Original Research

Effect of distinct sources of Ca^{2+} on cardiac hypertrophy in cardiomyocytes

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Abstract

It is believed that intracellular calcium (Ca^{2+}) overload can cause the cardiac hypertrophy, but it is possible that the Ca^{2+} entering the cytoplasm through distinct pathways will induce various effects on cardiomyocytes. The aim of the present study is to explore the effect of different sources of Ca^{2+} on cardiomyocyte hypertrophy. The cardiomyocytes isolated from neonatal Sprague–Dawley rats were treated with three agents (ionomycin, caffeine and angiotensin II [Ang II]) that increased the intracellular Ca^{2+} concentration via different pathways. Treatments with ionomycin, caffeine and Ang II for 24 h caused a significant increase in resting $[Ca^{2+}]_i$ by 108.0 $\pm$ 7.8%, 102.0 $\pm$ 6.9% and 59.8 $\pm$ 3.3%, respectively. Caffeine and Ang II increased the cell surface area of cardiomyocytes and the mRNA level of atrial natriuretic peptide, brain natriuretic peptide and β-myosin heavy chain, but ionomycin did not. Moreover, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) activity and the amplitudes of the twitch $[Ca^{2+}]_i$ transients were reduced in the caffeine-treated group and Ang II-treated group. Furthermore, cardiomyocyte hypertrophy induced by caffeine was inhibited by cyclosporin A (CsA) and KN93, whereas cardiomyocyte hypertrophy induced by Ang II was inhibited by KN93, but not CsA. Our results show that cardiomyocyte hypertrophy is associated with SERCA2a activity, contractile performance and signaling pathways of CaMKII and/or calcineurin, whereas the Ca^{2+} overload is not sufficient to cause the cardiomyocyte hypertrophy.

Keywords: Ca^{2+} overload, cardiomyocyte, hypertrophy, CaMKII, calcineurin

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Introduction

Intracellular calcium (Ca^{2+}) is considered as a pivotal ion during the process of excitation–contraction coupling (ECC) in the heart. In addition to being an intracellular second messenger, Ca^{2+} is also very important in regulating gene transcription, energy balance and hypertrophic growth in the heart.[1] It has been shown that a continual increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) can produce cardiac hypertrophy and even dysfunction.[2] Sei *et al.*[3] found that treatment of cultured neonatal ventricular myocytes with the calcium channel agonist Bay K8644 alone induced hypertrophic marker gene expression by elevating the $[Ca^{2+}]_i$. Their results further demonstrated that Ca^{2+} overload could induce the marker gene expression only via the calmodulin (CaM)-dependent pathway. Many types of pathways, such as the entry of extracellular Ca^{2+} through L-type Ca^{2+} channels (LTCC) or store-operated Ca^{2+} entry (SOCE), release of intracellular Ca^{2+} stores from the cardiac ryanodine receptor (RyR2) or the inositol 1,4,5-triphosphate receptor (IP_3R2), can increase $[Ca^{2+}]_i$. It has been shown that angiotensin II (Ang II) can induce Ca^{2+} overload by combination with a G-protein-coupled receptor and then cardiomyocyte hypertrophy via the IP_3R.[4] Caffeine and ionomycin can also increase $[Ca^{2+}]_i$, and lead to Ca^{2+} overload by the different mechanisms.[5,6] However, there have been few studies on the effects of caffeine and ionomycin on hypertrophy of the cultured neonatal ventricular myocytes. These facts make us wonder whether Ca^{2+} overload, induced by different pathways, is necessary to fully activate the hypertrophic program.

Ca^{2+} overload can activate two major calcium-dependent signaling pathways: CaM–CaMKII–HDAC and CaM–calcineurin–NFAT.[1] Both of these pathways are implicated in the development of cardiac hypertrophy.[7–9] Ca^{2+}/CaM-dependent protein kinase II (CaMKII) can modulate ECC in cardiomyocytes through phosphorylation of several Ca^{2+} regulatory proteins, including LTCC, RyR2, SERCA2a and phospholamban.[9–11] Calcineurin can directly dephosphorylate the nuclear factor of activated T-cells (NFAT) in the cytoplasm and promote its translocation

into the nucleus to induce hypertrophic gene expression.[7,12] Data from two separate groups indicated that pathological Ca^{2+} could leak from the sarcoplasmic reticulum (SR) via the RyR2, which enhanced the activity of calcineurin.[13,14] Similarly, Kim *et al*.[15] demonstrated that caffeine-induced Ca^{2+} release led to the activation of CaMKII. These studies suggested that Ca^{2+} release through the RyR2 might induce cardiac hypertrophy due to the activation of calcineurin and/or CaMKII. Wu *et al*.[16] reported that CaMKII might only respond to the Ca^{2+} released via the IP_3R2 and be insensitive to the contractile Ca^{2+} in cardiac myocytes. It is obvious that the Ca^{2+} associated with hypertrophy appears to be released via the RyR2, the IP_3R2 or SOCE.[17] Therefore, we assumed that distinct sources of Ca^{2+} might differ in the regulation of cardiac hypertrophy. In this study, we developed three types of Ca^{2+} overload models with cultured neonatal rat ventricular cardiomyocytes using ionomycin, caffeine and Ang II, which all increase $[Ca^{2+}]_i$ mainly through SOCE, the RyR2 and the IP_3R2, respectively. Then we investigated the effects of the three agents on cardiac hypertrophy, especially the roles of calcineurin and CaMKII on cardiomyocyte hypertrophy induced by caffeine and Ang II.

Materials and methods

Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and was approved by the Animal Administrative Committee of Xi'an Jiaotong University (Xi'an, Shaanxi, China). Animals were supplied by the Laboratory Animal Center of the Fourth Military Medical University (Xi'an, Shaanxi, China).

Primary cultures of neonatal rat ventricular cardiomyocytes

The neonatal ventricular myocytes isolated from one-day-old Sprague–Dawley rats were prepared as follows. The neonates were anesthetized using isoflurane (1.5–2.0% vol/vol in air) until nociceptive reflexes induced by tail pinch were abolished. Then the anesthetized animals were disinfected with 75% ethanol and decapitated under aseptic conditions. The hearts were quickly removed and placed in ice-cold phosphate-buffered saline (PBS) containing 100 U/mL penicillin and 100 μg/mL streptomycin. After the atria were removed, the ventricles were minced into about 1-mm^3 fragments and dissociated with 0.1% collagenase II (Gibco, Invitrogen Corp, Grand Island, NY, USA) for 30 min at 37°C. After five minutes of centrifugation at 1000 rpm, the collagenase solution was replaced with Kraft–Brühe (KB) solution. After gentle shaking for 30 min, the cardiomyocytes were harvested and allowed to remain for 60 min at 37°C in a CO_2 incubator. The non-myocytes (fibroblasts and endothelial cells) adhered to the bottom of the culture dish, whereas the cardiomyocytes remained in the supernatant. Cardiomyocytes were collected and seeded at a density of 1×10^5 cells/cm^2 onto gelatin-coated coverslips that had been placed into 35-mm culture dishes.

Cardiomyocytes were incubated in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 15% fetal calf serum for 48 h. The medium was replaced with serum-free medium 24 h before treatment with ionomycin (1 μmol/L; Calbiochem, La Jolla, CA, USA), caffeine (1 mmol/L; Sigma, St Louis, MO, USA) or Ang II (100 nmol/L; Calbiochem).

Cell viability

Cell viability was assessed by trypan blue exclusion assay performed in each treated group. Trypan blue stain (Sigma) was prepared fresh as a 0.4% solution in 0.9% sodium chloride. The cells were washed in PBS twice, and were suspended in 0.25% trypsin (Sigma) for five minutes and centrifuged at 1000 rpm for five minutes. Supernatants were removed and pellets were re-suspended in 100 μL 0.4% trypan blue solution and incubated for five minutes. Cells were microscopically counted in a hemocytometer, and the cell viability was expressed as a percentage of the trypan blue-negative cells.

Calcium imaging

The cardiomyocytes were placed on a 22×22 mm^2 glass coverslip, and the coverslip was fixed to a chamber that was mounted on an inverted microscope (IX71; Olympus, Tokyo, Japan) and perfused with normal Tyrode solution. The chamber comprised two parts. The support base consisted of a shallow sloped compartment made of magaluma, and the cover part was made of teflon with a silicone rubber ring attached to its inferior surface that sealed the coverslip. The coverslip served as the bottom of the chamber. The $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent dye Fura-2/AM (Sigma).[18] Fura-2 is a ultraviolet-excited Ca^{2+} indicator that allows ratiometric measurement. In free Ca^{2+}, fura-2 shows a broad excitation spectrum between 300 and 400 nm, with a peak at approximately 363 nm. On Ca^{2+} binding, the excitation peak increases in intensity and also shifts to shorter wavelengths. For ratiometric measurements, excitation at 340 and 380 nm has usually been preferred.[18] If the dye is excited at 340 nm, Ca^{2+} binding will produce an increase in fluorescence, whereas a decrease in fluorenscence is observed when the dye is excited at 380 nm. Fura-2 fluorescence was excited alternately at 340 and 380 nm using the Polychrome V monochrometer (TILL Photonics GmbH, Gräfelfing, Germany), and the excitation light was focused on the cells via a $\times40$ oil objective (NA = 1.35; U/340; Olympus). Emitted fluorescence was collected at 510 nm by a high-speed, cooled CCD camera (C9100; Hamamatsu Photonics, Hamamatsu City, Japan), and recorded with SimplePCI software.

The $[Ca^{2+}]_i$ were calculated using the formula: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]\times\beta$,[18] where R is the ratio of fluorescence of the cell at 340 to the fluorescence at 380 nm. R_{min} is the ratio of fluorescence at zero calcium and R_{max} is the ratio at saturated calcium concentration. K_d is the

dissociation constant of fura-2 for calcium and β is the ratio of fluorescence of fura-2 at 380 nm in the presence of ethylene glycol tetraacetic acid (EGTA) to that of its fluorescence in the presence of ionomycin. R_{max} and R_{min} were determined by the ionomycin method. In brief, R_{max} was calculated by adding ionomycin (10 μmol/L) and $CaCl_2$ (5 mmol/L), and R_{min} was determined by adding EGTA to the bathing solution. Fluorescence of the steady state was first monitored for 30 s. Then fluorescence of the Ca^{2+}-fura-2 mixture was measured for 20 s after addition of 10μmol/L ionomycin and 5 mmol/L $CaCl_2$ to obtain the maximum ratio value (R_{max}). Finally, the fluorescence of Ca^{2+}-free fura-2 was measured for 20 s after addition of 5 mmol/L EGTA to obtain the minimum ratio value (R_{min}).

Cardiomyocyte morphological analysis

Cardiomyocyte images were captured with a CCD (600ES-CU; Pixera, Los Gatos, CA, USA) camera fixed to an inverted microscope (Leica, Wetzlar, Germany). From each coverslip, 60 regular myocytes were selected at random and viewed at $\times$200 magnification. Cardiomyocytes were outlined and the cell surface area (CSA) was measured with SimplePCI software.

Protein content

Cultured myocytes were treated with the three agents for 24 h. The cells were washed with PBS and then treated with 10% trichloroacetic acid (Sigma) at 4°C for one hour to precipitate the protein.[19] The precipitates were dissolved in 0.15 N NaOH. Thereafter, the protein content was determined using a BCA protein assay kit (Beyotime, Haimen, Jiangsu, China).

RNA extraction, reverse transcription, and realtime polymerase chain reaction

Total RNA was isolated from cultured myocytes using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The integrity and purity of the extracted RNA was evaluated by the ratio of the absorbance at 260 nm to the absorbance at 280 nm. Single-stranded cDNA was synthesized using the Eppendorf PCR system (Eppendorf, Hamburg, Germany). Realtime polymerase chain reaction was performed on cDNA using 20 μL reaction volumes with SYBR Premix Ex Taq Takara (Takara, Dalian, China) using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The mixtures were heated to and held at 95°C for 30 s followed by 40 cycles at 95°C for five seconds, and 60°C for 30 s. All reactions were performed in triplicate, and the mRNA values were normalized to the 18s ribosome RNA, which is a housekeeping gene. We carried out a study to analyze the relative quantity of the expression data based on the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (C_{T,\ target} - C_{T,\ ER})_{Time\ x} - (C_{T,\ target} - C_{T,\ ER})_{Time\ 0}$. Time x is any time point and time 0 represents the 1$\times$ expression of the target gene normalized to the endogenous reference (ER).

Oligonucleotide primer sequences were as follows:

For rat atrial natriuretic peptide (ANP), forward: 5′ ATGGG CTCCTTCTCCATCAC 3′, reverse: 5′-TCTTCGGTACCGG AAGCTG-3′;

For rat brain natriuretic peptide (BNP), forward: 5′ GGG CTGTGACGGGCTGAGGTT 3′, reverse: 5′ AGTTTGTG CTGGAAGATAAGA 3′;

For rat β-myosin heavy chain (β-MHC), forward: 5′ CGCTC AGTCAGGCGGAT 3′, reverse: 5′ GCCCCAAATGC AGCCAT 3′;

For rat 18S, forward: 5′ GCCGCTAGAGGTGAAATTCTTG 3′, reverse: 5′ CTTTCGCTCTGGTCCGTCTT 3′.

Solutions

KB solution (mmol/L): KCl 85, K_2HPO_4 30, $MgSO_4$ 5, EGTA 1, Na_2ATP 5, pyruvate 5, creatin 5, taurin 20 and glucose 20 (pH = 7.3 with addition of KOH). The normal Tyrode solution (mmol/L): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES 5 (pH = 7.4 with the addition of NaOH). 0 Na^+ − 0 Ca^{2+} solution (mmol/L): 140 LiCl and 10 EGTA substituted for NaCl and $CaCl_2$ (pH = 7.4 with the addition of LiOH). The caffeine was also dissolved in 0 Na^+ − 0 Ca^{2+} solution.

Statistical analysis

All data are represented as the mean ± SEM. Statistical analyses were performed using an unpaired student's *t*-test or one-way analysis of variance (ANOVA). Student's *t*-test was applied for single comparisons with the control. Comparisons among more than two groups were performed using ANOVA, followed by Tukey's *post hoc* test. Data analysis was carried out by SPSS software version 11.5 (SPSS Inc, Chicago, IL, USA), and a value of $P < 0.05$ was considered as statistically significant.

Results

Intracellular Ca^{2+} transients and Ca^{2+} overload induced by different agents

Thirty single, spontaneously beating cells were selected randomly from each group after 24 h of incubation with 1 μmol/L ionomycin, 1 mmol/L caffeine or 100 nmol/L Ang II. The twitch $[Ca^{2+}]_i$ transients were detected by using the fura-2 ratio method (Figure 1a).[18] Compared with the control group, the resting $[Ca^{2+}]_i$ was significantly increased by 108.0 ± 7.8%, 102.0 ± 6.9% and 59.8 ± 3.3% after cardiomyocytes were treated with ionomycin, caffeine and Ang II, respectively (Figure 1b). These results suggested the success of the Ca^{2+} overload models for cardiomyocytes. In addition, the elevated resting $[Ca^{2+}]_i$ induced by caffeine and ionomycin was significantly higher than with Ang II. Figure 1c shows the amplitudes of $[Ca^{2+}]_i$ transients during systole, which were calculated by subtraction of the resting $[Ca^{2+}]_i$ from the peak value. Compared with the control group, the amplitudes of $[Ca^{2+}]_i$ transients were significantly decreased after the cardiomyocytes were

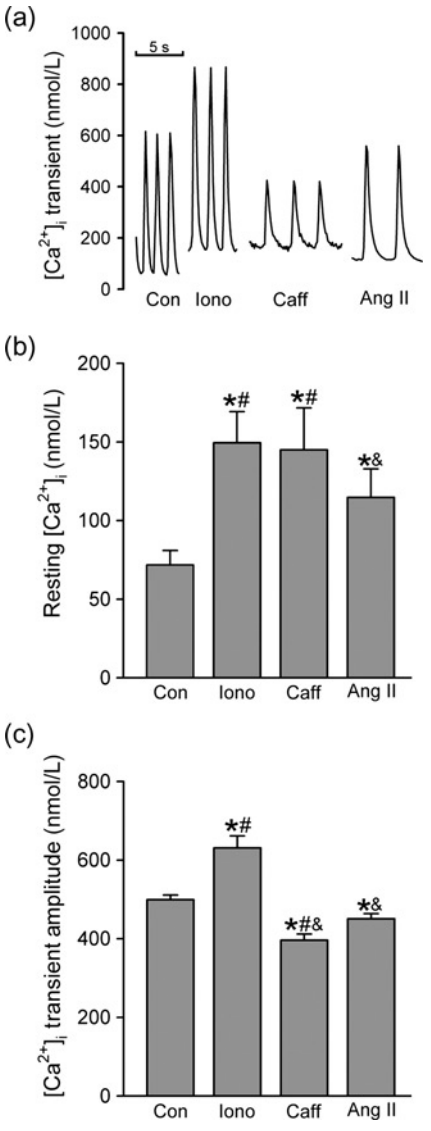

Figure 1 (a) Intracellular Ca^{2+} transients after cardiomyocytes were treated for 24 h, respectively, with 1 μmol/L ionomycin (Iono), 1 mmol/L caffeine (Caff), 100 nmol/L angiotensin II (Ang II) and normal Dulbecco's modified Eagle's medium (DMEM) (Con). $[Ca^{2+}]_i$ level baseline (b) and amplitudes of Ca^{2+} transients (c) after cardiomyocytes were treated for 24 h respectively with ionomycin (Iono), caffeine (Caff), angiotensin II (Ang II) and normal DMEM (Con). Data are presented as the mean $\pm$ SEM ($n = 30$ cells). $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus Ang II group and $^{\&}P < 0.05$ versus ionomycin group

treated with caffeine or Ang II. However, the amplitudes of $[Ca^{2+}]_i$ transients were significantly higher than the control group after the cardiomyocytes were treated with ionomycin.

Cardiomyocyte hypertrophy induced by caffeine and Ang II

We first examined the cell viability from each group using a trypan blue exclusion assay. Our results indicate no significant difference between the three agent-treated groups and the control group (data not shown). After 24 h of incubation with ionomycin, caffeine and Ang II, 60 cells were randomly selected for photography from each group. Figures 2a and b showed that exposure of cardiomyocytes to caffeine or Ang II resulted in a significant increase in CSA of 32.5% and 49.7% versus control, respectively, whereas ionomycin did not. Moreover, the surface area of the cells from the caffeine group was significantly lower by 11.4% compared with that from the Ang II group. The data of protein content also showed the same results (Figure 2c). ANP, BNP and β-MHC were used as markers of cardiomyocyte hypertrophy.[13] We also measured the mRNA expression after cardiomyocytes were cultured for 24 h with ionomycin, caffeine and Ang II (Figure 3). Changes of markers are consistent with the morphological results (Figure 2).

Assessment of SR Ca^{2+} content

In the condition of Na^+-free Ca^{2+}-free bath solution, $[Ca^{2+}]_i$ transients induced by 10 mmol/L caffeine can be used as assessment of the SR Ca^{2+} content.[20] Cardiomyocytes were perfused with normal Tyrode bath solution for 30 s and then rapidly switched to 0 Na^+ − 0 Ca^{2+} solution for another 30 s. Then 10 mmol/L of caffeine was added to release Ca^{2+} from the SR. The SR Ca^{2+} content (Figure 4a) was significantly reduced in cardiomyocytes treated with ionomycin, caffeine and Ang II ($P < 0.05$).

Altered function of Na^+/Ca^{2+} exchanger and SERCA2a

The half-decay time ($T_{1/2}$) of caffeine-induced Ca^{2+} ($C[Ca^{2+}]_i$) transients could be used as an index of the cardiac Na^+/Ca^{2+} exchanger (NCX1) function.[21] Application of high-concentration caffeine (10 mmol/L) in the bath solution caused a rapid Ca^{2+} increase due to Ca^{2+} release from the SR (Figure 4b). In the presence of 10 mmol/L caffeine, SERCA2a is inhibited[22] and other removal pathways (e.g. sarcolemmal Ca^{2+}-ATPase, mitochondrial Ca^{2+} uniporter) can be neglected.[23] Then the Ca^{2+} clearance from the cytoplasm is mainly through NCX1 in the plasma membrane. Figure 5 (left) showed that ionomycin, caffeine or Ang II reduced $T_{1/2}$ of $C[Ca^{2+}]_i$ transients by 72.68 $\pm$ 2.77%, 46.15 $\pm$ 2.13% and 49.84 $\pm$ 2.38% ($n = 12$, $P < 0.05$), respectively, which demonstrated a higher level of NCX1 function.

In the rat cardiomyocyte, most (>90%) of Ca^{2+} is taken back into the SR via SERCA2a immediately after contraction.[22] So the $T_{1/2}$ of spontaneous $[Ca^{2+}]_i$ transients ($S[Ca^{2+}]_i$) reflects Ca^{2+} removal rate from the cytoplasm and can be used to evaluate SERCA2a activity.[20,24] Figure 1a indicated the $[Ca^{2+}]_i$ transients under these conditions. Accordingly, Figure 5 (right) showed that treatment with caffeine or Ang II prolonged the $T_{1/2}$ of $S[Ca^{2+}]_i$ by 43.94 $\pm$ 7.35% and 29.92 $\pm$ 5.38%, respectively, and ionomycin had no effect on it ($n = 12$, $P < 0.05$). This indicates that the function of SERCA2a is impaired in the cardiomyocytes treated with caffeine or Ang II.

CaMKII and calcineurin in cardiomyocytes treated with Ang II and caffeine

Both CaMKII and calcineurin are key transducers of Ca^{2+} signaling. They can induce cardiac hypertrophy through

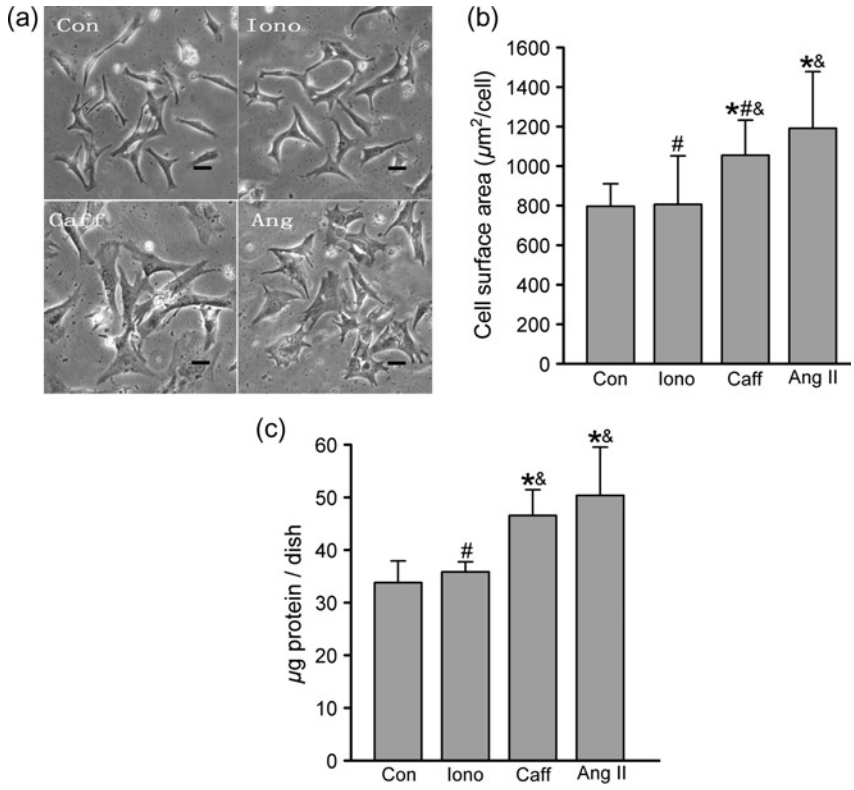

Figure 2 Changes of cell surface area after exposure of cultured neonatal rat cardiomyocytes to ionomycin, caffeine, angiotensin II and normal Dulbecco's modified Eagle's medium. Cells were observed with phase-contrast microscopy (a). The photographs were captured using a $\times 20$ objective, and the magnification bar corresponds to 20 μm. Effects of the three agents on (b) cell surface areas and (c) protein content were determined. The data are represented as the mean $\pm$ SEM ($n = 60$ cells). $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus Ang II group and $^{\&}P < 0.05$ versus ionomycin group

different but overlapping signaling pathways.[7,8] To study the effects of CaMKII and calcineurin on cardiomyocyte hypertrophy induced by caffeine and Ang II, we examined the roles of their specific inhibitors in the mRNA expression of β-MHC. The cardiomyocytes were preincubated with the calcineurin inhibitor cyclosporin A (CsA) (2 μmol/L; Calbiochem), and/or the CaMKII inhibitor KN93 (1 μmol/L; Calbiochem) for 30 min and then treated with 1 μmol/L ionomycin, 1 mmol/L caffeine or 100 nmol/L Ang II for 24 h. In the caffeine group (Figure 6a), CsA or KN93 caused the β-MHC mRNA level to be reduced significantly, but the β-MHC mRNA level was still higher than the control group. Meanwhile, β-MHC mRNA level did not drop to the level of control group when both CsA and KN93 were used. In Ang II group (Figure 6b), KN93 significantly suppressed Ang II-induced β-MHC mRNA level, whereas CsA did not show a significant inhibitory effect on Ang II-induced β-MHC mRNA level. When both calcineurin and CaMKII were inhibited by CsA and KN93, the β-MHC mRNA levels of the two groups were suppressed to a greater degree especially in the Ang II group. The data demonstrated that caffeine activated calcineurin and CaMKII, and AngII only activated CaMKII. The results of this study are consistent with another recent report demonstrating that treatment with Ang II failed to increase calcineurin concentration within 24 h.[25] In the ionomycin group, CsA and KN93 did not induce significant changes in the β-MHC mRNA level as ionomycin did not induce cardiomyocyte hypertrophy (Figure 6c).

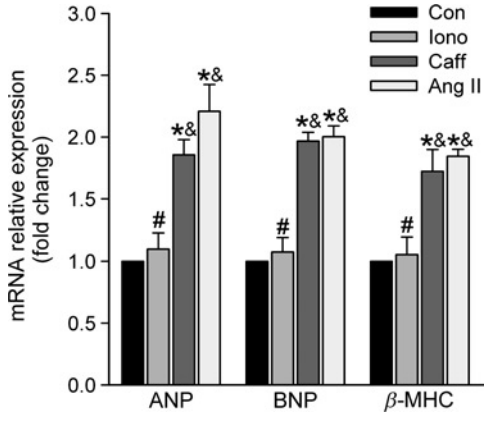

Figure 3 mRNA level of selected marker genes for cardiac hypertrophy after the cardiomyocytes were treated for 24 h, respectively, with ionomycin, caffeine, angiotensin II and normal Dulbecco's modified Eagle's medium. Data were represented as fold change (relative to Con) normalized to the 18S ribosome RNA. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; β-MHC, β-myosin heavy chain; Ang II, angiotensin II. Data are shown as the mean $\pm$ SEM. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus Ang II group and $^{\&}P < 0.05$ versus ionomycin group

Discussion

In this study, we utilized three agents to elevate the $[Ca^{2+}]_i$ in cultured neonatal cardiomyocytes, and then investigated

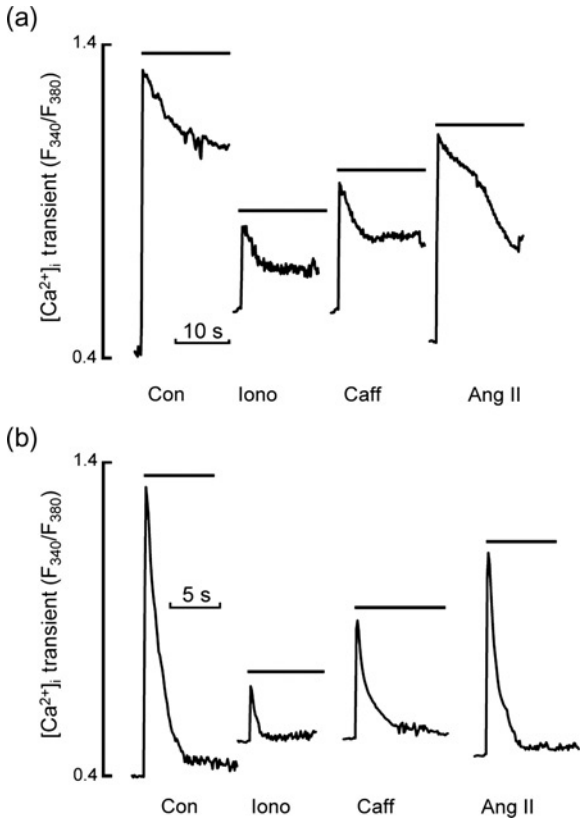

Figure 4 Traces of caffeine-induced $[Ca^{2+}]_i$ transient (C $[Ca^{2+}]_i$) estimated in 0 Na^+ − 0 Ca^{2+} solution (a) and in normal Tyrode solution (b). The mark bars indicated the presence of 10 mmol/L caffeine. The experiments were performed after the cardiomyocytes were treated for 24 h with ionomycin, caffeine, angiotensin II and normal Dulbecco's modified Eagle's medium, respectively

the induced cardiomyocyte hypertrophy. Our results show that Ca^{2+} overload induced by caffeine or Ang II can lead to cardiomyocyte hypertrophy through the signaling pathways of CaMKII and/or calcineurin, whereas ionomycin

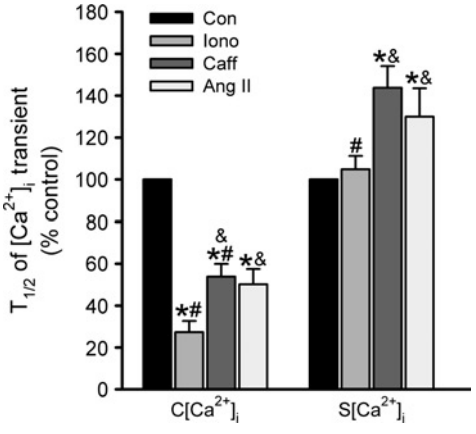

Figure 5 Changes of the half-decay time ($T_{1/2}$) of caffeine-induced $[Ca^{2+}]_i$ transients (C$[Ca^{2+}]_i$) and spontaneous $[Ca^{2+}]_i$ transients (S$[Ca^{2+}]_i$) after the cardiomyocytes were respectively treated for 24 h with ionomycin, caffeine, angiotensin II (Ang II) and normal Dulbecco's modified Eagle's medium. Data are normalized to control and are represented as the mean ± SEM. $^*P < 0.05$ versus control, $^{\#}P < 0.05$ versus corresponding Ang II group and $^{\&}P < 0.05$ versus corresponding ionomycin group

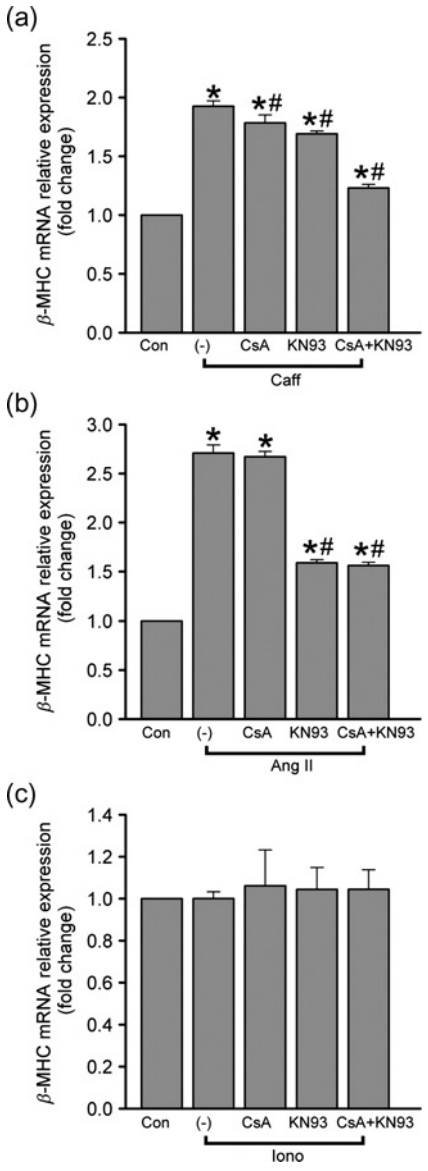

Figure 6 Changes of β-myosin heavy chain (β-MHC) mRNA expression after pretreatment with the calcineurin inhibitor cyclosporin A (CsA) and/or the CaM-dependent protein kinase II (CaMKII) inhibitor KN93. The cultured cardiomyocytes were subjected to 1 mmol/L caffeine (a), 100 nmol/L Ang II (b) or 1 μmol/L ionomycin (c) for 24 h in the presence or absence of pretreatment with CsA and/or KN93. Values are presented as the mean ± SEM. $^*P < 0.05$ versus control; $^{\#}P < 0.05$ versus caffeine treatment (a) or Ang II treatment (b) without any inhibitors. Ang II, angiotensin II

shows no significant effect on CSA of the cardiomyocytes and the hypertrophic marker genes. We additionally find that the Ca^{2+} overload alone is not sufficient to cause the cardiomyocyte hypertrophy and the Ca^{2+} overload-induced hypertrophy is related to the activity of SERCA2a, CaMKII and calcineurin.

Analysis of Ca^{2+} overload induced by the agents

Ionomycin is a type of antibiotic produced by streptomyces and it can make the extracellular Ca^{2+} enter the cells. The sustained exposure to ionomycin would eventually lead to Ca^{2+} overload in cardiomyocytes.[6] Our results also showed that ionomycin elevated $[Ca^{2+}]_i$ by 108.0%

(Figure 1b) and reduced the Ca^{2+} concentration in the SR by 38.2% (Figure 4a). The amplitude of the twitch $[Ca^{2+}]_i$ transients was significantly increased versus the control group (Figure 1c).

Caffeine is widely used to study Ca^{2+} handling during ECC.[26] A well-known property of caffeine is that it is capable of increasing the open probability of the RyR2 channel, and elevating the diastolic $[Ca^{2+}]_i$.[5] As shown in the caffeine group, the resting $[Ca^{2+}]_i$ (Figure 1b) was significantly higher, whereas the amplitude of the twitch $[Ca^{2+}]_i$ transients (Figure 1c) and the Ca^{2+} content of the SR (Figure 4a) were reduced. These results are in agreement with other previous reports.[26,27]

Ang II is well recognized as a stimulator of cardiac hypertrophy via the type 1 Ang II receptor (AT1) *in vivo* or *in vitro*.[28] Stimulation of AT1 with Ang II activates phospholipase C, produces IP_3, and induces Ca^{2+} release from the intracellular Ca^{2+} stores. Our results showed that Ang II could induce Ca^{2+} overload (Figure 1b) and reduce SR Ca^{2+} content (Figure 4a). The amplitude of the twitch $[Ca^{2+}]_i$ transients in the Ang II group was also depressed significantly (Figure 1c).

After treatment with ionomycin, caffeine or Ang II for 24 h, all of the three agents induced intracellular Ca^{2+} overload, and ionomycin and caffeine induced much more Ca^{2+} overload than Ang II (Figure 1b).

Analysis of Ca^{2+} clearance

Ca^{2+} entering the cytoplasm from the extracellular environment or from the SR should eventually return to the extracellular milieu or intracellular storage organelles. The two major systems capable of pumping Ca^{2+} out of the cardiomyocyte or into the SR are the NCX1 in the plasma membrane and the SERCA2a in the SR. Accumulating evidence suggested that the activity of SERCA2a was depressed in hypertrophied cardiomyocytes.[29,30] The $T_{1/2}$ of $C[Ca^{2+}]_i$ and $S[Ca^{2+}]_i$ transients were used to assess the function of the NCX1 and the activity of SERCA2a, respectively.[24] Our results show that the function of the NCX1 is significantly enhanced after treatment of cardiomyocytes with the three agents and the activity of the SERCA2a is reduced in the caffeine and Ang II groups but not in the ionomycin group (Figure 5). According to the results of Ca^{2+} measurements, we conclude that the cardiomyocyte hypertrophy induced by caffeine and Ang II exhibits similar features to those of previous investigations, which was accompanied by the smaller amplitudes of $[Ca^{2+}]_i$ transients, the reduced SR Ca^{2+} content, the depressed SERCA2a function and the enhanced NCX1 activity. However, in the ionomycin group, the amplitudes of $[Ca^{2+}]_i$ transients are higher than control, and the function of SERCA2a is unaltered. All these phenomena demonstrate that cardiomyocyte hypertrophy induced by Ca^{2+} overload is much related to the altered function of SERCA2a.

Hypertrophic responses to the three agents

Ca^{2+} overload can induce the cardiac hypertrophy by CaM-dependent pathways. Both CaMKII and calcineurin are Ca^{2+}/CaM-dependent enzymes, which have been shown to play important and often synergistic roles in transcriptional regulation in cardiomyocytes. A growing body of evidence demonstrated that either CaMKII or calcineurin was sufficient to induce cardiomyocyte hypertrophy via CaM–CaMKII–HDAC and CaM–calcineurin–NFAT signaling pathways, respectively.[1,8,12]

It was found that Ca^{2+} release from the SR near the nucleus could selectively activate nearby CaM and CaMKII. Moreover, increasing evidence indicated that the IP_3R2s were mainly localized in the nuclear envelope that was highly conterminous with the SR in ventricular myocytes, providing uniform driving force for local Ca^{2+} release through RyR2 and IP_3R2.

Our results show that all of the three agents can produce Ca^{2+} overload, but ionomycin cannot induce hypertrophy of the cardiomyocytes. According to the present studies, ionomycin failed to induce cardiomyocyte hypertrophy, mainly due to the failure of its Ca^{2+} overload to activate either the CaMKII or calcineurin pathway. Caffeine and Ang II caused the Ca^{2+} release close to the nucleus through RyR2 and IP_3R2, respectively, and then led to the activation of CaMKII and/or calcineurin. In contrast, ionomycin elevated $[Ca^{2+}]_i$ via the influx of extracellular Ca^{2+}, so it failed to activate either CaMKII or calcineurin.

It was reported that the local Ca^{2+} release from the IP_3R2 could activate both CaMKII and calcineurin.[4,16] In contrast to the reports, we find that Ang II only activates CaMKII, at least in the early stage cardiac hypertrophy. Indeed, the activated CaMKII could phosphorylate calcineurin, which antagonized NFAT nuclear translocation.[31] In addition, NFAT maybe was phosphorylated by several counteracting kinases, such as mitogen-activated protein kinase and protein kinase A, and this phosphorylation directly reduced the effects of calcineurin.[32]

Previous studies suggested that transgenic overexpression of CaM caused cardiac hypertrophy, whereas expression of a CaM mutant could not induce hypertrophy.[33] Furthermore, the CaM antagonist W7 also completely blocked hypertrophy of primary cultured cardiomyocytes in response to the elevated $[Ca^{2+}]_i$.[3] These results demonstrated that CaM was sufficient and necessary for the induction of Ca^{2+}-mediated cardiac hypertrophy. In our studies, the ionomycin-induced Ca^{2+} overload fails to activate the two CaM-dependent enzymes, CaMKII and calcineurin, and therefore cannot trigger hypertrophic response. Thus, the indexes involved in hypertrophy in the ionomycin group, such as CSA, protein content and marker genes, are unaltered compared with the control.

Limitations of the study

Further studies are clearly needed to elucidate the mechanism by which the cardiomyocytes discriminate between elevations in Ca^{2+} associated with long-term hypertrophic stimuli and normal fluctuations in $[Ca^{2+}]_i$ concentrations involved in ECC. Moreover, the sources of Ca^{2+} to selectively activate CaMKII and calcineurin also need to be investigated in the future.

Conclusion

The present experiments indicate that increased Ca^{2+} release via the IP_3R2 or the RyR2 can induce cardiac hypertrophy in cultured cardiomyocytes but not all the aberrant elevations of the $[Ca^{2+}]_i$ can do so. The Ca^{2+} overload alone is not sufficient to induce cardiomyocyte hypertrophy, but the origin of the Ca^{2+}, the contractile profile of the cardiomyocyte and the type of the activated signaling pathways are important in the development of cardiomyocyte hypertrophy.

Author contributions: JZ and HX designed the research and wrote the manuscript. HX established the Ca^{2+} overload models, conducted the imaging experiment and measured the protein content. YZ, JW and LS contributed to the assay of gene expression. HX and JS analyzed data and performed statistical analysis. They have participated sufficiently in this work.

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