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Imbalance between CaM kinase II and calcineurin activities impairs caffeine-induced calcium release in hypertrophic cardiomyocytes

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ABSTRACT

Cardiac hypertrophy impairs Ca^{2+} handling in the sarcoplasmic reticulum, thereby impairing cardiac contraction. To identify the mechanisms underlying impaired Ca^{2+} release from the sarcoplasmic reticulum in hypertrophic cardiomyocytes, we assessed Ca^{2+}-dependent signaling and the phosphorylation of phospholamban, which regulates Ca^{2+} uptake during myocardial relaxation and is in turn regulated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calcineurin. In cultured rat cardiomyocytes, treatment with endothelin-1, angiotensin II, and phenylephrine-induced hypertrophy and increased CaMKII autophosphorylation and calcineurin expression. The calcineurin level reached its maximum at 72 h and remained elevated for at least 96 h after endothelin-1 or angiotensin II treatment. By contrast, CaMKII autophosphorylation, phospholamban phosphorylation, and caffeine-induced Ca^{2+} mobilization all peaked 48 h after these treatments. By 96 h after treatment, CaMKII autophosphorylation and phospholamban phosphorylation had returned to baseline, and caffeine-induced Ca^{2+} mobilization was impaired relative to baseline. A similar biphasic change was observed in dystrophin levels in endothelin-1-induced hypertrophic cardiomyocytes, and treatment with the novel CaM antagonists DY-9760e and DY-9836 significantly inhibited the hypertrophy-induced dystrophin breakdown. Taken together, the abnormal Ca^{2+} regulation in cardiomyocytes following hypertrophy is in part mediated by an imbalance in calcineurin and CaMKII activities, which leads to abnormal phospholamban activity.

1. Introduction

Cardiac hypertrophy is an adaptive response of the heart to various intrinsic and extrinsic stimuli. Over time, however, cardiac hypertrophy is detrimental to Ca^{2+} handling in the sarcoplasmic reticulum, thereby impairing cardiac contraction. Sustained cardiac hypertrophy facilitates the development of heart failure [1,2]. Understanding the molecular

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mechanisms underlying impairment of sarcoplasmic reticular Ca^{2+} handling following cardiac hypertrophy will be important for producing novel therapeutic strategies for hypertrophy-induced heart failure.

Ca^{2+} regulates diverse cellular functions such as excitation–contraction coupling in cardiomyocytes [3,4]. During excitation–contraction coupling, activation of voltage-dependent L-type Ca^{2+} channels causes Ca^{2+} influx into the cytoplasm and in turn triggers Ca^{2+} release from the sarcoplasmic reticulum via ryanodine receptors [5]. The increase in the intracellular Ca^{2+} is coupled to activation of troponin C, which induces muscle contraction through the interaction of myofilaments with actin filaments. Uptake of the cytosolic Ca^{2+} by the sarcoplasmic reticulum is essential to allow cardiac muscle to relax. The Ca^{2+} sequestration from the cytoplasm into the sarcoplasmic reticulum is achieved by sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) and a Na^{+}/Ca^{2+} exchanger in the plasma membrane. The SERCA2a form of SERCA has a central role in the reuptake of Ca^{2+} during myocardial relaxation, and phospholamban is a key regulator of SERCA2a activity. Dephosphorylated phospholamban tightly associates with SERCA2a and inhibits its Ca^{2+} pump activity; phospholamban phosphorylation at either Ser-16 or Thr-17 relieves this inhibition by dissociating phospholamban from SERCA2a. In general, decreased expression of SERCA2a or increased expression of phospholamban, or both, lead to impaired Ca^{2+} handling in the sarcoplasmic reticulum of hypertrophied myocardium [6,7].

Protein kinases and phosphatases regulate the Ca^{2+} handling that maintains the contraction and relaxation of the heart [8–11]. Increasing evidence from the use of pharmacologic inhibitors and genetic approaches suggests a causal relationship between the activity of Ca^{2+}/CaM-dependent protein kinase II (CaMKII) and cardiac hypertrophy. Recent studies have demonstrated that transgenic expression of either the nuclear isoform CaMKIIδ_B or the cytoplasmic isoform, CaMKIIδ_C, induces cardiac hypertrophy [12,13]. CaMKII is also involved in the development of cardiac hypertrophy in cultured cardiomyocytes [14–16]. Zhu et al. reported that endothelin-1 increases CaMKII activity in cardiomyocytes, and pretreatment with the CaMKII inhibitor KN62 suppresses endothelin-1-induced cardiomyocyte hypertrophy. Ramirez et al. reported that expression of the CaMKIIδ_B isoform in neonatal rat ventricular myocytes increased expression of atrial natriuretic factor, including that mediated by phenylephrine. In addition, hypertrophied cardiomyocytes from hypertensive rats have higher expression of CaMKIIδ [17]. The increase in CaMKII expression is reversed by angiotensin converting enzyme inhibitor and is accompanied by a regression of the cardiac hypertrophy [18]. Additionally, the Ca^{2+}/CaM-dependent protein phosphatase calcineurin is critical for the cardiac hypertrophic response. For example, in transgenic mice expressing an active mutant of calcineurin in heart, the constitutively active calcineurin is sufficient to induce massive cardiac hypertrophy [10,19]. Consistent with this observation, calcineurin inhibitors such as cyclosporine A and FK506 prevent angiotensin II- or phenylephrine-induced cardiac hypertrophy [20,21]. Likewise, adenoviruses expressing calcineurin inhibitors such as Cain or AKAP inhibit calcineurin activity and in turn attenuate angiotensin II- and phenylephrine-induced hypertrophy in cultured cardiomyocytes [22,23].

CaMKII and calcineurin mediate the phosphorylation and dephosphorylation of phospholamban, respectively [24]. Phosphorylation of Thr-17 on phospholamban by CaMKII activates SERCA2a by relieving its inhibition, thereby enhancing Ca^{2+} uptake into the sarcoplasmic reticulum [25,26] and accelerating relaxation of the heart. Phospholamban phosphorylation also contributes to increased contraction by elevating the concentration of the stored Ca^{2+} in the sarcoplasmic reticulum. Conversely, calcineurin directly dephosphorylates phospholamban or promotes its dephosphorylation by protein phosphatase 1 in the sarcoplasmic reticulum [27,28], leading to decreased SERCA2a activity. Thus, SERCA2a activity is regulated in opposite ways by CaMKII and calcineurin activities through phospholamban phosphorylation.

Dystrophin is a structural protein that forms a submembranous cytoskeletal network in skeletal muscle and cardiac muscle by creating links between intracellular actin filaments and extracellular laminin through dystrophin-associated proteins [29]. Dystrophin mRNA and protein accumulate in response to cardiac hypertrophy [30]. Moreover, the vulnerability of dystrophin-deficient myocardium to mechanical stress suggests that dystrophin plays a critical role in maintaining the integrity of the sarcolemma against shear stress and pressure overload [31–34]. Indeed, cleavage of dystrophin by an enteroviral protease 2A leads to cytoskeletal disruption and postmyocarditis cardiomyopathy. Recently, several studies suggested that translocation and cleavage of myocardial dystrophin leads to the progression of heart failure [35,36]. Dystrophin is known to be a CaM-binding protein [37–39]. We recently documented that DY-9760e, a novel CaM antagonist, inhibited calpain-induced breakdown of fodrin and calpastatin, which are also CaM-binding proteins [40]. Thus, we hypothesize that DY-9760e could also inhibit dystrophin breakdown following hypertrophy.

To understand the molecular mechanisms of impaired Ca^{2+} regulation following cardiac hypertrophy, we measured CaMKII autophosphorylation and calcineurin level in endothelin-1- and angiotensin II-induced hypertrophy. We found that an imbalance between CaMKII and calcineurin levels triggers abnormal phospholamban phosphorylation with a concomitant decrease in caffeine-induced Ca^{2+} release. The breakdown of dystrophin confirmed the impairment of Ca^{2+} handling following endothelin-1-induced hypertrophy, and DY-9760e and its active metabolite DY-9836 were able to rescue the dystrophin breakdown.

2. Materials and methods

2.1. *Ventricular cardiomyocyte primary cultures*

Neonatal ventricular myocytes were isolated from hearts of 1–3-day-old Wistar rats by collagenase digestion and cultured according to the method of Waspe et al. [41]. Briefly, neonatal rats were decapitated and their hearts removed immediately. Ventricles were separated from atria and washed in Hank's balanced salt solution (137.0 mM NaCl, 5.4 mM KCl, 0.4 mM

KH_2PO_4, 0.3 mM $Na_2HPO_4{\cdot}12H_2O$, 6.12 mM glucose, 4.2 mM $NaHCO_3$), and myocytes were dissociated from the ventricles by serial digestion with 0.1% trypsin and 0.05% DNase I in Hank's balanced salt solution. After each digestion, the digested cardiomyocytes were collected and suspended in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 0.02% trypsin inhibitor to inhibit further digestion by trypsin. Cells were collected by centrifugation (4 °C, 1000 × *g* for 10 min). After the supernatant was removed, DMEM containing 10% FBS was added. The cells were gently agitated and then plated on uncoated 90-mm culture dishes. The plates were allowed to stand for 30 min in a CO_2 incubator at 37 °C to remove nonmyocytes attached to the culture plates. The unattached myocytes were collected and plated at 1–2 × 10^6 cells per 35-mm dish and incubated with DMEM and 10% FBS in a humidified incubator with 5% CO_2 at 37 °C for 24 h. The cells were cultured in serum-free DMEM for 24 h before treatment with endothelin-1 (100 nM), angiotensin II (100 nM), or phenylephrine (10 μM).

2.2. Immunocytochemistry

Cultured myocytes were plated on polyethyleneimine-coated cover glass at a density of 1–2 × 10^5 cells per coverslip (Ø12 mm). After incubation in the presence or absence of endothelin-1, phenylephrine, or angiotensin II for 48 h, the cultured cells were washed three times in phosphate-buffered saline (PBS; pH 7.4) and fixed in 4% formaldehyde. After permeabilization with 0.1% Triton X-100 in PBS, the fixed cells were incubated with 1% bovine serum albumin in PBS for 30 min. For cell size measurement, cells were incubated for 3 h at room temperature with Rhodamine-conjugated Phalloidin (1:300; Molecular Probes, Eugene, OR) in PBS containing 1% BSA. After cell images were taken under an Olympus fluorescence microscope, the cell surface area of the cells measured quantitatively using NIH Image program 1.63 software. All cells from randomly selected fields in three independent cultured plates were examined for each condition (at least 100 myocytes per group). The cell surface area in control cells was expressed as 100% and compared with those in the treated cells.

2.3. Intracellular Ca^{2+} measurement using Fura-2

Neonatal ventricular myocytes were cultured on glass coverslips and maintained in the growth medium. After stimulation with 100 nM endothelin-1 for 48 h or 96 h, myocytes were loaded with the Ca^{2+}-sensitive dye Fura-2 acetoxymethyl ester (2.5 μM) for 30 min before measurement of Ca^{2+} levels in a chamber on the stage of an inverted microscope. Cells were perfused with normal Tyrode solution containing 150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5.6 mM glucose, and 5 mM HEPES at 37 °C. When Ca^{2+} fluorescence levels came to the steady state, 10 mmol/l caffeine was applied for 10 s through small perfusion pipe. The amplitude of the caffeine-induced Ca^{2+} transient was used as an index of sarcoplasmic reticulum Ca^{2+} content [42]. Changes in caffeine-induced Ca^{2+} release from the sarcoplasmic reticulum were determined using a ratio of the fluorescence emission at 510 nm in response to excitation at 340 nm and 380 nm.

2.4. Immunoblotting analysis

Cultured cells were washed with cold PBS and stored at −80 °C until immunoblotting analyses were performed. Frozen myocytes were homogenized in buffer containing 50 mM Tris–HCl (pH 7.4), 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 100 nM calyculin A, 50 μg/ml trypsin inhibitor, and 1 mM dithiothreitol. Insoluble material was removed by a 10 min centrifugation at 15,000 × *g*. After determination of the protein content in each supernatant fraction using Bradford's solution, samples containing equivalent amounts of protein were applied to a 7.5–12% acrylamide denaturing gel [43]. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the proteins were transferred to an Immobilon PVDF transfer membrane for 2 h at 70 V. The membranes were blocked in 30 mM Tris–HCl (pH 7.4), 100 mM NaCl, and 0.1% Tween 20 containing 5% fat-free milk for 1 h at room temperature, then incubated overnight at 4 °C with antibodies against CaMKII [44] and phospho-CaMKIIδ [45] (rabbit polyclonal antibodies; 1:2000 dilution), calcineurin [45] (rabbit polyclonal antibody; 1:2000), phospho-phospholamban (Thr-17; rabbit polyclonal antibody; 1:1000, Santa Cruz Biotechology, Santa Cruz, CA, and Ser-16; rabbit polyclonal antibody; 1:1000, Upstate, Lake Placid, NY), PP1 and PP2A [46] (rabbit polyclonal antibody; 1:1000), SERCA2 (mouse monoclonal antibody; 1:1000, Sigma, St. Louis, MO), dystrophin (mouse monoclonal antibody; 1:1000, Chemicon, Temecula, CA), or β-tubulin (mouse monoclonal antibody; 1:10,000, Sigma, St. Louis, MO). After washes, the membranes were further incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. The immunoreactive proteins on the membrane were visualized with an enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK). The images were scanned and analyzed semiquantitatively using NIH Image and Image Gauge Software (Fujifilm, Tokyo, Japan).

2.5. Statistical analysis

Data are represented as mean ± S.D. of three to four independent experiments. Multiple comparisons between experimental groups were performed by one-way analysis of variance (ANOVA), followed by a Dunnett's post hoc test. $P < 0.05$ was considered significant.

3. Results

3.1. Cardiac hypertrophy induced by angiotensin II, endothelin-1, and phenylephrine

We first confirmed that exposure to 100 nM of endothelin-1, angiotensin II, or 10 μM phenylephrine induces hypertrophy in cultured rat cardiomyocytes. After 48 h of stimulation, cells were stained with rhodamine phalloidin, which labels actin filaments. Fluorescence microscopy of stimulated cells showed a marked increase in cell surface areas (Fig. 1A). Endothelin-1, angiotensin II, and phenylephrine enlarged cell

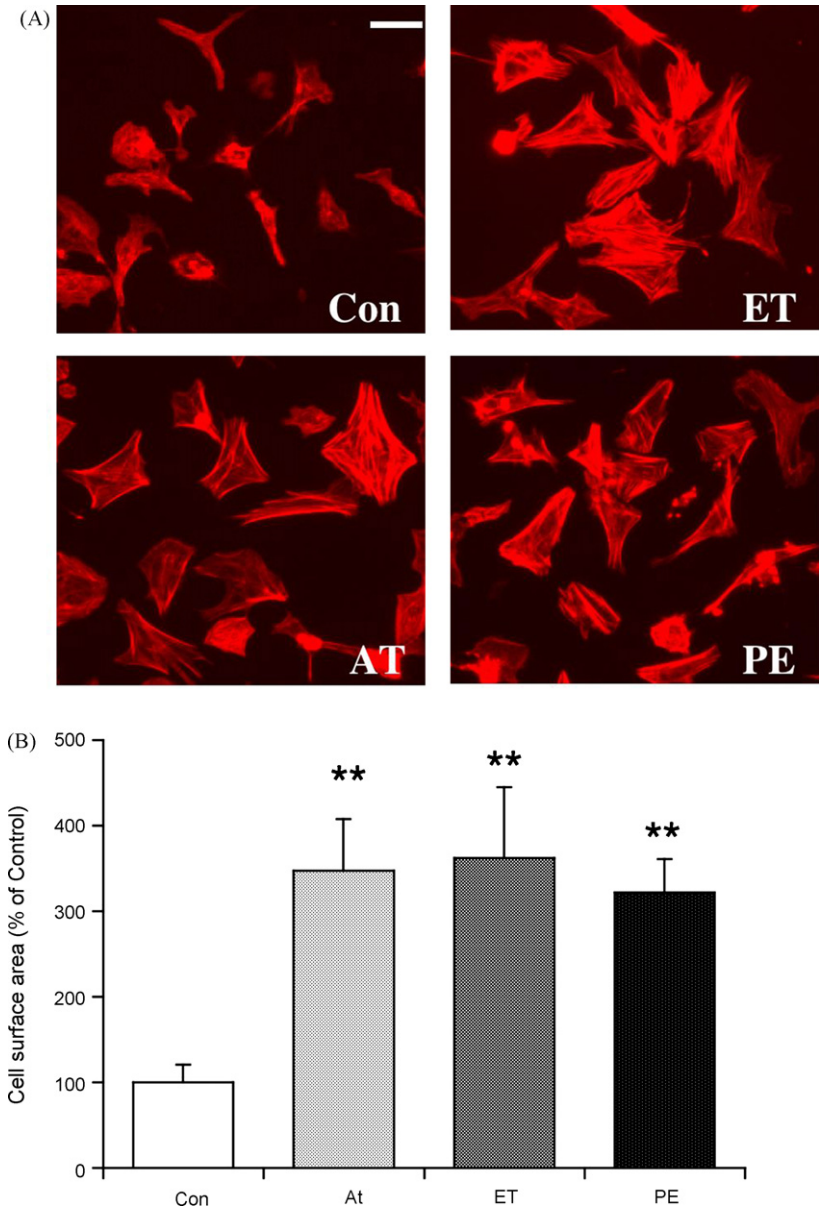

Fig. 1 – Changes of cell surface area after treatment of cultured neonatal rat cardiomyocytes with endothelin-1, angiotensin II, or phenylephrine for 48 h. After 24 h of culture with serum-free DMEM, cells were treated with endothelin-1 (100 nM), angiotensin II (100 nM), or phenylephrine (10 μM) for 48 h. (A) Cells were fixed with 4% paraformaldehyde, stained with a monoclonal antibody directed against rhodamine phalloidin, and processed for fluorescence microscopy as described in Section 2. One hundred cells from randomly selected fields in three wells were examined for each condition. Bar: 20 μm. (B) Cell size results are expressed as relative surface area standardized to the mean surface area of control cells in each experiment. Pooled data from three independent experiments are presented as mean ± S.D. ($n = 100$). $^{*}P < 0.05$ vs. control. Con, control; ET, endothelin-1; AT, angiotensin II; PE, phenylephrine.

surface areas of cardiomyocytes by 3.6-, 3.4-, and 3-fold relative to control, respectively (Fig. 1B).

3.2. *Increased CaMKII autophosphorylation and calcineurin level following cardiac hypertrophy*

We first assessed CaMKII autophosphorylation, which produces the active form of CaMKII, and the level of calcineurin protein in hypertrophied cardiac myocytes 48 h after treatment with 100 nM endothelin-1, angiotensin II, or 10 μM phenylephrine. Because the 54 kDa isoform of CaMKIIδ is predominantly expressed in the rat heart [47], we performed an immunoblot with an antibody against autophosphorylated CaMKIIδ using cell extracts from endothelin-1-, angiotensin II-, and phenylephrine-treated myocytes. Autophosphorylation of CaMKIIδ increased 1.3-, 1.5-, and 1.3-fold after treatment with endothelin-1, angiotensin II, and phenylephrine, respectively, without a change in the total

CaMKII protein level (Fig. 2A). Likewise, expression of the 60 kDa calcineurin protein increased about twofold after treatment with endothelin-1, angiotensin II, or phenylephrine (Fig. 2B).

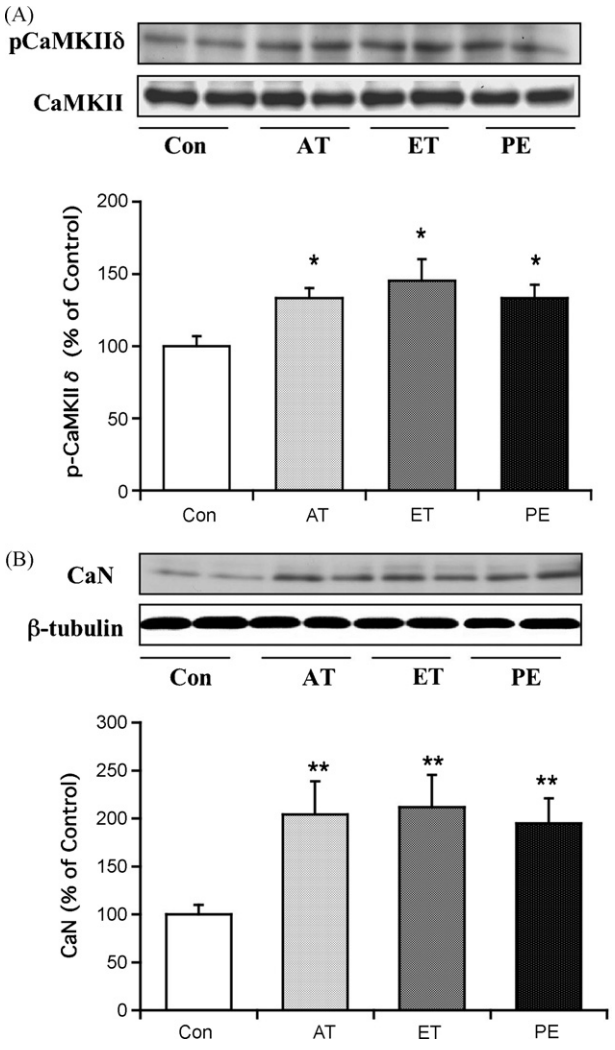

Fig. 2 – Changes in expression of CaMKII and calcineurin after treatment of rat neonatal cardiomyocytes with endothelin-1, angiotensin II, or phenylephrine for 48 h. (A) Cultured cardiomyocytes were treated without (control) or with endothelin-1 (100n M), angiotensin II (10 nM), or phenylephrine (10 μM) for 48 h. Extracts were prepared and blotted at the indicated times. Immunoblots performed with an anti-CaMKII antibody showed equal protein loading. Quantitative analysis of the 54 kDa phosphorylated CaMKIIδ band was performed by densitometric analysis of the immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. *P < 0.01 vs. control cells. (B) Representative image of an immunoblot performed using an anti-calcineurin antibody and the same samples described above. Quantitative analysis of the 60 kDa calcineurin band was performed by densitometric analysis of immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. **P < 0.01 vs. control cells. CaN, calcineurin.

3.3. *Temporal changes in calcineurin protein and CaMKIIδ autophosphorylation*

We next assessed temporal changes in the levels of calcineurin protein and CaMKII autophosphorylation in cell extracts obtained from cultured cardiomyocytes. In the continuous presence of endothelin-1 (100 nM), calcineurin levels gradually increased with a peak at 72 h and remained elevated at least until 96 h, whereas, the amounts of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) had no change (Fig. 3A). Likewise, treatment with angiotensin II (100 nM) significantly increased calcineurin level at least until 96 h. By contrast, the increased autophosphorylation of CaMKIIδ was transient and peaked at 48 h, returning to the basal level within 96 h (Fig. 4). These results suggest that the balance of calcineurin and CaMKII activities changes in the late phase of cardiac hypertrophy.

3.4. *Phospholamban phosphorylation during development of hypertrophy*

Because CaMKII and calcineurin regulate phospholamban phosphorylation in the cardiac sarcoplasmic reticulum, we next assessed phosphorylation of phospholamban at and Ser-16 (phosphorylation site for protein kinase A) and Thr-17 (phosphorylation site for CaMKII) in cell extracts obtained from cultured cardiomyocytes following endothelin-1 and angiotensin II stimulation. Consistent with the change in CaMKIIδ autophosphorylation, treatment with 100 nM endothelin-1 or angiotensin II increased phospholamban Thr-17 phosphorylation 2.6- and 2.3-fold, respectively. With both treatments, the phosphorylation peaked at 48 h and returned to baseline within 96 h after stimulation (Fig. 5A). By contrast, the phosphorylation of phospholamban at Ser-16 by protein kinase A and levels of SERCA2a protein in cardiomyocytes did not change during stimulation with either endothelin-1 or angiotensin II (Fig. 5B).

3.5. *Changes in sarcoplasmic reticular Ca^{2+} content following cardiac hypertrophy*

To verify the pathological relevance of the dephosphorylation of phospholamban Thr-17 at 96 h after endothelin-1 treatment, we measured the Ca^{2+} content of the sarcoplasmic reticulum by assessing caffeine-induced Ca^{2+} release in cultured cardiomyocytes at 48 h in control cells and at 48 h and 96 h in endothelin-1-treated cells. The Fura-2 ratio at 340/380 nm was not significantly different between the three groups at baseline. When Ca^{2+} release from the sarcoplasmic reticulum was triggered by the application of 10 mM caffeine, the transient Ca^{2+} elevation was significantly enhanced at 48 h and suppressed 96 h after endothelin-1 treatment (Fig. 6A). The integrative volumes of the Ca^{2+} transient at 48 h increased 1.5-fold compared to control cells and was significantly reduced at 96 h compared to that in control cells and cells treated with endothelin-1 for 48 h (Fig. 6B).

3.6. *DY-9760e and DY-9836 inhibit heart failure-induced dystrophin breakdown*

Since dystrophin, a membrane associated cytoskeletal protein, has pivotal role in contraction of cardiomyocytes and its

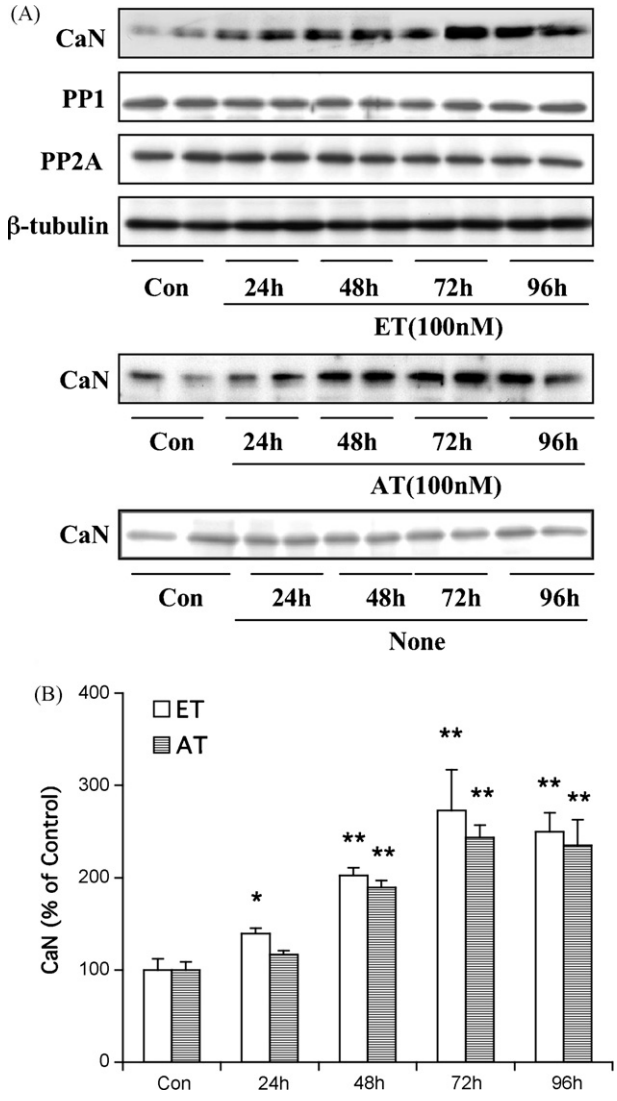

Fig. 3 – Time course of calcineurin level in rat neonatal cardiomyocytes following endothelin-1 or angiotensin II stimulation. (A) Representative image of an immunoblot using anti-calcineurin, -PP1 and -PP2A antibodies and extracts of cultured cardiomyocytes treated without (control) or with endothelin-1 (100 nM), angiotensin II (100 nM) from 24 h to 96 h. (B) Quantitative analysis of the 60 kDa calcineurin levels was performed by densitometric analysis of immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. *P < 0.05; **P < 0.01 vs. control cells. Con, control; ET, endothelin-1; AT, angiotensin II; CaN, calcineurin.

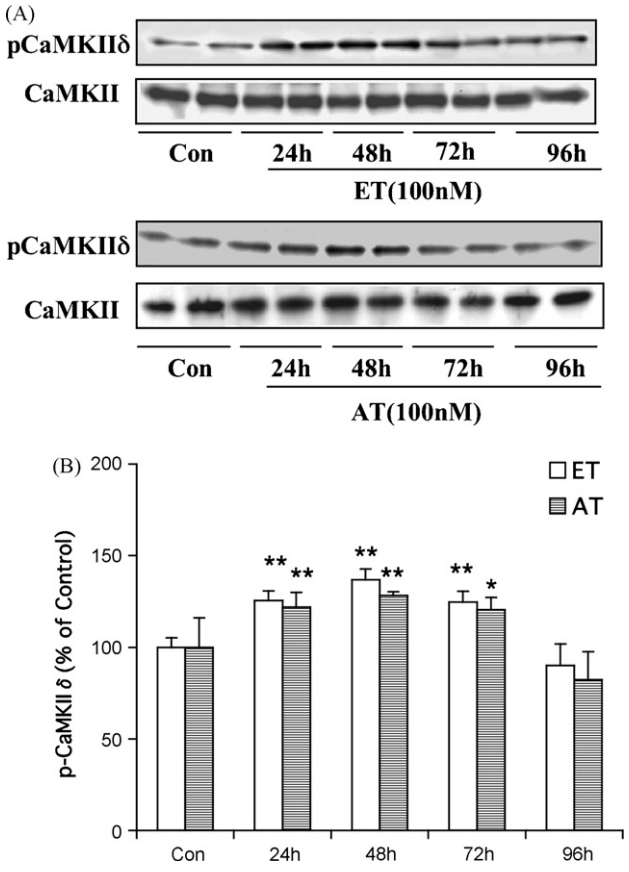

Fig. 4 – Time course of CaMKII autophosphorylation in rat neonatal cardiomyocytes following endothelin-1 or angiotensin II stimulation. (A) Representative image of an immunoblot using an anti-phospho-CaMKIIδ antibody after treatment of cultured cardiomyocytes without (control) or with endothelin-1 (100 nM) or angiotensin II (100 nM) from 24 h to 96 h. (B) Quantitative analysis of the CaMKII autophosphorylation was performed by densitometric analysis of immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. *P < 0.05; **P < 0.01 vs. control cells. Con, control; ET, endothelin-1; AT, angiotensin II.

breakdown is associated with hypertrophy-induced cardiac dysfunction [35,36], we tested whether endothelin-1-induced hypertrophy is associated with induction of dystrophin during a compensatory phase of hypertrophy. We also verified whether dystrophin is proteolyzed after prolonged incubation with endothelin-1 in cardiomyocytes. The level of 430 kDa dystrophin in cell extracts obtained from cardiomyocytes was elevated 24 h and 48 h after endothelin-1 treatment as compared with control cells, most likely a compensatory effect of endothelin-1-induced hypertrophy. However, the dystrophin level was markedly reduced at 96 h compared to both the control and the endothelin-10-treated cells at 48 h (Fig. 7A).

To rescue dystrophin from calpain-induced proteolysis, we treated cells with the CaM antagonist DY-9760e and its active metabolite DY-9836. Treatment with DY-9760e or DY-9836 alone had no effect on the dystrophin level after a 96 h incubation, but as expected, both compounds significantly rescued dystrophin from breakdown in a dose-dependent manner in endothelin-1-treated cells (Fig. 7B).

4. Discussion

Cardiac hypertrophy and in turn heart failure are associated with abnormal intracellular Ca^{2+} regulation and impaired

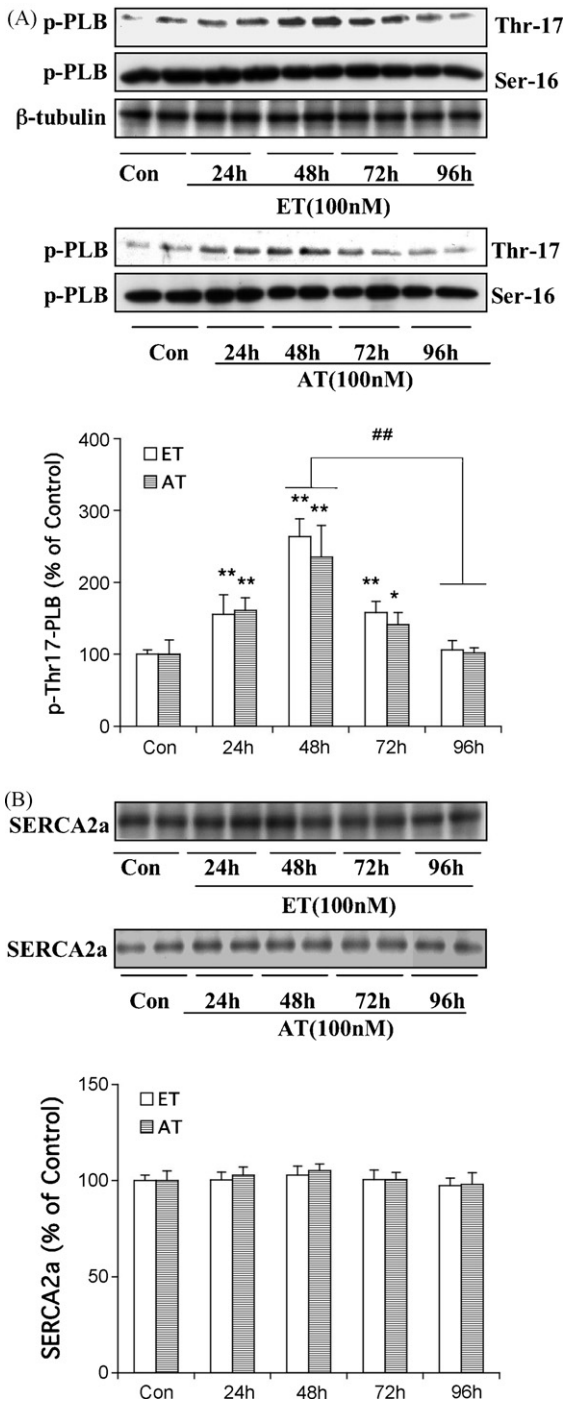

Fig. 5 – Stimulation with agonists induces changes in levels of phospho-phospholamban and SERCA2a expression in rat neonatal cardiomyocytes. (A) Phosphorylation of phospholamban both in Thr-17 and Ser-16 was determined by treating of cultured myocytes without (control) or with endothelin-1 (100 nM) or angiotensin II (100 nM), followed by extraction and immunoblotting at the indicated times from 24 h to 96 h. Immunoblots with an anti-β-tubulin antibody showed equal protein loading. Quantitative analysis of the 22 kDa phosphorylated phospholamban at Thr-17 was performed by densitometric analysis of the immunoblots. Data are mean ± S.D. of four independent experiments performed in triplicate. *P < 0.05; **P < 0.01 vs. control cells. ##P < 0.01 vs. 48 h. (B) Representative image of an immunoblot using an anti-SERCA2a antibody and the same samples described above. Quantitative analysis of the 110 kDa SERCA2a bands was performed by densitometric analysis of the immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. Con, control; ET, endothelin-1; AT, angiotensin II; p-PLB, phospho-phospholamban.

myocardial contractility [48,49]. Ca^{2+}/CaM-dependent enzymes, including calcineurin and CaMKIIδ, play critical roles in the development of cardiac hypertrophy and heart failure [50–52]. Calcineurin and CaMKIIδ also regulate Ca^{2+} content in the sarcoplasmic reticulum by controlling phospholamban phosphorylation. However, the precise mechanism by which calcineurin and CaMKIIδ affect Ca^{2+} stores during the development of cardiac hypertrophy has remained unclear. Here, to determine how Ca^{2+} regulation becomes impaired during the development of hypertrophy, we assessed temporal changes in the protein levels of calcineurin, activated CaMKIIδ, and phosphorylated phospholamban in endothelin-1- and angiotensin II-induced hypertrophy. The most important observations were that (1) the change in CaMKIIδ autophosphorylation (active form) is biphasic following endothelin-1 and angiotensin II treatments, in that it increased until 48 h and was reduced to baseline by 96 h after treatment; (2) by contrast, the calcineurin level progressively increased until at least 96 h; (3) the timing of dephosphorylation of phospholamban during 96 h of treatment with endothelin-1 was closely correlated with the timing of impaired caffeine-induced Ca^{2+} release from the sarcoplasmic reticulum; and (4) dystrophin was broken down after 96 h of treatment with endothelin-1, suggesting that Ca^{2+}-dependent calpain is activated during decompensation of cardiomyocytes in the late phase of endothelin-1-induced hypertrophy. Taken together, the increased calcineurin level and reduced CaMKIIδ autophosphorylation at 96 h after endothelin-1 and angiotensin II treatments likely mediate the dephosphorylation of phospholamban phosphorylation at Thr-17 seen at 96 h. Because the protein level of SERCA2a remained unchanged until 96 h, the impairment of caffeine-induced release of Ca^{2+} from the sarcoplasmic reticulum is unlikely to be due to effects on SERCA2a; rather, the reduced phosphorylation of phospholamban is likely to cause the impaired Ca^{2+} release.

Numerous studies have defined a pivotal role for Ca^{2+} as a trigger of the cardiac hypertrophy produced by endothelin-1, angiotensin II, phenylephrine, and Ca^{2+} channel agonists [53–55]. Our data here showed that cardiac hypertrophy induced by all three agonists is associated with increased calcineurin level and CaMKIIδ autophosphorylation, although autophosphorylation of CaMKII subsequently decreases whereas the level of calcineurin does not. We first speculated that the inverse regulation of phospholamban function by CaMKII autophosphorylation and calcineurin levels triggers the abnormal Ca^{2+} regulation in hypertrophic cardiomyocytes and that the disturbed balance of CaMKII and calcineurin levels can trigger heart failure. To test our hypothesis, we

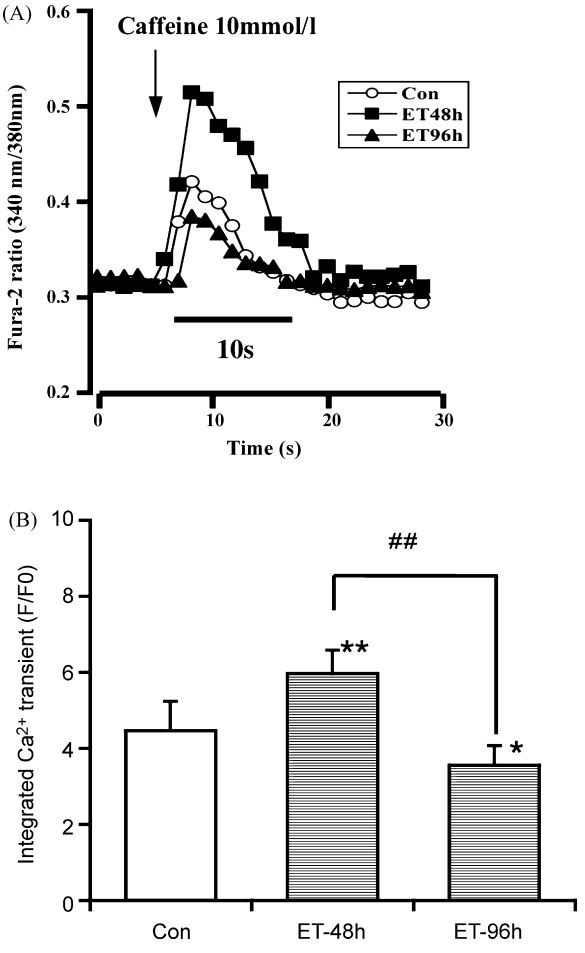

Fig. 6 – Caffeine-induced Ca^{2+} release after treatment of cultured myocytes without (control) or with endothelin-1 for 48 h or 96 h. (A) Original traces show the current either in control conditions or 48 h or 96 h after the application of 10 mmol/l caffeine application in the presence of 100 nM endothelin-1. The arrow indicates the time of caffeine administration. The amplitude of the caffeine-induced Ca^{2+} transient can be used as an index of SR Ca^{2+} content. (B) Mean amplitudes of Ca^{2+} transients in cardiac myocytes after treatment without (control) or with endothelin-1 for 48 h or 96 h. F/F0, fluorescence intensity/background fluorescence levels; Con, control; ET, endothelin-1. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control cells, $^{\#\#}P < 0.01$ vs. 48 h.

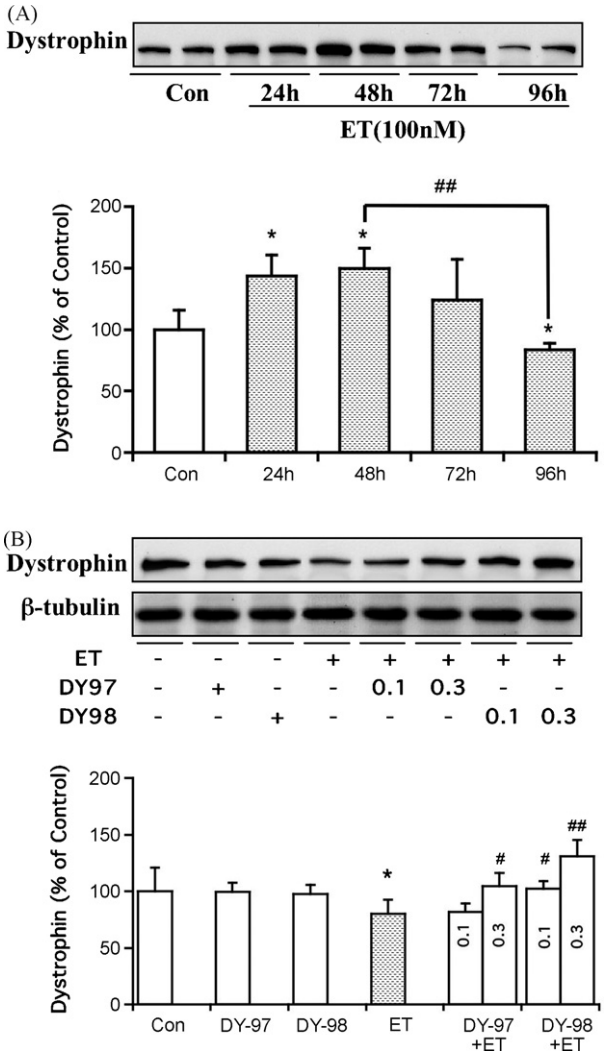

Fig. 7 – The dystrophin breakdown during development of hypertrophy and its inhibition by novel CaM antagonists. (A) The dystrophin level was determined by quantifying the 430 kDa immunoblotting band after treatment of cultured myocytes without (control) or with endothelin-1 from 24 h to 96 h. Immunoblots with anti-β-tubulin antibody show equal protein loading. Quantitative analysis of the 430 kDa band was performed by densitometric analysis of immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. $^{*}P < 0.05$ vs. control cells. $^{\#\#}P < 0.01$ vs. 48 h. (B) Effect of DY-9760e or DY-9836, novel CaM antagonist on dystrophin degradation in cultured cardiomyocytes. Myocytes were treated with DY-9760e or DY-9836 (0.1–0.3 μM) in the absence (Con) or presence of endothelin-1 for 96 h. Immunoblotting with an anti-β-tubulin antibody showed equal amounts of loaded protein in each lane. Quantitative analysis of the levels of 430 kDa dystrophin protein was performed by densitometric analysis of immunoblots. Data are mean ± S.D. of three independent experiments performed in triplicate. $^{*}P < 0.05$ vs. control cells. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. treatment with endothelin-1 alone. ET, endothelin-1; DY-97, DY-9760e; DY-98, DY-9836.

made models of heart failure in culture by incubating cardiomyocytes with endothelin-1 or angiotensin II for prolonged times. Although our model is useful for defining decompensatory mechanisms, the cardiomyocytes tend to die after 96 h due to the degradation of dystrophin and activation of calpain. Therefore, we could only evaluate the intracellular events in heart failure in the first 96 h.

Accumulating evidence has suggested that the disruption of dystrophin precedes the progression to cardiac dysfunction in cardiac hypertrophy in vivo [35,36]. In the failing heart, both mechanical and humoral stresses trigger the breakdown of dystrophin and dystrophin-associated protein complexes. As

expected, dystrophin was first elevated by endothelin-1 treatment as a compensatory effect of hypertrophy, but prolonged incubation with endothelin-1 reversed this effect, triggering dystrophin breakdown. Because the protein levels of other cardiomyocyte components, such as β-tubulin, SERCA2a, and total CaMKIIδ, did not change over 96 h, the specific dystrophin breakdown by calpain likely mediates the pathological events leading to heart failure. Dystrophin is a 430 kDa elongated protein and is essential for the maintenance of membrane integrity in cardiomyocytes and skeletal myocytes. Deficits in dystrophin cause Duchenne muscular dystrophy, a progressive, lethal disease [39]. The influence of CaM-binding to dystrophin has been investigated, but the results are controversial. There are two consensus CaM-binding sequences, one located in the N-terminal part of dystrophin and the other in the C-terminal part [56]. Bonet-Kerrache et al. [57] reported that the N-terminal fragment of dystrophin binds to CaM in a Ca^{2+}-dependent manner, but native dystrophin does not directly bind to CaM. Notably, the N-terminal part of dystrophin can interact with the F-actin network, thereby serving as an anchor protein for F-actin, which is involved in the control of membrane cell shape [58]. The interaction between F-actin and dystrophin is stimulated in the presence of Ca^{2+}/CaM. Although the relevance of CaM-binding to the C-terminal part of dystrophin has not been investigated, the C-terminal part of dystrophin contains a domain that associates with α-syntrophin and phosphorylation sites for CaMKII. In this context, CaM-binding to dystrophin likely affects the function of dystrophin in maintaining the membrane integrity of cardiomyocytes.

We recently documented that another submembranous cytoskeletal F-actin-binding protein, fodrin, is cleaved by calpains following brain or heart ischemia [40,59]. Binding of Ca^{2+}/CaM to fodrin promotes its susceptibility to calpain-induced breakdown, and a CaM inhibitor, DY-9760e, inhibits this breakdown of fodrin by calpain. We therefore speculated that CaM-binding to dystrophin might also promote its breakdown by calpain. Consistent with our hypothesis, the CaM inhibitor DY-9760e and its metabolite DY-9836 inhibited the dystrophin breakdown that occurs in endothelin-1-induced hypertrophy. We have also documented the cardioprotective action of DY-9760e in ischemic heart [60] and the inhibitory action of DY-9760e and DY-9836 on dystrophin breakdown likely contributes in part to their cardioprotective action. We plan to further investigate the precise molecular mechanism underlying CaM-binding to dystrophin in future studies. The primary structural functions of dystrophin are to link the cytoskeleton of the cardiomyocyte to the extracellular matrix and to maintain sarcolemmal integrity. For instance, dystrophin-deficient cardiomyocytes are abnormally vulnerable to mechanical stress-induced injury, thereby leading to a loss of sarcolemmal integrity and contractile dysfunction [35,61], and human failing myocardium loses immunostaining for the N-terminal region of dystrophin [62]. Therefore, calpain-mediated dystrophin breakdown likely triggers the loss of membrane integrity during development of heart failure. In this context, inhibition of dystrophin breakdown by DY-9760e and DY-9836 is potentially relevant to inhibit the development of heart failure.

The activation of calpain during prolonged exposure to endothelin-1 also suggests that Ca^{2+} regulation is abnormal in cardiomyocytes. Consistent with our hypothesis, Gwathmey et al reported intracellular Ca^{2+} transients with a marked decline phase in failing human myocardium [63]. In the early phase of endothelin-1 and angiotensin II treatment, until at least 48 h, balanced increases in the activities of CaMKIIδ and calcineurin enhanced phospholamban phosphorylation, thereby increasing the Ca^{2+} content in the sarcoplasmic reticulum and triggering elevated caffeine-induced Ca^{2+} release. After prolonged exposure to endothelin-1 (72–96 h), however, an imbalance of calcineurin/CaMKIIδ activities developed due to the vulnerability of CaMKIIδ to Ca^{2+} overloading. This imbalance in turn triggered a marked decrease in phospholamban phosphorylation, which was associated with a disturbance of sarcoplasmic reticular Ca^{2+} regulation and dystrophin breakdown. Abnormal Ca^{2+} regulation in the sarcoplasmic reticulum is a characteristic of hypertrophied and failing myocardium that elicits decreased expression of SERCA2a and/or increased expression of phospholamban [6,7]. In the present study, SERCA2a protein expression did not change significantly throughout agonist treatment, whereas phospholamban phosphorylation rose and then fell in the presence of endothelin-1 or angiotensin II. Therefore, we postulate that the abnormal Ca^{2+} regulation in the sarcoplasmic reticulum of cultured cardiomyocytes is caused by dephosphorylation of phospholamban. In parallel with these data, decreased phosphorylation of phospholamban Thr-17 was also found in a heart failure model [64]. Thus, the inhibition of phospholamban dephosphorylation may be helpful in preventing phenotypic progression of heart failure. In the present study, we confirmed that phospholamban phosphorylation is positively regulated by CaMKII and negatively regulated by calcineurin, and that sustained phospholamban dephosphorylation through elevation of calcineurin phosphatase activity is pivotal for the abnormal Ca^{2+} regulation in the sarcoplasmic reticulum. In addition, as a consequence of reduced Ca^{2+} uptake activity caused by the phospholamban dephosphorylation, the basal Ca^{2+} levels may slightly increase in cardiomyocytes. Notably, half-maximal activation of CaMKII requires 1 μM free Ca^{2+}, but less than 0.1 μM calcineurin [65]. The increased phospholamban phosphorylation 48 h after endothelin-1 and angiotensin II treatments is likely to be a compensatory effect of hypertrophy, thereby maintaining the contractility of cardiomyocytes even after hypertrophy. However, decreased CaMKIIδ activity after persistent stimulation with endothelin-1 and angiotensin II possibly disrupts the balance between phosphorylation and dephosphorylation of phospholamban. Because of excess phospholamban dephosphorylation, the Ca^{2+} regulation by the sarcoplasmic reticulum becomes dysfunctional and cardiac contractility cannot be maintained, thereby causing heart failure.

In conclusion, an imbalance of CaMKII and calcineurin activities are elicited by prolonged exposure to endothelin-1 and angiotensin II in cultured cardiomyocytes. The imbalance of CaMKII/calcineurin activities in the cardiomyocytes in turn impairs sarcoplasmic reticular Ca^{2+} regulation in part through phospholamban dephosphorylation. At the same time, dystrophin breakdown after prolonged exposure to endothelin-1

mediates the loss of membrane integrity associated with cardiac hypertrophy. We recently reported the cardioprotective effect of a novel CaM compound, DY-9760e, in heart ischemia [60]. The rescue of dystrophin breakdown by DY-9760e and its active metabolite DY-9836 strongly suggests that these compounds are cardioprotective in endothelin-1- and angiotensin II-induced cardiac hypertrophy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.08.022.

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