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Dynamic Alterations in the $Ca_V1.2$/CaM/CaMKII Signaling Pathway in the Left Ventricular Myocardium of Ischemic Rat Hearts

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Cardiac L-type calcium channel ($Ca_V1.2$), calmodulin (CaM), and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) form the $Ca_V1.2$/CaM/CaMKII signaling pathway, which plays an important role in maintaining intracellular Ca^{2+} homeostasis. The roles of CaM and CaMKII in the regulation of $Ca_V1.2$ in Ca^{2+}-dependent inactivation and facilitation have been reported; however, alterations in this signaling pathway in the heart after myocardial ischemia (MI) had not been well characterized. In this study, we investigated the dynamic changes in $Ca_V1.2$, CaM, and CaMKII mRNA and protein expression levels in the left ventricles of the heart following MI in rats. The MI model was induced by ligating the left anterior descending coronary artery; the rats were divided into the following five groups: the 6 h post-MI group (MI-6h), 24 h post-MI group (MI-24h), 1 week post-MI group (MI-1w), 2 weeks post-MI group (MI-2w), and the sham group. The mRNA levels were measured by quantitative real-time polymerase chain reaction and the protein expression was determined by western blotting and immunohistochemistry. There were no observable differences in the $Ca_V1.2$ mRNA and protein levels at the early stages of MI, but these levels decreased at MI-2w. Both the mRNA and protein levels of CaM increased at MI-6h, peaked at MI-24h, and then reduced to normal levels at MI-2w. CaMKII mRNA and protein levels decreased at MI-6h and reached their lowest level at MI-24h. Taken together, these data demonstrate that there are dynamic changes in the $Ca_V1.2$/CaM/CaMKII signaling pathway following MI injuries, which suggests that different therapeutic regimens should be used at different time points after MI injuries.

Introduction

Myocardial ischemia (MI) is a complex pathophysiological process that involves various factors and pathways; one generally accepted initiation mechanism is Ca^{2+} overload. The voltage-dependent L-type Ca^{2+} channel (LTCC) is the major route for Ca^{2+} to enter cells. It had to control diverse physiological functions, including gene transcription, rhythmic firing, synaptic transmission, hormone secretion, and excitation-contraction coupling (Parkash, 2008). Four members of the Ca_V1 family ($Ca_V1.1$–$Ca_V1.4$) are present in the LTCC; $Ca_V1.2$ is mainly present in cardiomyocytes (Davies *et al.*, 2007; Yang *et al.*, 2011). In addition to physiological functions, changes in density or functions of the LTCC have been implicated in a variety of cardiovascular diseases, including atrial fibrillation (Grandi *et al.*, 2011), heart failure (He *et al.*, 2001), and ischemic heart disease (Aggarwal and Boyden, 1995). LTCC antagonists were widely used to treat cardiovascular diseases for decades. For example, they are frequently used to treat and manage ischemic heart disease. Additionally, these antagonists have been used as antiarrhythmic drugs (class IV) for supraventricular arrhythmias and to reduce blood pressure in patients with hypertension in order to decrease the risks of a major cardiovascular event (Fujino *et al.*, 2005).

To date, many studies have elucidated the regulatory mechanisms of the LTCC. The functions of the LTCC are tightly controlled by protein kinase A (PKA) through phosphorylation mediated by β-adrenergic receptor and protein kinase C (PKC) through different signaling mechanisms based on different isoforms (Kamp and Hell, 2000). The LTCC is also regulated by calmodulin (CaM) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which induce dramatic changes in Ca^{2+} signaling. Our previous studies showed that CaM and CaMKII play distinct roles in $Ca_V1.2$ regulations in which the CaMKII phosphorylation site is slowly dephosphorylated during rundown of the channels; the effects of CaM are attenuated over time. Phosphorylation by CaMKII facilitates and prolongs the effects of CaM on the channel, and both CaMKII

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and CaM are required to reverse the run-down of the LTCC (Hao *et al.*, 2008, 2009).

CaM is the primary intracellular Ca^{2+}-binding protein and consists of two lobes with two Ca^{2+}-binding sites in each lobe. CaM changes its conformation when Ca^{2+} is bound; therefore, it has been considered a Ca^{2+} sensor in various Ca^{2+} signaling cascades. CaMKII contains both Ca^{2+}- and CaM-binding sites that are essential for signal transduction. CaMKII is activated through elevations in intracellular Ca^{2+} levels and, in turn, adjusts Ca^{2+} homeostasis in many ways. Autophosphorylation of CaMKII could activate inhibitory phosphorylation sites, which prevents further activation of CaMKII. Recent studies have highlighted the roles of CaM and CaMKII in the regulation of $Ca_V1.2$, especially in Ca^{2+}-dependent inactivation (CDI) and Ca^{2+}-dependent facilitation (CDF). CaM is necessary and could possibly be sufficient for the mediation of CDF and CDI in the LTCC, whereas CaMKII only affects their time courses. Thus, CaM is a key molecule in bifurcating the Ca^{2+} signal to CDF or CDI, and CaMKII plays a modulatory role in both pathways (Nie *et al.*, 2007). However, it cannot be predicted how $Ca_V1.2$, CaM, and CaMKII change in the left ventricular myocardium during MI. Based on previous results, we hypothesize that the $Ca_V1.2$/CaM/CAMKII signaling pathway might be involved in myocardial impairments during MI.

To date, changes in the $Ca_V1.2$/CaM/CaMKII signaling pathway following MI have not been well documented. Many clinical studies have suggested that treatment timing for myocardial infarction is vital for healing. Thus, this study was conducted to examine the dynamic changes in the $Ca_V1.2$/CaM/CaMKII signaling pathway in the left ventricular myocardium following MI in rats. Our current data demonstrated that the $Ca_V1.2$/CaM/CaMKII signaling pathway changes differently following MI at different stages, suggesting that the current therapeutic regimen should be modified for different time points following MI.

Materials and Methods

MI model preparation

Experiments were performed using Wistar rats weighing 250–300 g, supplied by the Laboratory Animal Centre of China Medical University (SCXK Liao, No. 2008-0005). All animals received humane care in compliance with the *Principles of Laboratory Animal Care* (NIH publication No. 85-23, revised 1985). All animal experiments were approved by the Animal Care and Use Committee of China Medical College.

Rats were anesthetized by intraperitoneal injection with 1% sodium pentobarbital (40 mg/kg) and subsequently placed in supine position. Three electrocardiogram (ECG) limb electrodes were then connected. Under sterile conditions, the rats' hearts were exposed with a 2-cm left lateral thoracotomy. The left anterior descending coronary artery was completely ligated with a 3/8 circular needle followed by a 5/0 silk thread through the shallow layer of the myocardium. To make it completely consistent, the ligation procedure should be finished in a fixed position with similar strength. If the ligation was not firm, then the rat would be excluded from the test group. The heart was subsequently put back into the thoracic cavity, and the chest wall was sewn up. Induction of MI was successful when ST-segment elevation continued for 30 min (Zhao *et al.*, 2009). The percentage for the infarct was measured by the quantitative histochemical staining method (Harnarayan *et al.*, 1970). The ratio of the infarct area to total heart area was $38.36\% \pm 8.59\%$. Specimens were collected from the infarct area of left ventricular anterior wall after ligation of coronary artery.

Experimental animals were randomly divided into four groups: 6 h post-MI group (MI-6h), 24 h post-MI group (MI-24h), 1 week post-MI group (MI-1w), and 2 weeks post-MI group (MI-2w). The sham group rats underwent the same procedure except for the ligation process. Infection after operation was prevented through the use of the antibiotic penicillin at 100,000 U/day (i.p. injection for 3 days). The rats were killed at similar times and their blood was collected from the abdominal aorta; then, the heart was immediately removed and the infarcted left ventricular tissues were harvested at different time points after the operation.

Lactate dehydrogenase determination

Lactate dehydrogenase (LDH) release in the blood serum was evaluated at different time points after MI. LDH levels were determined by a colorimetric method according to the manufacturer's instructions.

Hematoxylin-eosin staining

Rats were sacrificed at the designated time points and their hearts were quickly harvested from the thoracic cavity and immersed in paraformaldehyde for 48 h. Fixed tissues were embedded in paraffin and 5-μm-thick histological sections were prepared. The sections were mounted onto slides and stained with hematoxylin and eosin (HE). To avoid air oxidation and stain fading, the sections were mounted with gum for microscopic examination and photography. The morphological features of myocardial tissue were observed under a light microscope for analysis of myocardium injuries.

Quantitative real-time polymerase chain reaction analysis of $Ca_v1.2$, CaM, and CamKII mRNA expression

Total RNA was extracted from ~50 mg of myocardial tissue samples using Trizol reagent (Invitrogen); 0.5 μg of total RNA was used for first-strand cDNA synthesis with the RT reagent kit (TaKaRa). The reaction conditions were followed based on the instructions in the manufacturer's protocols: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. Then, quantitative real-time polymerase chain reaction (qRT-PCR) was performed after RT with dilutions of cDNA template with SYBR premix Ex Taq II (TaKaRa). The reaction conditions were as follows: 95°C for 30 s, 95°C for 5 s, and 60°C for 30 s, 40 cycles. The following primer sequences were used: *Cacna1c*, sense 5′-gatgcaagacgctatgggctatga g-3′ and antisense 5′-gcatgctcatgttcggggtttgt c-3′; *Cam*, sense 5′-atggctgaccagctg ac-3′ and antisense 5′-ctttgcagtcatcat c-3′; *Camk2*, sense 5′-ggttcacc gacgagtatc ag-3′ and antisense 5′-gtcttcaaagagttcgcc ac-3′; and *Gapdh*, sense 5′-caacgacccttcattga cc-3′ and antisense 5′-gaagacgccagtagactc ca-3′. Each primer pair produced a

single band after amplification with a single melting curve. Standard curves of the purpose gene were generated with reverse-transcribed RNA from the heart tissue and analyzed using quintupled serial dilutions. Concentrations were determined by comparing the *Ct* values of the samples to the standard curves. The results have been expressed as relative expression compared with the Gapdh expression.

Western blot analysis of $Ca_V1.2$, CaM, and CaMKII protein expression

Proteins were extracted from ~100 mg of myocardial tissue samples followed by tissue homogenization and sonification in RIPA lysis buffer with a cocktail of proteinase inhibitors. Two well-known phosphatase inhibitors, NaF and Na_3VO_4, were used in this study. Total protein levels were determined using a Bradford protein assay kit. Aliquots of protein extracts from the samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to the polyvinylidene difluoride membrane (Millipore) by electrophoresis. Membranes were blocked with 5% non-fat milk blocking buffer in Tris-buffered saline with Tween 20 (TBST) for 1 h at room temperature and incubated with the indicated primary antibodies overnight at 4°C. The following primary antibodies were prepared according to manufacturer's instructions: mouse anti-CaM (1:500; Santa Cruz), mouse anti-CaMKII (1:500; Santa Cruz), rabbit anti-p-CaMKII (1:500; Santa Cruz), and rabbit anti-$Ca_V1.2$ (1:500; Sigma). Unbound antibodies were removed by repeated washing in TBST. Horseradish peroxidase-conjugated sheep anti-rabbit or sheep anti-mouse immunoglobulin IgG (1:5000) was used as the secondary antibody; the membranes were incubated for 2 h at room temperature. The immunoreactive proteins were detected by enhanced chemiluminescence. The optical density of each protein band was analyzed with the Fluor Chem V2.0 software.

Immunohistochemistry analysis of $Ca_V1.2$, CaM, and CaMKII proteins

The left ventricles were immersed in 4% paraformaldehyde for 48 h and embedded in paraffin blocks. Five-micrometer sections were cut, deparaffinized, rehydrated, and incubated in 3% H_2O_2 at 37°C for 25 min. Slides were treated with citric acid buffer at 95°C for 10 min, and

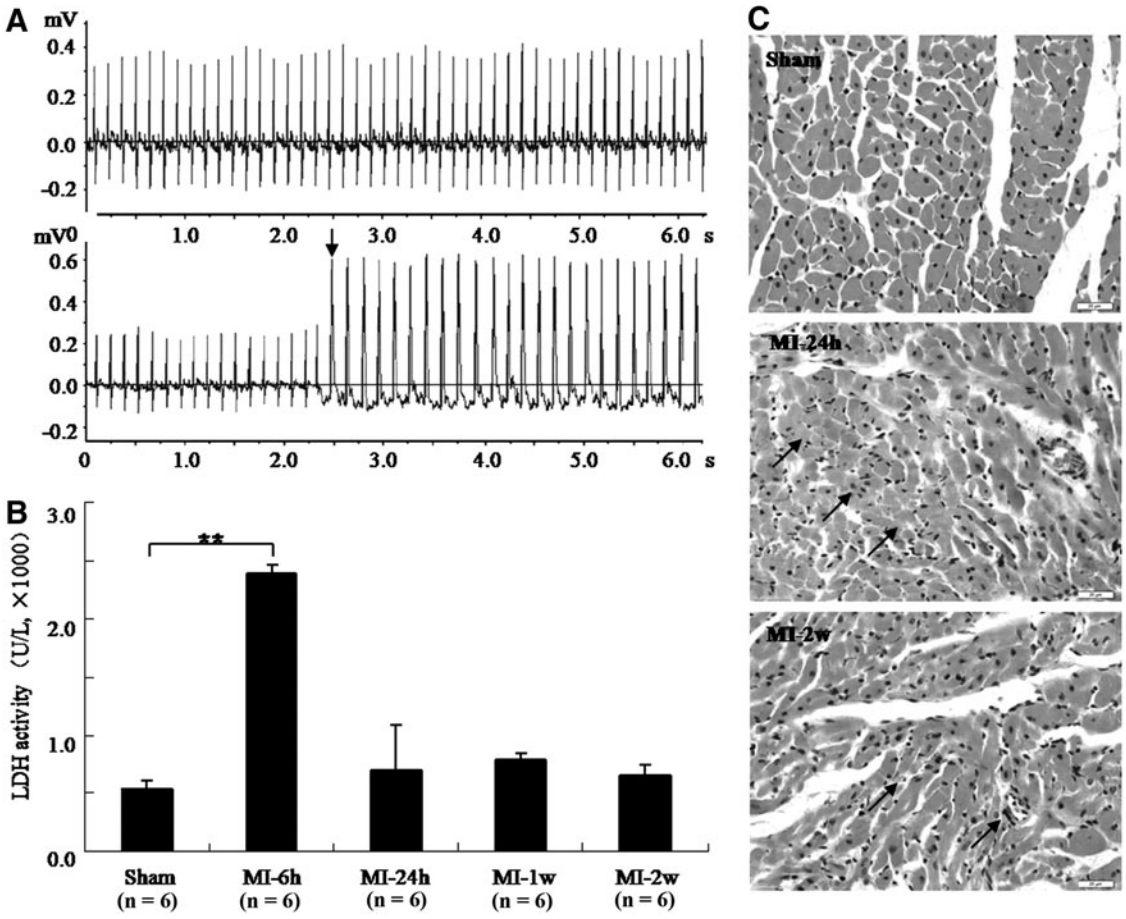

FIG. 1. Pathologic changes in myocardial ischemia (MI) rats. Change in electrocardiograms (ECGs) of MI rats. **(A)** Normal ECG was detected in normal rats (upper panel) and a marked ST-segment elevation was detected in MI rats (lower panel). Note: arrow shows the ST-segment elevation. **(B)** Levels of lactate dehydrogenase (LDH) released in rat serum during MI (**$p < 0.01$). **(C)** Representative slides showing hematoxylin-eosin staining in the left ventricular myocardium of rats (400×). The interstitial edema, neutrophil infiltration, and myocardial disorganization were observed at MI-24h (shown as arrows). Increased neutrophil infiltration and hyperplastic fibroblast infiltration were observed at MI-2w (shown as arrows).

non-specific binding was blocked using goat serum in PBS buffer at 37°C for 30 min. The sections were then incubated with primary antibodies against rat CaM, CaMKII, or $Ca_V1.2$ overnight at 4°C. Slides were then incubated with biotinylated secondary antibody at 37°C for 30 min. The antibody complex was detected with 30-min incubation in a streptavidin-biotin-horseradish peroxidase complex at 37°C. Color was developed with diaminobenzidine, which generated a brown reaction byproduct. Slides were counterstained with hematoxylin for 5 min, and mounted with gum. The preparations were examined under a light microscope.

Statistical analysis

The data are presented as mean ± standard error. Statistical analysis was performed with the SPSS13.0 statistical software followed by ANOVA; $p < 0.05$ was considered statistically significant for differences between the sham and experimental groups.

Results

Pathological changes of MI in the left ventricular myocardium

ECGs were recorded before and after coronary artery ligation and successful infarction was confirmed by ST-segment elevation (Fig. 1A). A 4.5-fold increase in LDH released into the serum was also observed at MI-6h, compared with that of the sham group; levels returned to normal at MI-24h (Fig. 1B). Variable degrees of myocardial injury at MI-24h and MI-2w were observed through HE staining (Fig. 1C). In the sham group, the myocardial cells were well arranged, with clear cellular nuclei, and the cytoplasm was uniformly stained. In MI-24h group, acute heart injury characterized with neutrophil infiltration, interstitial edema, and partial rupture was observed. It was revealed that there was marked increase of neutrophil infiltration and hyperplastic fibroblast infiltration in the MI-2w heart tissues.

Pathological changes differed between the early and later stages of MI injury. In the early stages, ST-segment elevation and increased LDH release were apparent, and HE staining showed acute myocardium injury. In the later stages, LDH release approached normal levels, and HE staining showed chronic myocardium injury. Therefore, the pathological changes differed between different stages of MI injury.

Messenger RNA and protein expression of $Ca_V1.2$, CaM, and CaMKII in the left ventricular myocardium of MI rats

Four different time points were selected to evaluate the changes in mRNA and protein levels after MI. The mRNA levels of $Ca_V1.2$ were evaluated by qRT-PCR (Fig. 2A); $Ca_V1.2$ mRNA levels in the left ventricle of MI rats did not change in MI-6h or MI-24h group, but were lower at MI-2w than the levels of the sham group (0.65 ± 0.05 vs. 1.00 ± 0.04, respectively; $p < 0.05$). Next, changes in CaM mRNA and protein levels after MI were evaluated. CaM mRNA expression in the left ventricles of MI rats was significantly higher than that in the sham group at MI-6h (2.44 ± 0.10 vs. 1.00 ± 0.06, respectively; $p < 0.01$) and peaked at MI-24h (3.11 ± 0.10 vs. 1.00 ± 0.06, respectively; $p < 0.01$); levels were similar to those of the sham group at MI-2w (1.35 ± 0.14 vs. 1.00 ± 0.06, respectively; Fig. 2B). Since phospho-CaMKII has been shown to regulate the activity of $Ca_V1.2$, we later examined the expression of CaMKII and phospho-CaMKII in the hearts of rats with MI. Our data showed that CaMKII decreased significantly at MI-6h and MI-24h. CaMKII mRNA expression in the left ventricles of MI rats was significantly lower than that of the sham group at MI-6h (0.28 ± 0.07 vs. 1.00 ± 0.08, respectively; $p < 0.01$); it reached its lowest level at MI-24h (0.11 ± 0.09 vs. 1.00 ± 0.08, respectively; $p < 0.01$) and then recovered to 60% of the sham levels at MI-2w (0.60 ± 0.04 vs. 1.00 ± 0.08, respectively; $p < 0.01$; Fig. 2C).

Similarly, western blot results showed that protein levels did not change at MI-6h and MI-24h, but that the levels

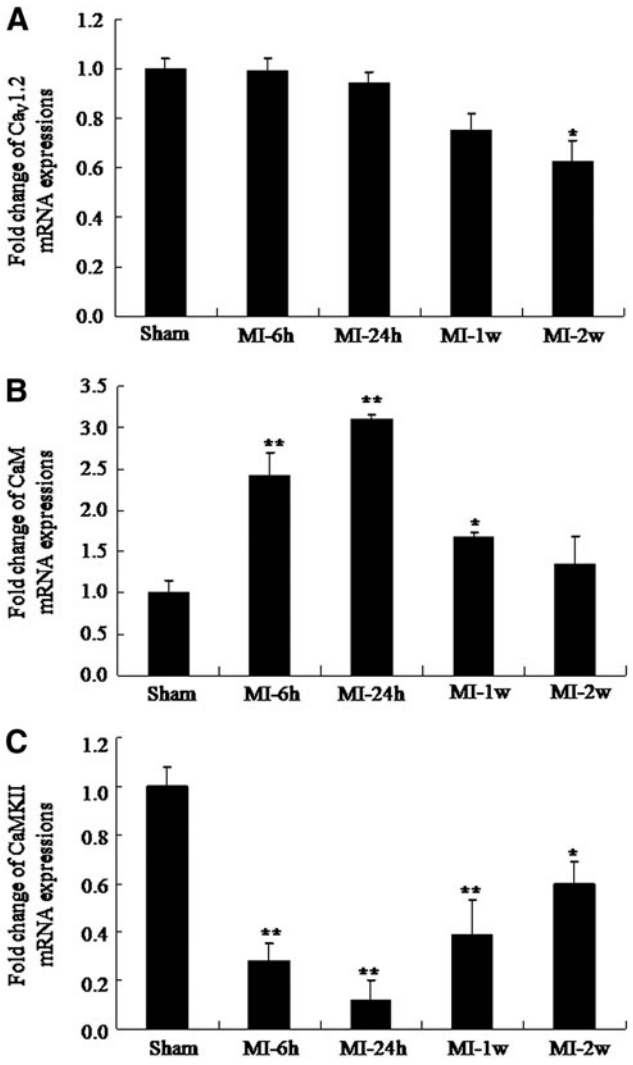

FIG. 2. Dynamic change in Cacna1c, Cam, and Camk2 mRNA expression in the left ventricular tissue of MI rats. **(A)** Cacna1c mRNA levels (mean ± standard error [SE], $n = 6$, $^*p < 0.05$ compared with sham group). **(B)** Cam mRNA levels (mean ± SE, $n = 6$, $^*p < 0.05$ $^{**}p < 0.01$ compared with sham group). **(C)** Camk2 mRNA levels (mean ± SE, $n = 6$, $^*p < 0.05$ $^{**}p < 0.01$ compared with sham group).

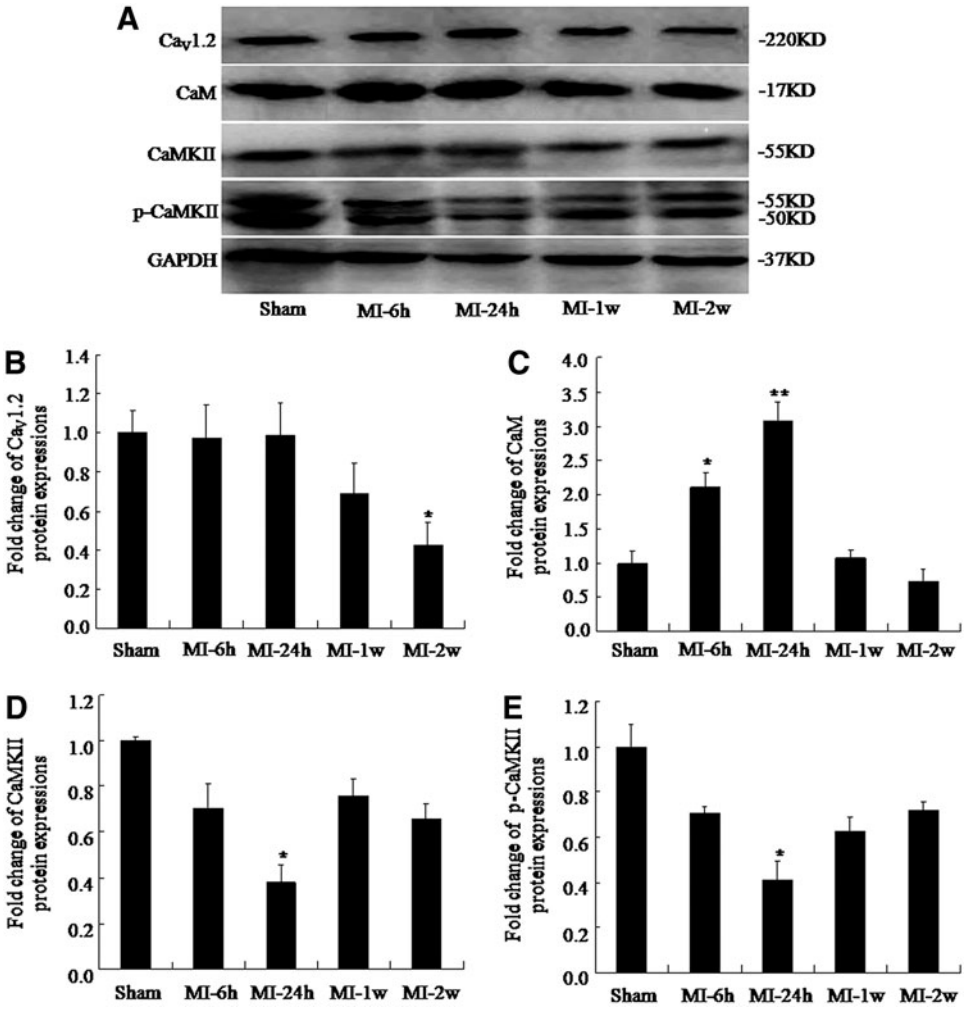

FIG. 3. Dynamic change in $Ca_V1.2$, calmodulin (CaM), calmodulin-dependent protein kinase II (CaMKII), and phospho-CaMKII protein expression in the left ventricular tissue of MI rats. **(A)** Representative protein bands of $Ca_V1.2$, CaM, CaMKII, and phospho-CaMKII in MI rats. **(B)** $Ca_V1.2$ protein levels (mean ± SE, $n = 6$, $^*p < 0.05$ compared with sham group). **(C)** CaM protein levels (mean ± SE, $n = 6$, $^*p < 0.05$ $^{**}p < 0.01$ compared with sham group). **(D, E)** CaMKII and phospho-CaMKII protein levels (mean ± SE, $n = 6$, $^*p < 0.05$).

decreased at MI-2w (0.43 ± 0.17 vs. 1.00 ± 0.12, respectively; $p < 0.05$; Fig. 3A, B). These findings indicate that $Ca_V1.2$ mRNA and protein levels post-MI did not change in the early stages, but markedly decreased during the later stages of MI injury. Protein levels of CaM were also higher than that of the sham group at MI-6h (2.10 ± 0.50 vs. 1.00 ± 0.38, respectively; $p < 0.05$) and peaked at MI-24h (3.06 ± 0.65 vs. 1.00 ± 0.38; $p < 0.01$); the levels were similar to those of the sham group at MI-2w (0.72 ± 0.39 vs. 1.00 ± 0.38, respectively; Fig. 3A, C). The protein levels of CaMKII and phospho-CaMKII were significantly lower than the sham group levels at MI-24h (0.41 ± 0.03 vs. 1.00 ± 0.04 and 0.37 ± 0.08 vs. 1.00 ± 0.02, respectively; $p < 0.05$) and then recovered to 72% and 66% at MI-2w compared with sham levels (0.72 ± 0.01 vs. 1.00 ± 0.04 and 0.66 ± 0.07 vs. 1.00 ± 0.02, respectively; Fig. 3A, D, E). However, we found no differences between phospho-CaMKII and total CaMKII levels ($p > 0.05$). The GAPDH signal confirms that unequal loading of the samples did not account for the differences observed.

Immunohistochemical staining for CaM showed that more scattered brown color dye within the cytoplasm was observed at MI-24h compared with that for the sham group (Fig. 4A). These results showed that CaM levels increased significantly at the early stages, but gradually recovered to normal levels at the later stages of MI. The levels of CaM mRNA and protein that differed at different time points after MI injury would influence $Ca_V1.2$ in different ways throughout the course of MI. Immunostaining for phospho-CaMKII showed that there was lesser brown color dye within the cytoplasm at MI-24h compared with that for the sham group (Fig. 4B). These results suggested that the decreased expression of CaMKII and phospho-CaMKII at early stages of MI was, at least in part, a negative feedback mechanism to reduce Ca^{2+} overload induced by increased CaM levels.

Discussion

The present study demonstrated that $Ca_V1.2$ mRNA and protein levels did not change at the early stages of MI (MI-6h and MI-24h), but decreased significantly at the later stages (MI-1w and MI-2w) in rats. Consistent with our data, the Ca^{2+} channel α1c subunit expression decreased dramatically in the ventricle in ischemic hypertrophic myocardium at 6 weeks after MI (Huang *et al.*, 2008; Goonasekera *et al.*, 2012). In the failing mouse heart, the investigators consistently observed a significant reduction in α1c protein after 8 weeks of pressure overload stimulation (Goonasekera *et al.*, 2012). Since $Ca_V1.2$ levels decreased at

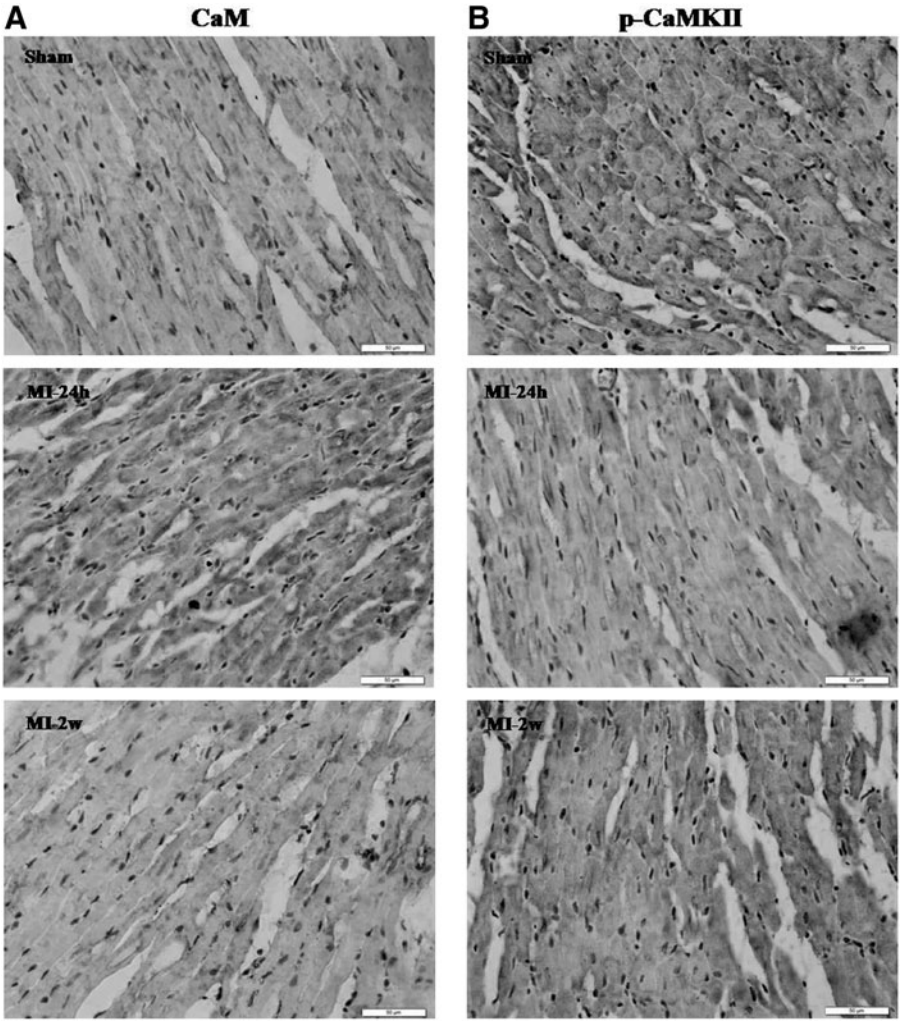

FIG. 4. Expression of CaM and phospho-CaMKII in the left ventricular myocardium of rats (400×). **(A)** Very slim-stained CaM in cell membrane and cytoplasm in sham while increased staining in the cytoplasm regions was observed in the MI-24h. **(B)** Immunohistochemical expression of phospho-CaMKII showed slim stain in the cytoplasmic region in the MI-24h group.

the later stages of MI, LTCC blockers have either no effect or can negatively affect the survival and cardiovascular events at this time point in clinical trials (Mahe *et al.*, 2003). An increase in cytoplasmic Ca^{2+} concentration in myocardial cells with hypoxia has been reported (Song *et al.*, 2005; Liu *et al.*, 2009), and intracellular Ca^{2+} overload is an important cause for damage and dysfunction in the myocardium (Tang *et al.*, 2011; Dhalla *et al.*, 2012). As $Ca_V1.2$ is involved in the major route for Ca^{2+} to enter into myocardial cells, the decreased expression of $Ca_V1.2$ and the overload of intracellular Ca^{2+} seemed to be paradoxical, which suggests that intracellular Ca^{2+} overload of MI may be related to other regulatory proteins of $Ca_V1.2$, such as Na^+/Ca^{2+} exchanger, Na^+/H^+ exchanger, RyR, and SERCA (Zima and Blatter, 2006; Vittone *et al.*, 2008). CaMKII may regulate a variety of Ca^{2+}-dependent proteins, such as LTCC, intracellular Ca^{2+} release ryanodine receptor channels, and phospholamban (PLB). LTCC opening is primarily initiated by cell membrane depolarization, but opening probability is also regulated by phosphorylation and this is the mechanism by which CaMKII increases Ca^{2+} entry. CaMKII phosphorylation causes LTCCs to enter a high-activity gating mode (Dzhura *et al.*, 2000). Ryanodine receptor 2 (RYR2) was a CaMKII-regulated protein that was involved in the regulation of the intranuclear Ca^{2+} concentration. Currie *et al.* (2004) have reported that CaMKII may promote activation of RYR2 of the sarcoplasmic reticulum (SR), thereby increasing RyR2 Ca^{2+} sensitivity and spontaneous release of Ca^{2+} from internal stores. The PLB was also defined as a CaMKII-regulated phosphoprotein (Sag *et al.*, 2007). CaMKII could cause increased PLB phosphorylation at T17, which might help preserve SR Ca^{2+} and make PLB unable to inhibit SERCA2a, thereby increasing Ca^{2+} reuptake into intracellular stores (Schwoerer *et al.*, 2008). In this study, our findings revealed that there was a significant reduction of CaMKII levels in the acute phase of ischemia, which might decrease the activation of LTCC and RYR2 and intracellular Ca^{2+} concentration. Phospho-CaMKII may promote activation of RYR2 of the SR, thereby increasing RyR2 Ca^{2+} sensitivity and spontaneous release of Ca^{2+} from internal storage (Currie *et al.*, 2004). Further, phospho-CaMKII could cause increased PLB phosphorylation at T_{17}, which might help preserve SR Ca^{2+}, and disabled PLB's ability to inhibit SERCA2a, thereby increasing Ca^{2+} reuptake into intracellular stores (Schwoerer *et al.*, 2008). In this study, our findings revealed a significant reduction of CaMKII levels in the acute phase of ischemia, which might decrease the activation of LTCC and RYR2 and intracellular Ca^{2+} concentration.

The cyclic adenosine monophosphate (cAMP)–dependent PKA, a multifunctional enzyme that could be modulated by intracellular Ca^{2+} concentrations, may phosphorylate several proteins, such as LTCC and PLB and RyR2. The phosphorylation of LTCC causes the Ca^{2+} influx and a Ca^{2+}-induced Ca^{2+} release, thereby increasing intracellular Ca^{2+} concentrations. The phosphorylation of PLB accelerates Ca^{2+} uptake into the SR. The phosphorylation of RyR2 may increase RyR2 Ca^{2+} sensitivity and the spontaneous release of Ca^{2+} from internal storage (Leineweber *et al*., 2006). Previous studies demonstrated that β-adrenoceptor is generally activated during MI (Schomig *et al*., 1995; Ungerer *et al*., 1996). Noradrenaline binds to β-adrenoceptor to activate stimulating adenylate cyclase G protein (Gs). Then, adenylate cyclase is activated by Gs, which turns adenosine triphosphate (ATP) to cAMP. The cAMP in turn activates PKA and increases intracellular Ca^{2+} concentrations, thereby activating CaM/CaMKII (Lu *et al*., 2012). In this study, the results indicated that the reduction of CaMKII levels is a negative feedback regulator of cellular Ca^{2+} overload, which could reduce the extent of ischemic myocardial injury.

In our study, CaM mRNA and protein levels considerably increased at the early stages of MI and recovered to normal levels at the later stages in rats with MI, which suggested that CaM changed in different modalities during MI to regulate $Ca_V1.2$ in different ways. Our previous study has shown that the run-down of $Ca_V1.2$ activity is reversed by CaM and ATP (Xu *et al*., 2004). The direct binding of CaM with the IQ motif of the Ca^{2+}-channel α1-subunit has been reported to be involved in the molecular mechanism by which it exerts its Ca^{2+}-dependent effects, namely, CDI and CDF. Based on our previous and current findings, we propose that, under the condition of MI, the relative increase in CaM in cardiomyocytes at the early stages may facilitate $Ca_V1.2$ channel activities through CDF and enhance Ca^{2+} inflow into cells, while increased $[Ca^{2+}]$ may result in CDI to avoid Ca^{2+} overload and myocardial damage. Consistent with our results, inhibition of CaM was found to be essential for cardioprotection in the ischemic heart (Afanas'ev *et al*., 2006; Fukunaga *et al*., 2006). In this study, decreased $Ca_V1.2$ expression and Ca^{2+} overload can be explained by the increased CaM expression. Therefore, our findings have established a link between increasing CaM and MI, and have defined an essential role of CDF due to CaM.

In this current study, CaMKII and phospho-CaMKII mRNA and protein levels had considerably decreased at the early stages of MI in rats and inclined to return to normal levels at the later stages, which shows the dynamic changes during MI and regulation of $Ca_V1.2$ in different ways. CaMKII is activated through increases in intracellular Ca^{2+}/CaM, which in turn maintains Ca^{2+} homeostasis. In our previous study, CaM and CaMKII had distinct roles in the reversal of run-down; in that, CaM served to activate the channel and CaMKII-mediated phosphorylation facilitated the effects of CaM. Dzhura *et al.* (2000) reported that the single LTCC activity could be facilitated by CaM in inside-out patches, but only in the presence of CaMKII. The changes in the expression of CaMKII and CaM may be related to transcription initiation and mRNA accumulation. Further, altered degradation of proteins may also be involved in the expression of CaMKII and CaM. Here, we propose another possible mechanism in which decreased levels of CaMKII and phosphor-CaMKII may serve as a negative feedback to regulate CDF that was caused by CaM to avoid Ca^{2+} influx through the LTCC in MI. The CDF and CDI are two continuous processes accompanied by an increase in cellular Ca^{2+}. The CDF was first induced and then followed by an increase in Ca^{2+} channel open probability; then, the CDI immediately appeared. However, the excess of CDF might lead to Ca^{2+} overload and inhibit the occurrence of CDI (Pitt *et al.*, 2001). Uemura has suggested a molecular role of CaMKII in the ischemic heart, which appeared to be characteristic features reversible and immediate translocation of substantial amounts of CaMKII, and reduced autophosphorylation (Uemura *et al.*, 2002). In addition, the dynamic changes in CaMKII and phospho-CaMKII can also regulate other ion channels of cardiomyocytes, such as potassium and sodium channels (Mori *et al.*, 2000; El-Haou *et al.*, 2009), which have been shown to be involved in Ca^{2+} homeostasis in cells.

In conclusion, this study is the first, to our knowledge, to show dynamic changes in the $Ca_V1.2$/CaM/CaMKII signaling pathway in rats with MI. The most interesting finding was that the expression of $Ca_V1.2$ did not change at early stages, but decreased during later stages of MI. CaM, an important regulator protein of the LTCC, was upregulated at the early stages of MI. In contrast, CaMKII was downregulated at the early stages of MI. Thus, it would be reasonable to conclude that decreased expression of CaMKII might serve as a negative feedback to inhibit Ca^{2+} overload. The $Ca_V1.2$/CaM/CaMKII signaling pathway is a promising therapeutic target for preventing or alleviating cardiac function after MI; we propose that current clinical therapeutic regimens should be modified depending on the time point after MI.

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Disclosure Statement

The authors have declared that no competing interests exist.

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