed below.

In the RyR2, CaM inhibits channel activity at both diastolic (nM to sub- μM) and systolic (μM to sub-mM) cytoplasmic $[\text{Ca}^{2+}]$ s [1,2]. In the light of the above activation-link hypothesis, it indicates that the CaM that is bound to the CaMBD of RyR2 interferes with the CaMBD/CaMLD interaction. Thus, in the conditions that prevail in a diastolic phase, namely at sub-threshold $[\text{Ca}^{2+}]$ (nM to sub- μM) and in the presence of physiological concentration of Mg^{2+} (1 mM), the RyR2-bound CaM will prevent the activation-link formation to stabilize the closed state of RyR2 Ca^{2+} channels. At above-threshold $[\text{Ca}^{2+}]$ (in a systolic phase), the RyR2-bound CaM will prevent excessive channel activation (buffering effect) and facilitate post-systolic relaxation.

A common syndrome underlying many different types of cardiac disease is that the RyR2 Ca^{2+} channel opens erroneously in an otherwise resting state: the pathological state of RyR2 known as 'diastolic Ca^{2+} leak'. It is also known that in the pathological conditions, the threshold concentration of the cytoplasmic Ca^{2+} for channel activation is considerably reduced, i.e. increased sensitivity to the channel activating Ca^{2+} and the threshold concentration of the cytoplasmic Mg^{2+} for channel inhibition is considerably increased, i.e. reduced sensitivity to the inhibitory Mg^{2+} [39,40]. According to the above hypothesis, such pathological states are produced by two mutually related mechanisms: (a) increased Ca^{2+} affinity of Ca^{2+} -dependent formation of the activation-link and (b) reduced Mg^{2+} affinity of Mg^{2+} inhibition of activation-link formation. The aberrant formation of activation-link produced by these mechanisms (a and b) will weaken the CaM binding. Such conditions will result in a sustained de-stabilization of the closed state of the channel, causing the sustained diastolic Ca^{2+} leak, which ultimately leads to the development of various cardiac disorders, as shown in recent studies. Thus, according to the studies by Xu *et al.* of the transgenic mice carrying CPVT R2474S/+ mutation in RyR2 [12], beta-adrenergic stimulation of RyR2 (mimicry of catecholaminergic stimulation that causes lethal arrhythmia) induced a significant reduction in the affinity of CaM binding to the CPVT RyR2, as determined by the binding assay of a fluorescent photo-affinity CaM probe, CaM-SANPAH. Similar fluorescent photo-affinity CaM binding assay of the ventricular pacing-induced canine heart failure model by Ono *et al.* [11] has shown that in the RyR2 of failing hearts the CaM-affinity is about 20% of that of normal RyR2. Furthermore, according to an earlier study by Meissner and his colleagues [10], transgenic mice containing RyR2 unable to bind CaM, developed hypertrophy and early death. All of these reports are consistent with the view that the weakened CaM/RyR2 interaction and eventual dissociation of CaM from the RyR2, produces various types of cardiac disease, including CPVT, cardiac hypertrophy and heart failure.

The aberrant activation-link formation, weakened CaM binding and eventual CaM dissociation will cause a series of intracellular events that ultimately lead to the development of cardiac disease. Prevention of the aberrant activation link formation will then prevent the pathological events and the development of cardiac disorder. In order to test this prediction, we have employed the *in vitro* cell model of cardiac hypertrophy. Neurohormonal stimulation of cultured neonatal cardiomyocytes causes hypertrophy, characteristic changes in gene expression [13–16] and in protein signaling [17–19]. The strong advantage of this cellular disease model is that because of rather rapid development of disease states, it is possible to investigate various intracellular events during the process of development of hypertrophy in a relatively short time. In addition, we have made an important improvement of this system by creating the capability to introduce various key peptides and antibodies into the living cell using a protein carrier. These added capabilities have permitted us to manipulate conformational states of RyR2 to induce and prevent hypertrophy. Thus, as shown in our recent reports [20,21], pharmacological agonist of hypertrophy ET-1, as well as DPc10 (a central domain peptide that causes aberrant inter-domain interaction between the N-terminal domain and the central domain of RyR2), developed hypertrophy, and dantrolene that corrects the aberrant N-terminal domain/central domain interaction, prevented the development of the ET-1 or peptide-induced hypertrophy. In our more recent study of the neonatal cardiomyocyte model, we have shown that during the ET-1-induced development of hypertrophy, the frequency of spontaneous Ca^{2+} transients increases causing cytoplasm-to-nucleus translocation of CaM and CaMKII [21]. These findings suggest that hypertrophic stimulus applied to the cell surface receptor produces first conformational mal-regulation of RyR2, and aberrant activation of RyR2 Ca^{2+} channels and aberrant intracellular Ca^{2+} events, and then the pathogenic signal elicited in the RyR2 is transduced to the nuclear transcriptional sites via the nuclear translocation of the signaling proteins such as CaM and CaMKII.

The present study has shed more light into the sequential intracellular molecular events during the development of hypertrophy in the neonatal cardiomyocytes. As shown here, several conspicuous intracellular events induced by ET-1, such as (a) partial dissociation of CaM from the RyR2, (b) significant increase of the frequency of spontaneous Ca^{2+} transients, (c) nuclear translocation of the cytoplasmic CaM, (d) nuclear translocation of the cytoplasmic CaMKII, and (e) import of N-FAT into the nucleus. We postulate that pro-hypertrophy stimulus applied to the cell surface receptor produces first an abnormal formation of the activation-link between the CaMBD and CaMLD, weakening CaM binding to the RyR2 and dissociating the RyR2-bound CaM. This leads to diastolic Ca^{2+} leak, which is reflected upon the increased frequency of spontaneous Ca^{2+} transients. The pattern of aberrant cytoplasmic Ca^{2+} events produced by conformational disorders in RyR2 is sensed and memorized by the CaM/CaMKII system. This message is then transmitted to the nucleus as a pathogenic signal when CaM and then CaMKII move into the nucleus for the development of hypertrophy. This process is mediated by the well-known pro-hypertrophic signal transduction pathway: calcineurin-mediated pathway, in which Ca^{2+} /CaM-dependent activation of calcineurin dephosphorylates N-FAT, which thereby translocates dephosphorylated N-FAT to the nucleus to activate a hypertrophy gene program [25–27,18,19]. In view of well-established mechanism of CaMKII/HDAC-mediated activation of pro-hypertrophic/pro-heart failure program [17], we postulate that the observed nuclear translocation of CaMKII may be involved in this pathogenic route.

One of the most important aspects of the present study is the finding that the antibody directed to the CaMBD inhibited all of the aforementioned sequential events from (b) through (e), and prevented hypertrophy to develop. This indicates that binding of the antibody to the CaMBD produces a ‘molecular wedging’ effect on the CaMBD/CaMLD interaction, and prevented aberrant formation of activation-link in a diastolic phase. Consequently it prevented the subsequent events that would have led to the development of hypertrophy. Thus, the present data provide a strong evidence that the aberrant formation of the activation-link between the CaMBD and the CaMLD is one of early key steps in the signal transduction pathway that eventually leads to the activation of hypertrophy program. We should also mention that the aberrant CaMBD/CaMLD interaction is a new target of therapeutic treatment and that the prevention of it is a new strategy for developing the method of treatment.

Recent conformational probe studies of RyR1 and RyR2 in normal and diseased states by Ikemoto and his colleagues have shown that tight interaction (‘zipping’) between the N-terminal domain and the central domain of RyR stabilizes the closed state of the channel, while unzipping of the interacting domains opens the channel [41]. In the diseased conditions, aberrant domain unzipping takes place in an otherwise resting state [42,43]. Interestingly, therefore, it seems that the pathological formation of the CaMBD/CaMLD activation-link described in the present study and the previously described aberrant unzipping of the interacting N-terminal domain/central domain pair are allosterically coupled. If this is the case, therapeutic agents that correct either aberrant formation of the CaMBD/CaMLD activation link or aberrant unzipping of the N-terminal/central domain pair will be equally effective, and combined application of the two types of therapeutic agents will produce synergetic effects. In support of this concept, dantrolene that binds to the N-terminal domain [44] and corrects aberrant unzipping of the N-terminal/central domain pair inhibited diastolic Ca^{2+} leak, and nuclear translocation of CaM and CaMKII, and then prevented the development of hypertrophy in the ET-1 treated neonatal rat cardiomyocyte [21], and in the presence of added CaM, dantrolene exerted more potent channel inhibition effect [45].

In conclusion, the antibody, which is directed to the CaMBD and has an wedging effect on the CaMBD/CaMLD interaction within RyR2, inhibited pathogenic intracellular events, such as cytoplasmic Ca^{2+} overload, nuclear translocation of the putative pathogenic signal transducing proteins (CaM and CaMKII), and nuclear import of N-FAT, and prevented the development of hypertrophy in the neonatal cardiomyocytes. This supports the notion that aberrant tight interaction between the CaMBD and the CaMLD of RyR2 is a key pathogenic mechanism occurring in an early step of the pathogenic pathways of cardiac hypertrophy. An important question to be addressed in the future research is whether the similar mechanism operates in the development of cardiac hypertrophy of human patients and animal models.

Abbreviations used

Ab	antibody
CaM	calmodulin
CaMBD	calmodulin binding domain
CaMBP	calmodulin binding peptide
CaMKII	calmodulin kinase II
CaMLD	calmodulin like domain
CPVT	catecholaminergic polymorphic ventricular tachycardia
ET-1	endothelin-1
HDAC	histone de-acetylase
N-FAT	nuclear factor of the activated T-cell
RyR	ryanodine receptor.

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Figure 1. Calmodulin dissociates from RyR2 during the development of hypertrophy in neonatal rat cardiomyocytes by ET-1

The neonatal rat cardiomyocytes were incubated for 24 h in the presence (hypertrophy model) or in the absence (control) of 0.1 μ M ET-1. Both the control and the hypertrophied cardiomyocytes were treated with 2 % formaldehyde for 2 min to perform in vivo protein-protein cross-linking. **A.** The cells were harvested and analyzed by Western blot using anti-RyR2 antibody (34C) to detect RyR2 and anti-CaM antibody to detect the CaM associated with RyR2. **B.** Densitometric analysis of the immuno-staining intensity of the CaM that co-migrated with RyR2 (determined as the density ratio CaM/RyR2) indicates that about 70 % of the RyR2 bound CaM was dissociated in the ET-treated and hypertrophied cardiomyocytes ($p < 0.01$).

Figure 2. ET-1 induces abnormal Ca^{2+} -transients in neonatal rat cardiomyocytes and anti-CaMBD antibody prevents it

After 24 h incubation, the control and the ET-1 treated cells, loaded with anti-CaMBD antibody or without loading, were subjected to confocal Ca^{2+} imaging to follow the development of spontaneous Ca^{2+} -transients using cell permeable Ca^{2+} indicator fluorescence dye Fluo-4 AM introduced to the cardiomyocyte culture. The average frequency of Ca^{2+} transients per min ($\pm$ SD) obtained from 4–5 experiments indicated in each panel of the four groups of cells: Control, ET-1-untreated cell; ET-1, ET-1 treated cell; ET-1 + Anti-CaMBD Ab, ET-1 treated cell with incorporated anti-CaMBD antibody; Anti-CaMBD Ab alone, control cell with incorporated anti-CaMBD antibody. Note that in the ET-1 treated cells the frequency of spontaneous Ca^{2+} transients showed tremendous increase resulting in the appearance of trains of Ca^{2+} transients, and that the anti-CaMBD antibody that had been introduced into the cells by mediation of BioPORTER prevented the occurrence of the abnormal Ca^{2+} transients developed in the ET-1 treated cells. Inset :

Neonatal cardiomyocytes were harvested and analyzed by Western blot using anti-CaMBD Ab to show that the antibody can detect RyR2.

Figure 3. During the development of hypertrophy in cardiomyocytes, CaM and CaMK-II are translocated (re-distributed) from cytoplasm to the nucleus, and anti-CaMBD antibody prevents CaM/CaMKII translocation

Left panel. Confocal images of immuno-staining patterns of four groups of cells (Control, ET-1-untreated cell; ET-1, ET-1 treated cell; ET-1 + Anti-CaMBD Ab, ET-1 treated cell with incorporated anti-CaMBD antibody; Anti-CaMBD Ab alone, control cell with incorporated anti-CaMBD antibody), showing the staining pattern with anti-CaM monoclonal antibody, with anti-CaMKII monoclonal antibody. The bar shows a 25 μm scale. **Right panel.** Summary of densitometric analysis of the distribution of immuno-staining intensity between the cytoplasm and the nucleus determined using ImageJ program. Note that ET-1 treatment caused significant increase in the cell size (hypertrophic growth) and redistribution of the immuno-staining intensity of both CaM and CaMKII staining from the cytoplasm to the nucleus. Importantly, anti-CaMBD antibody prevented the nuclear translocation of CaM and CaMKII in the ET-1 treated cells. Anti-CaMBD antibody that was introduced in the control cells produced no appreciable effect.

Figure 4. The transcription factor N-FAT is translocated from the cytoplasm to the nucleus in the ET-1 treated cells, and it is prevented by anti-CaMBD antibody
Left panel. Confocal images of the four groups of cells (Control, ET-1-untreated cell; ET-1, ET-1 treated cell; ET-1 + anti-CaMBD Ab, ET-1 treated cell with incorporated anti-CaMBD antibody; Anti-CaMBD Ab alone, control cell with incorporated anti-CaMBD antibody) immuno-stained with anti-N-FAT monoclonal antibody. The bar shows a 25 µm scale. **Right panel.** Summary of densitometric analysis of the distribution of immuno-staining intensity between the cytoplasm and the nucleus determined using ImageJ program. The immuno-staining intensity of N-FAT shifted from the cytoplasm to the nucleus in the ET-1 treated and hypertrophied cells, and anti-CaMBD antibody incorporated into the cell prevented

nuclear translocation of N-FAT, indicating that the nuclear import of N-FAT, a well-established mechanism of pathogenesis of cardiac hypertrophy in *in vivo* models and human patients, is used also as a pathogenic mechanism of hypertrophic growth of neonatal cardiomyocytes.

Figure 5. ET-1-induced intracellular events resulted in a significant increase in the cell size of neonatal cardiomyocytes, and anti-CaMBD antibody prevented the cell size increase

A. Histogram of cell size distribution of the four groups of cells (Control, ET-1-untreated cell; ET-1, ET-1 treated cell; ET-1 + Anti-CaMBD Ab, ET-1 treated cell with incorporated anti-CaMBD antibody; Anti-CaMBD Ab alone, control cell with incorporated anti-CaMBD antibody) was determined by measuring the areas of the individual cells (immuno-stained with anti- α -actinin antibody) using ImageJ program. **B.** Summary of the average cell sizes of the four groups of cells and SD obtained from 3 experiments. The data indicate that the anti-CaMBD antibody prevented the development of hypertrophy in the neonatal cardiomyocytes. ns, statistically not significant.