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Effects of Calmodulin-dependent Protein Kinase Ⅱ Inhibitor, KN-93, on Electrophysiological Features of Rabbit Hypertrophic Cardiac Myocytes*

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Summary: Cardiac hypertrophy is an independent risk factor for sudden cardiac death in clinical settings and the incidence of sudden cardiac death and ventricular arrhythmias are closely related. The aim of this study was to determine the effects of the calmodulin-dependent protein kinase (CaMK) Ⅱ inhibitor, KN-93, on L-type calcium current ($I_{Ca,L}$) and early after-depolarizations (EADs) in hypertrophic cardiomyocytes. A rabbit model of myocardial hypertrophy was constructed through abdominal aortic coarctation (LVH group). The control group (sham group) received a sham operation, in which the abdominal aortic was dissected but not coarcted. Eight weeks later, the degree of left ventricular hypertrophy (LVH) was evaluated using echocardiography. Individual cardiomyocyte was isolated through collagenase digestion. Action potentials (APs) and $I_{Ca,L}$ were recorded using the perforated patch clamp technique. APs were recorded under current clamp conditions and $I_{Ca,L}$ was recorded under voltage clamp conditions. The incidence of EADs and $I_{ca,L}$ in the hypertrophic cardiomyocytes were observed under the conditions of low potassium (2 mmol/L), low magnesium (0.25 mmol/L) Tyrode's solution perfusion, and slow frequency (0.25−0.5 Hz) electrical stimulation. The incidence of EADs and $I_{ca,L}$ in the hypertrophic cardiomyocytes were also evaluated after treatment with different concentrations of KN-92 (KN-92 group) and KN-93 (KN-93 group). Eight weeks later, the model was successfully established. Under the conditions of low potassium, low magnesium Tyrode's solution perfusion, and slow frequency electrical stimulation, the incidence of EADs was 0/12, 11/12, 10/12, and 5/12 in sham group, LVH group, KN-92 group (0.5 μmol/L), and KN-93 group (0.5 μmol/L), respectively. When the drug concentration was increased to 1 μmol/L in KN-92 group and KN-93 group, the incidence of EADs was 10/12 and 2/12, respectively. At 0 mV, the current density was 6.7±1.0 and 6.3±0.7 $PA \cdot PF^{-1}$ in LVH group and sham group, respectively (P>0.05, n=12). When the drug concentration was 0.5 μmol/L in KN-92 and KN-93 groups, the peak $I_{Ca,L}$ at 0 mV was decreased by (9.4±2.8)% and (10.5±3.0)% in the hypertrophic cardiomyocytes of the two groups, respectively (P>0.05, n=12). When the drug concentration was increased to 1 μmol/L, the peak $I_{Ca,L}$ values were lowered by (13.4±3.7)% and (40±4.9)%, respectively (P<0.01, n=12). KN-93, a specific inhibitor of CaMKII, can effectively inhibit the occurrence of EADs in hypertrophic cardiomyocytes partially by suppressing $I_{Ca,L}$, which may be the main action mechanism of KN-93 antagonizing the occurrence of ventricular arrhythmias in hypertrophic myocardium.

Key words: calmodulin-dependent protein kinase Ⅱ; KN-93; myocardial hypertrophy; electrophysiology; perforated patch recording techniques

Myocardial hypertrophy is a common pathological process that occurs in several cardiovascular diseases such as hypertension, hypertrophic cardiomyopathy, aortic stenosis and myocardial infarction. Clinically, various inducing factors, including ischemia, bradycardia and severe electrolyte imbalance (low potassium or magnesium), may increase the risk of ventricular arrhythmias and sudden death. Changes in the electrophysiological characteristics of cardiomyocytes (such as action potential and QT interval prolongation, and calcium homeostasis imbalance) are the fundamental mechanisms responsible for arrhythmias. Preliminary studies[1, 2] have shown that the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) Ⅱ signal transduction pathway might play an important role in the occurrence of ventricular arrhythmias induced by hypertrophic cardiomyopathy. The aim

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of this study was to determine changes of L-type calcium current ($I_{Ca,L}$) and incidence of early after-depolarizations (EADs) in rabbit hypertrophic cardiomyocytes under low potassium and magnesium conditions, and to evaluate the effects of a specific inhibitor of CaMK Ⅱ, KN-93, on $I_{Ca,L}$ and EADs in hypertrophic cardiomyocytes. The electrophysiological effects of KN-93 on hypertrophic cardiomyocytes and the mechanism(s) involved were elucidated.

1 MATERIALS AND METHODS

1.1 Reagents and Solution Composition

KN-92, KN-93, DMSO, BSA, HEPES, EGTA, Na_2ATP, protease E, verapamil and β-escin were purchased from Sigma (USA), and type Ⅰ collagenase was purchased from Gibco (USA). Other reagents were analytically pure. Normal Tyrode's solution, Tyrode's solution without calcium, Tyrode's solution containing 0.2 mmol/L calcium, and pipette solution for recording action potential and $I_{Ca,L}$ in single cells were prepared according to a previous study[3]. The composition of low potassium and magnesium Tyrode's solution was as follows: 2 mmol/L KCl, 0.25 mmol/L $MgSO_4$, 139.2 mmol/L NaCl, 0.33 mmol/L NaH_2PO_4, 1.8 mmol/L $CaCl_2$, 10 mmol/L glucose and 10 mmol/L HEPES. The pH value was adjusted to 7.3 using NaOH. β-escin solution was prepared as follows: 50 mmol/L stock solution of β-escin dissolved in water was diluted immediately prior to use to 25 μmol/L using pipette solution.

1.2 Creation of Left Ventricular Hypertrophy Model

Twenty female New Zealand rabbits, weighing 2.0−2.5 kg each, were provided by the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology (China). The rabbits were randomly divided into two groups: sham-operated group (sham) and left ventricular hypertrophy (LVH) group (n=10 each group). The LVH model was established in LVH group through abdominal aortic coarctation according to a previous study[4]. The animals in sham group were subjected to the same as LVH group, except for the abdominal aortic coarctation. All operations were performed under the aseptic conditions. After each operation, penicillin (800 000 U) was injected intramuscularly for 3 days to prevent infection. The rabbits were fed on a normal diet for 8 weeks.

1.3 Ultrasound Examination

Transthoracic echocardiography was performed prior to the operation and 8 weeks later. The animals were anesthetized with 20% urethane (1 g/kg) through the auricular vein, the chest hair was removed and they were fixed in the supine position prior to placement of three EGG leads. Echocardiography was performed with a Vivid 7 dimension cardiovascular ultrasound system (GE). The selected probe head was 10 S, the image depth was adjusted to 3.0−5.0 cm and the probe frequency was 11.4 MHz. The sector scan angle was reduced as much as possible. After two-dimensional echocardiography, M-mode ultrasound was used to measure septal thickness (SP), left ventricular posterior wall thickness (PW) and left ventricular end-diastolic dimension (LVEDd). Left ventricular (LV) mass was calculated according to the Devereus formula[5]: LV mass=1.04×[(LVEDd+PW+SP)^3–LVEDd^3].

1.4 Isolation of Single Ventricular Myocyte

After 8 weeks of feeding, the rabbits were weighed and then anesthetized with 3% sodium pentobarbital (30 mg/kg) and 30% urethane (300 mg/kg) through intravenous injection. The animals received anticoagulant treatment with heparin (1000 U/kg). After thoracotomy, the heart was removed rapidly and placed in calcium-free Tyrode's solution at 4°C to cause immediate cardiac arrest. Aortic retrograde catheterization was performed and Langendorff perfusion was initiated with Tyrode's solution bubbled with 95% O_2−5% CO_2. The heart was then perfused with calcium-free Tyrode's solution for 3−5 min, followed by perfusion with calcium-free Tyrode's solution (70 mL) containing 40 mg type Ⅰ collagenase, 5 mg protease E, and 10 mg bovine serum albumin (BSA) for 30 min to digest the cardiac muscle. After removal of atrial muscle and the right ventricle, myocardial tissue from the free wall of the left ventricle was collected and shredded, and subsequently placed in low-calcium Tyrode's solution for 5 min of warm incubation. The supernatant was collected and a single ventricular myocyte suspension was obtained. The single cells were preserved in normal Tyrode's solution containing 0.025% BSA and 200 U/mL ampicillin, and incubated at room temperature for 1 h. The preservation solution was placed in a l-mL chamber. After the cells adhered to the wall, the chamber was placed under an inverted microscope for the selection of clearly-striated, rod-shaped cardiomyocytes with a granule-free surface and no contractions. The experiment was performed at room temperature (25°C).

1.5 Perforated Patch Clamp Recording and Grouping

The perforated patch clamp technique was used to record $I_{Ca,L}$ in voltage clamp mode and action potentials in current clamp mode[6]. The EPC-9 patch clamp amplifier was connected to the computer via 12-bit A/D and D/A converter, and the collection of the stimulation signals and the current input signals were controlled using the Pulse+Pulsefit 8.5 software. Electrodes were drawn out from neutral glass in two steps using the microelectrode maker. The electrode tip was first immersed into the normal pipette solution for a few seconds, and subsequently a pipette solution containing β-escin with a final concentration of 25 μmol/L was loaded in a retrograde fashion into the end of electrode. After positive pressure was applied and the electrode was immersed into water, the resistance was 3−5 MΩ. The liquid junction potential was compensated. The micro-adjustment control was adjusted until a resistance seal greater than 1 GΩ formed between the electrode tip and the cell membrane surface. The capacitive current and leakage current were compensated. Due to the effects of β-escin, Rs was <20 MΩ after about 10 min. Slow capacitance compensation and series resistance compensation (50% to 80%) were adjusted after perforation formation in order to reduce the instantaneous charge and discharge current and clamping errors. During the measurement of capacitance, a slope stimulation of 0.4 V/s was applied. The current was measured and the equation Cm=I/(dV/dt) was used for calculations, in which, Cm is membrane capacitance, I is current, and dV/dt is the voltage slope. In order to eliminate the between-cell errors, the current value was presented as the current density ($pA•pF^{-1}$). The signals

were filtered through a fourth-order Bessel low-pass filter at a cut-off frequency of 1 kHz, and the signals were sampled at a frequency of 5 kHz. The collected data were stored in a computer hard-drive (Macintosh, Quadra, 650, Germany) for offline measurements.

Both the sham and LVH groups were superfused with normal Tyrode's solution and low potassium and magnesium Tyrode's solution. In KN-93 and KN-92 groups, hypertrophic cardiomyocytes were incubated with Tyrode's solution containing different concentrations of KN-93 (final concentrations: 0.5 and 1.0 μmol/L) and KN-92 (final concentrations: 0.5 and and 1.0 μmol/L) for 10 min, followed by low potassium and magnesium Tyrode's superfusion. KN-92 is structurally similar to KN-93 but has no inhibitory effects on CaMK Ⅱ, and this drug was used to control any non-specific effect of KN-93. Changes in $I_{Ca,\,L}$ and the incidence of EADs were observed in all groups under superfusion with low potassium and magnesium Tyrode's solution and low-frequency electrical stimulation. Twelve cardiomyocytes were studied in each group.

1.6 Statistical Analysis

Statistical analysis was performed using SPSS (version 13.0) software. All quantitative data were shown as $\bar{x}\pm s$. Patch-clamp data were analyzed using origin 7.5 data analysis software and graphing software. Quantitative data were analyzed with a *t*-test and one-way ANOVA, and qualitative data were analyzed with a Fisher's exact test. $P<0.05$ indicates a statistically significant difference.

2 RESULTS

2.1 Myocardial Hypertrophy and Electrical Capacitance of Cell Membrane

Echocardiography revealed that the left ventricular wall was significantly thickened and the left ventricular weight was increased in LVH group as compared with sham group (table 1). The left ventricular ejection fraction (EF) was greater than 60% in all hypertrophic rabbit hearts 8 weeks after the operation, indicating that the hearts were at the stage of compensatory hypertrophy without developing heart failure. At the single cell level, the membrane capacitance in LVH group was 201±48 pF, which was significantly greater than that in sham group (141±25 pF; $P<0.01$, $n=12$), suggesting significant enlargement of the single cardiomyocyte in LVH group.

Table 1 Comparison of cardiac morphological parameters between sham and LVH groups ($\bar{x}\pm s$, $n=12$)

Groups	SP (mm)	PW (mm)	LVDEd (mm)	LVM (g)	LVM/body weight (g/kg)
Sham	3.0±0.2	3.0±0.3	10.9±0.6	3.7±0.2	1.7±0.2
LVH	4.2±0.3*	4.0±0.2*	11.0±0.9	5.9±0.2Δ	2.4±0.2Δ

Δ$P<0.05$, *$P<0.01$ *vs.* sham group

2.2 Effects of KN-93 on Incidence of EADs in Single Hypertrophic Cardiomyocyte

The patch clamp was placed under the current-clamp mode and given an outward current of 15 ms, and 900 pA at a frequency of 0.25−0.5 Hz. Under the condition of low potassium and magnesium Tyrode's superfusion, the action potential duration (APD) in all groups was significantly prolonged, and EADs were induced (fig. 1). The incidence of EADs in sham, LVH, KN-92 (0.5 μmol/L) and KN-93 (0.5 μmol/L) groups was 0/12, 11/12, 10/12 and 5/12, respectively. When the drug concentration was increased to 1 μmol/L, the incidence of EADs in KN-92 and KN-93 groups was 10/12 and 2/12, respectively (fig. 2). With continuous superfusion of low potassium and magnesium Tyrode's solution, the sham group showed only APD prolongation without any EADs. The incidence of EADs in LVH group was 91%, showing no significant difference from that in KN-92 group ($P>0.05$). In KN-93 group, the incidence of EADs was significantly reduced as compared with LVH group ($P<0.05$). These results suggest that the incidence of EADs was significantly increased in myocardial hypertrophy ($P<0.01$). Pretreatment with KN-93 significantly reduced the incidence of EADs in hypertrophic cardiomyocytes. This finding suggests that CaMK Ⅱ plays an important role in the genesis of EADs in hypertrophic myocardium.

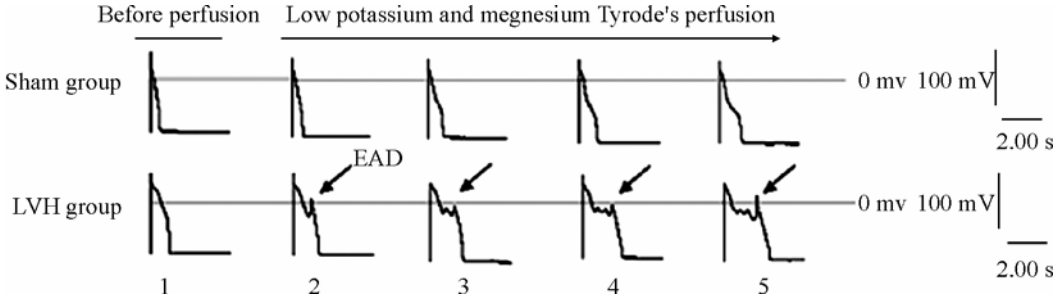

Fig. 1 A series of action potentials recorded from single cardiomyocyte under the current-clamp mode
EADs were induced with a stimulation frequency of 0.25 Hz and low potassium and magnesium Tyrode's superfusion. Arrows indicate EADs.

2.3 Changes of $I_{Ca,\,L}$ in Single Cardiomyocyte in Cardiac Hypertrophy

Changes in $I_{Ca,\,L}$ were measured using voltage clamp conditions in which the holding potential was set at −40 mV, and $I_{Ca,\,L}$ was recorded when a series of depolarizing pulses were applied [150 ms, 10 mV steps (from −40—+50 mV)]. The curve of the current amplitude versus the corresponding depolarized membrane

potentials (Ⅳ curve) was plotted (fig. 3). The $I_{Ca,L}$ values in LVH group were significantly greater than those in sham group. However, there was no significant difference in the current density (the current compared with the respective membrane capacitance). At 0 mV, the current density in LVH and sham groups was 6.7±1.0 and 6.3±0.7 pA•pF^{-1}, respectively (P>0.05, n=12).

2.4 Effects of KN-93 on $I_{Ca,L}$ in Hypertrophic Cardiomyocytes

To evaluate the effects of KN-93 and KN-92 on $I_{Ca,L}$, the holding potential was set at −40 mV, and $I_{Ca,L}$ was recorded when the depolarizing pulse was applied (150 ms, 0 mV). KN-92 and KN-93 at a dose of 0.5 μmol/L decreased the peak $I_{Ca,L}$ by (9.4±2.8)% and (10.5±3.0)% at 0 mV, respectively. Both KN-92 and KN-93 showed similar mild inhibitory effects on the $I_{Ca,L}$ of hypertrophic cardiomyocytes (P>0.05, n=12). When the concentration of both inhibitors was increased to 1 μmol/L, KN-92 reduced peak $I_{Ca,L}$ by (13.4%±3.7) and KN-93 reduced it by (40±4.9)% at 0 mV, respectively (P<0.01, n=12).

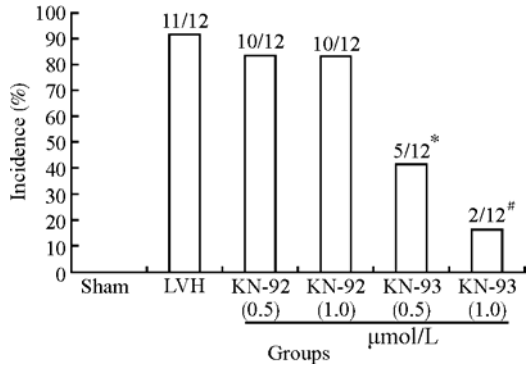

Fig. 2 Comparison of the incidence of EADs among all groups with low-frequency electrical stimulation (0.25−0.5 Hz) and low potassium and magnesium Tyrode's superfusion

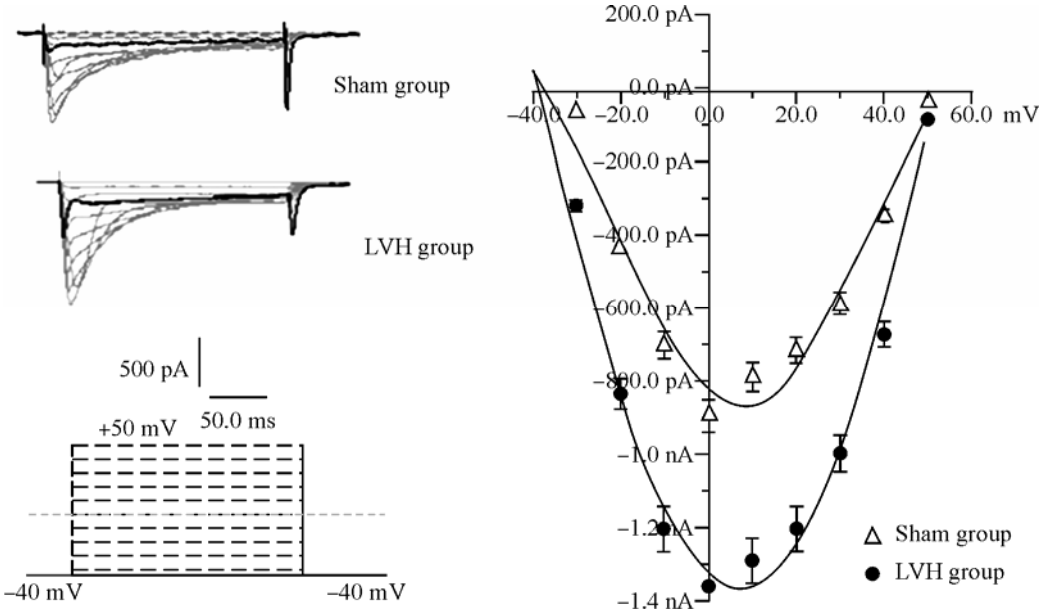

Fig. 3 Changes of $I_{Ca,L}$ in single cardiomyocyte in cardiac hypertrophy and the corresponding IV curve

3 DISCUSSION

In conventional whole-cell recording experiments, the intracellular fluid is perfused with the pipette solution and loss of the intracellular substances may occur, resulting in progressive loss ("running down") of the current being recorded. This "running down" of current is particularly problematic when recording $I_{Ca,L}$, which brought about the difficulty to record $I_{ca,L}$ and to evaluate how drugs affect this current. The present study used the perforated patch recording technique to overcome this shortcoming of whole-cell recording. In addition, β-escin used in this experiment is a saponin derivative, which is soluble in water. It is easy to use and does not affect the high-impedance seal. It interacts with cholesterol in the cell membrane lipids and forms channels that allow monovalent ions to pass through and effectively reduce the loss of current[7]. In this study, the $I_{Ca,L}$ was stabile over relatively long recording time, and its amplitude was reduced only by 8% after 30 min (n=12), ensuring accurate results that could be compared between the experimental and control groups.

Myocardial hypertrophy is a common pathological process of many cardiovascular diseases. Epidemiological studies have shown that incidence of sudden cardiac death is much higher in patients with myocardial hypertrophy than in the general population, and that the incidence of sudden cardiac death is closely related to EADs-triggered ventricular arrhythmia[8]. EADs refer to membrane potential oscillations that occur during phases 2 and 3 of the cardiac action potential. Recent studies suggest that the ion current basis of EADs may be the $I_{Ca,L}$[9, 10]. Pathological conditions such as excessively prolonged cardiac repolarization increase the opening of the L-type calcium channel (LTCC), resulting in increased calcium influx during the plateau phase of the action potential. This increase in intracellular calcium

triggers further oscillatory calcium release by the sarcoplasmic reticulum through positive feedback, leading to membrane oscillation and production of EADs. This study used low potassium, low magnesium perfusion and slow frequency electrical stimulation to stably induce a significantly prolonged APD in the hypertrophic cardiomyocytes at the single cardiomyocyte level, resulting in a significant increase in EADs. Additionally, the use of calcium antagonist, verapamil, effectively suppressed EADs. These results indirectly suggest that the incidence of EADs is indeed associated with the LTCC.

Previous studies found that in the model of long QT syndrome[11, 12] and the transgenic mouse model of cardiac hypertrophy[13], the Ca^{2+} dependent signaling molecule CaMK Ⅱ plays an important bridging role in mediating the QT interval prolongation and the occurrence of EADs and arrhythmia. The aforementioned models are often accompanied with prolonged APD and increased $[Ca^{2+}]_i$. The elevated intracellular calcium binds to calmodulin, resulting in phosphorylation of CaMK Ⅱ and significantly increasing its activity. The activated CaMK Ⅱ can phosphorylate the intracellular ion channels and calcium-dependent regulatory proteins such as LTCC[14, 15], increasing $I_{Ca,\ L}$, which may lead to $[Ca^{2+}]_i$ increase during the diastolic phase and trigger the occurrence of EADs. After the administration of specific CaMK Ⅱ inhibitory peptide AC3-Ⅰ, the probability of $I_{Ca,\ L}$ opening in the myocardial cells was significantly decreased, and the incidence of EADs and ventricular arrhythmia was also reduced accordingly. These findings suggest that the CaMK Ⅱ-induced EADs and ventricular arrhythmias in myocardial hypertrophy is closely related to phosphorylation of LTCC.

In this study, we used the model of pressure-overload cardiac hypertrophy and found that low potassium, low magnesium perfusion, and slow frequency electrical stimulation at the single-cell level could stably induce EADs in hypertrophic cardiomyocytes. We also found that the use of KN-93 significantly reduced the incidence of EADs. This suggests that the incidence of EADs in hypertrophic cardiomyocytes is closely related to the increase in the activity of CaMK Ⅱ. In addition, we found that the amplitude of $I_{Ca,\ L}$ in hypertrophic cardiomyocytes was significantly higher than that in normal myocardial cells, but the current density obtained by comparing the amplitude with their respective membrane capacitance showed no significant difference between the two cell types. KN-92 and KN-93 showed direct inhibitory effects on the $I_{Ca,\ L}$ of hypertrophic cardiomyocytes, and at the higher concentration tested the effects of KN-93 were greater than KN-92. Based on these results, we speculate that at low doses, KN-93 inhibits the occurrence of EADs in hypertrophic cardiomyocytes by reducing CaMK Ⅱ activity. However, at higher concentrations (1 μmol/L), the greater inhibition of $I_{Ca,\ L}$ by KN-93 and its stronger inhibitory effects on EADs may result from a direct effect of KN-93 on calcium channel as well as on CaMK Ⅱ activity.

Undoubtedly, there are many reasons for the occurrence of hypertrophy-induced ventricular arrhythmias, and multiple interacting factors may be important. However, in our study, the Ca^{2+}/CaMK Ⅱ signal transduction pathway played an important role in the genesis of EADs, and this pathway might be a novel target for the treatment of ventricular arrhythmias caused by EADs. In addition, understanding the mechanisms of arrhythmia at the signal transduction level could provide an effective gateway for the clinical treatment of arrhythmia in patients with myocardial hypertrophy.

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