

Original Article

Cardiac ablation of Rheb1 reduces sodium currents in infant mice

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Abstract: Objective: The Ras homolog enriched in brain gene (Rheb) is a center player within the insulin/Rheb/Mammalian Target of Rapamycin (mTOR) pathway, and plays a critical role in regulating cellular growth. Rheb^{-/-} embryos have been reported to die around midgestation, due to the defects of the development of the cardiovascular system. Recent studies from ours and another group consistently showed that Rheb1 was indispensable for the cardiac hypertrophic growth after early postnatal period. Besides that, we also found that Rheb1 a-MHC-Cre (cKO) mice exhibited ventricular tachycardia. However, the precise mechanism by which Rheb1 knockout causes ventricular arrhythmia in these mice is still unclear. Methods: Mouse cardiomyocytes were isolated using 10 days suckling Rheb1 cKO and wide type mice using Collagenase Type II. Sodium currents and L-type calcium currents were recorded using the whole-cell patch clamping technique. Results: The sodium current density of ventricular cardiomyocytes from Rheb1 cKO mice was decreased by about 60%. Significant left shift but no slope altered was observed in activation curve with $V_{1/2}$ values of -35.35 ± 1.12 mV for Rheb1 cKO group and -40.72 ± 1.18 mV for the controls. In addition, the area of window current, which refers the overlap of normalized activation and inactivation, was larger in Rheb1 cKO mice. Moreover, the sodium current, in general, was recovered much slower in Rheb1 cKO mice than that of the controls. However, L-type calcium currents were preserved in Rheb1 cKO mice. Conclusion: Sodium currents are decreased in Rheb1 cKO mice, which might be responsible for the phenotype of arrhythmia in Rheb1 cKO mice. Understanding the molecular composition of sodium ion channel complexes in the heart of these Rheb1 cKO mice will be critical to develop innovative and effective therapies for the treatment of cardiac arrhythmia.

Keywords: Rheb1, Arrhythmia, sodium currents, L-type calcium currents, patch clamp

Introduction

The Ras homolog enriched in brain gene (Rheb), a member of the Ras superfamily of G-proteins, is a center player in the insulin/Rheb/Mammalian Target of Rapamycin (mTOR) pathway, and plays a critical role in regulating cellular growth [1-3]. The insulin/Rheb1/mTOR pathway is activated by mTORC1 and responses to diverse protein synthesis and growth signals such as nutrients, energy, growth factors and even stress [4, 5]. Rheb1 contributes to the activation of mTORC1 but not mTORC2 [4, 5]. As a protein conserved from yeast to human, Rheb1 is ubiquitously expressed in mammalian and is highly enriched in brain, skeletal muscle and cardiac muscle [2, 6].

In adult cardiomyocytes, Rheb can activate growth and lead to cardiac hypertrophy via increasing mTORC1 activity [1, 3, 6]. Our previous study has shown that pharmacological inhibition of Rheb1-mTORC1 signaling protected the heart against adverse cardiac remodeling including myocardial infarction and hypertrophic cardiomyopathy [6]. Rheb^{-/-} embryos have been reported to die around midgestation, due to the defects of the development of the cardiovascular system [4]. In addition, Rheb1 is identified to be necessary for mTORC1 and myelination in postnatal brain development [3]. Recent studies from ours and another group consistently showed that Rheb1 was indispensable for the cardiac hypertrophic growth after early postnatal period [2, 5]. Besides that, we also

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found that Rheb1 a-MHC-Cre (cKO) mice exhibited several types of electrocardiographic abnormalities including P wave flattening, conduction block and ventricular tachycardia [2]. Importantly, ventricular arrhythmia only occurred in Rheb1 cKO mice but not in the control mice [2]. However, the precise mechanism by which Rheb1 knockout causes ventricular arrhythmia in these mice is still unclear [2].

Cardiac action potentials are formed and propagated through the coordinated activation of multiple ion channels whose functions are tightly regulated [7, 8]. The dysregulated function of any of these channels may disrupt action potential conduction, leading to arrhythmia [8, 9]. Among them, the voltage-gated sodium (Na) channels are responsible for generation of the rapid upstroke of the action potential, being central to the development of ventricular arrhythmias [10-14]. Moreover, increased Ca²⁺ influx via voltage-gated L-type Ca²⁺ channels has also been implicated in the development of arrhythmia in cardiac pathological remodeling [15-17]. However, whether the sodium currents and L-type calcium currents are regulated in these Rheb1 cKO mice is still unknown.

In the present study, we aim at investigating the electrophysiological basis for the ventricular arrhythmia caused by Rheb1 knockout.

Materials and methods

Generation of Rheb1 cKO mice

This study was approved by the ethical committee of Nanjing Medical School and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996). The Rheb1 cKO mice was generated as our previous described [2] and both the mutant mice and the wide-type mice of C57BL/6 genetic background were housed with 12 h dark/light cycles and with free access to food and water.

Mouse cardiomyocytes isolation

Mouse cardiomyocytes were isolated using 10 days suckling mice as previous described with some minor modifications [2, 18]. In brief, mice were killed by cervical dislocation, and hearts were quickly placed in Tyrode's solution containing NaCl 130 mM, KCl 5.4 mM, NaH₂PO₄

1.8 mM, MgSO₄ 1.2 mM, HEPES 5 mM, Taurine 20 mM, glucose 10 mM, CaCl₂ 1.8 mM (pH 7.3) at 4°C. Then the aorta was cannulated onto a modified Langendorff perfusion system quickly for coronary perfusion. The hearts were perfused with Tyrode's solution at 37°C for 1 min and then followed by Ca²⁺ free Tyrode's solution at a slow rate of 2 ml/min. After 5 minutes, Ca²⁺ free Tyrode's solution containing 150 U/ml Collagenase Type II (Worthington, Lakewood, USA) was followed for another 7-9 minutes until the hearts were palpably flaccid. After that, ventricles were cut into small pieces and gently agitated, and the cardiomyocytes were kept in KB solution containing L-glutamic acid 70 mM, KOH 50 mM, KCl 40 mM, taurine 20 mM, KH₂PO₄ 20 mM, MgCl₂ 3 mM, EGTA 0.5 mM, D-glucose 10 mM, HEPES 10 mM (pH 7.3) at room temperature for patch clamp experiment.

Patch clamp recording

Cardiomyocytes were transferred to a chamber perfused with bath solution at a constant rate of 1 ml/min. Pipettes were pulled by Pipette Puller Model P-1000 (Sutter instrument Co, USA). Currents recording was performed with an Axopatch 200B amplifier (Molecular Devices, Union City, CA). To minimize voltage errors due to series resistance, pipettes had resistance of 1-2 MΩ, and capacitance and series resistance were adjusted to 85%. Data expressing large series resistance (> 15 MΩ) and Tau constants (> 1.5 ms) were rejected. All the experiments were implemented at room temperature (20-22°C).

Current recording and pulse protocols

The biophysics characteristics of sodium currents were recorded according to voltage-clamp protocols [19]. For the recording of sodium currents, the bath solution contained NaCl 5 mM, Choline Chloride 130 mM, CaCl₂ 1.8 mM, MgCl₂ 1.8 mM, TEA-Cl 1 mM, HEPES 10 mM, glucose 10 mM (pH 7.4). The pipettes solution contained CsOH 130 mM, Aspartic acid 130 mM, NaCl 2.5 mM, CaCl₂ 1 mM, MgCl₂ 1 mM, EGTA 10 mM, TEA-Cl 10 mM, HEPES 10 mM (pH 7.4). To eliminate L/T type calcium currents, nisoldipine 0.5 μM and NiCl₂ 0.2 mM were added. Nisoldipine was dissolved in dimethyl sulfoxide (DMSO, the final concentration was less than 0.2% in the experiments) to make a stock solution of 10 mmol/L. To record L-type calcium cur-

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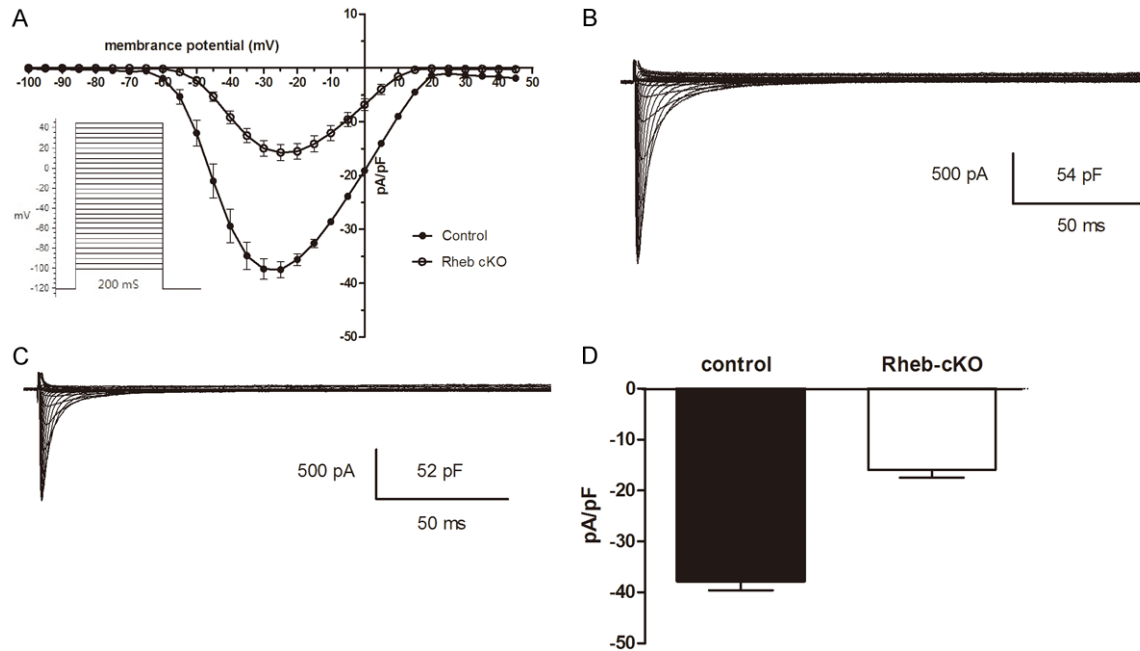


Figure 1. Sodium currents are decreased in Rheb1 cKO mice. (A) Peak current-voltage curves (B) and (C) Sodium current traces of the control and Rheb1 knockout cardiomyocytes, respectively (D) Current density.

rents, solutions were prepared as previous described [16]. The external solution contained CaCl_2 2 mM, MgCl_2 1 mM, tetraethylammonium chloride 135 mM, 4-aminopyridine 15 mM, glucose 10 mM, and HEPES 10 mM (pH 7.3). The pipette solution contained cesium aspartate 100 mM, CsCl 20 mM, MgCl_2 1 mM, Mg-ATP 2 mM, GTP 0.5 mM, EGTA 5 mM, and HEPES 5 mM (pH 7.3). These solutions distinguished the L-type calcium currents from other membrane currents and the $\text{Na}^+\text{-Ca}^{2+}$ exchanger. After cell membrane was ruptured, cell membrane capacitance was specifically calculated by integrating the capacitive transient current in response to a 5-mV depolarizing pulse. For the recording of L-type calcium currents, pre-pulse (-40 mV, 300 ms) was executed to exclude fast sodium current and T-type calcium currents. Details of pulse protocols were given in Figures. Before any recording, at least 5 minutes were permitted for cell dialysis in each experiment.

The kinetics of steady-state voltage dependence of activation, inactivation and recovery from inactivation of sodium channels were fitted as previous described [10]. In brief, the cell was held at a holding potential of -120 mV, and was depolarized to a test potential of +45 mV (step width 5 mV, step duration 200 ms) for probing the activation of sodium currents.

Meanwhile, the maximum current was used to calculate the current density. The steady-state voltage dependence of inactivation was determined by a 200 ms pre-pulse between -100 and +25 mV (in 5 mV intervals), and was followed by depolarization to -30 mV.

To determine the time dependent recovery from inactivation, a twin-pulse protocol was applied. Cells were depolarized to -30 mV for 50 ms followed by repolarizing to -120 mV for a variable periods, then followed by a test pulse to -30 mV to measure current amplitude. At each pulse, the peak sodium current was normalized to the pre-pulse current.

Statistical analysis

For patch clamping experiments, data were analyzed by utilizing pCLAMP v10.3 (Molecular Devices, Union City, CA), and the figures were generated by using GraphPad Prism v5.0 (GraphPad Software, Inc, USA). Steady-state activation or inactivation were fit with the Boltzmann equation, $I/I_{\text{max}} = [1 + \exp((V - V_{1/2})/k)]^{-1}$, where $V_{1/2}$ was half-maximal activation or inactivation, and k was slope. Recovery from inactivation was matched with two-exponential function: $I/I_{\text{max}} = A_f \times [1 - \exp(-t/\tau_f)] + A_s \times [1 - \exp(-t/\tau_s)]$, where t is the recovery time interval and τ refer to

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amplitudes and time constants. Results were presented as mean \pm SEM. Statistical comparisons were made using the student's test. *P* values less than 0.05 were considered to be statistically significant.

Results

Sodium currents are decreased in Rheb1 cKO mice

To determine sodium current density in cardiomyocytes from Rheb1 cKO mice and wide-type mice, whole cell patch clamp recording was made on freshly isolated ventricular cardiomyocytes. Representative sodium current traces were shown in **Figure 1B** and **1C**, indicating that Rheb1 knockout cardiomyocytes exhibited smaller sodium current density. As indicated in **Figure 1**, the sodium current density of ventricular cardiomyocytes from Rheb1 cKO mice was decreased by about 60%.

To further investigate the effects of Rheb1 knockout in the activation and inactivation kinetics of sodium channels in ventricular cardiomyocytes, 30 test pulses from -100 mV to +45 mV at a holding potential of -120 mV were stimulated and the curves were fitted by Boltzmann function. **Figure 2A** shows the normalized activation and inactivation curves of sodium currents. Significant left shift but no slope altered was observed in activation curve with $V_{1/2}$ values of -35.35 ± 1.12 mV for Rheb1 cKO group and -40.72 ± 1.18 mV for the controls (**Table 1**). However, no obvious changes were observed in fitted steady-state voltage dependence of inactivation curves (**Table 1**). **Figure 2B** showed that the area of window current, which refers the overlap of normalized activation and inactivation, was larger in Rheb1 cKO mice, suggesting that the persistent component of sodium current was increased, which would increase sodium conductance over a wide range of cardiac membrane potentials.

To determine the effects of Rheb1 knockout in the time dependent recovery from inactivation, a twin-pulse protocol was used. Followed by repolarization -120 mV for a variable period, cardiomyocytes were depolarized to -30 mV for 50 ms to measure current status. The recovery from inactivation curves were fitted by a double exponential function as shown in **Figure 2C**. The sodium current, in general, was recovered

much slower in Rheb1 cKO mice than that of the controls. In cardiomyocytes from Rheb1 cKO mice, the time parameters of fast (τ_1) and slow (τ_2) time component were 11.59 ± 1.00 ms and 86.46 ± 4.04 ms respectively, compared to 6.90 ± 0.49 ms and 73.02 ± 1.44 ms in the controls (**Table 1**).

L-type calcium currents are preserved in Rheb1 cKO mice

As Rheb-cKO mice has the phenotype of heart failure, we further explored the general properties of L-type calcium channels in these mice. A slight increase of L-type calcium currents was found in Rheb-cKO mice, but the changes, even the maximum current from every test pulse, did not achieve statistical significance (**Figure 3**). These results indicate that L-type calcium currents are preserved in Rheb-cKO mice.

Discussion

Previously we have reported that Rheb1 cKO mice led to heart failure, ventricular arrhythmia and premature death at infant stages [2]. However, little is known about the electrophysiological basis of ventricular arrhythmia caused by Rheb1 knockout. The major findings of the present study are as follows. Firstly, sodium current density of ventricular cardiomyocytes from Rheb1 cKO mice is decreased. Secondly, the persistent component of sodium current is increased in ventricular cardiomyocytes from Rheb1 cKO mice. Thirdly, the sodium current, is recovered much slower in Rheb1 cKO mice. Lastly, L-type calcium currents are preserved in Rheb1 cKO mice. Overall, to the best of our knowledge, here we firstly show that sodium currents are decreased in Rheb1 cKO mice, which might be responsible for the phenotype of arrhythmia observed in Rheb1 cKO mice.

Voltage-gated sodium channels contribute to the generation and propagation of cardiac action potentials [9, 20]. Voltage-gated sodium channels are composed with heteromeric of an α -subunit and one or two ancillary β -subunits [13, 14, 21]. Being one of the center players responsible for the genesis of cardiac arrhythmia, sodium currents were paid special attention in the present study [9]. A large number of sodium channel mutations have been identified in multiple arrhythmia syndromes, includ-

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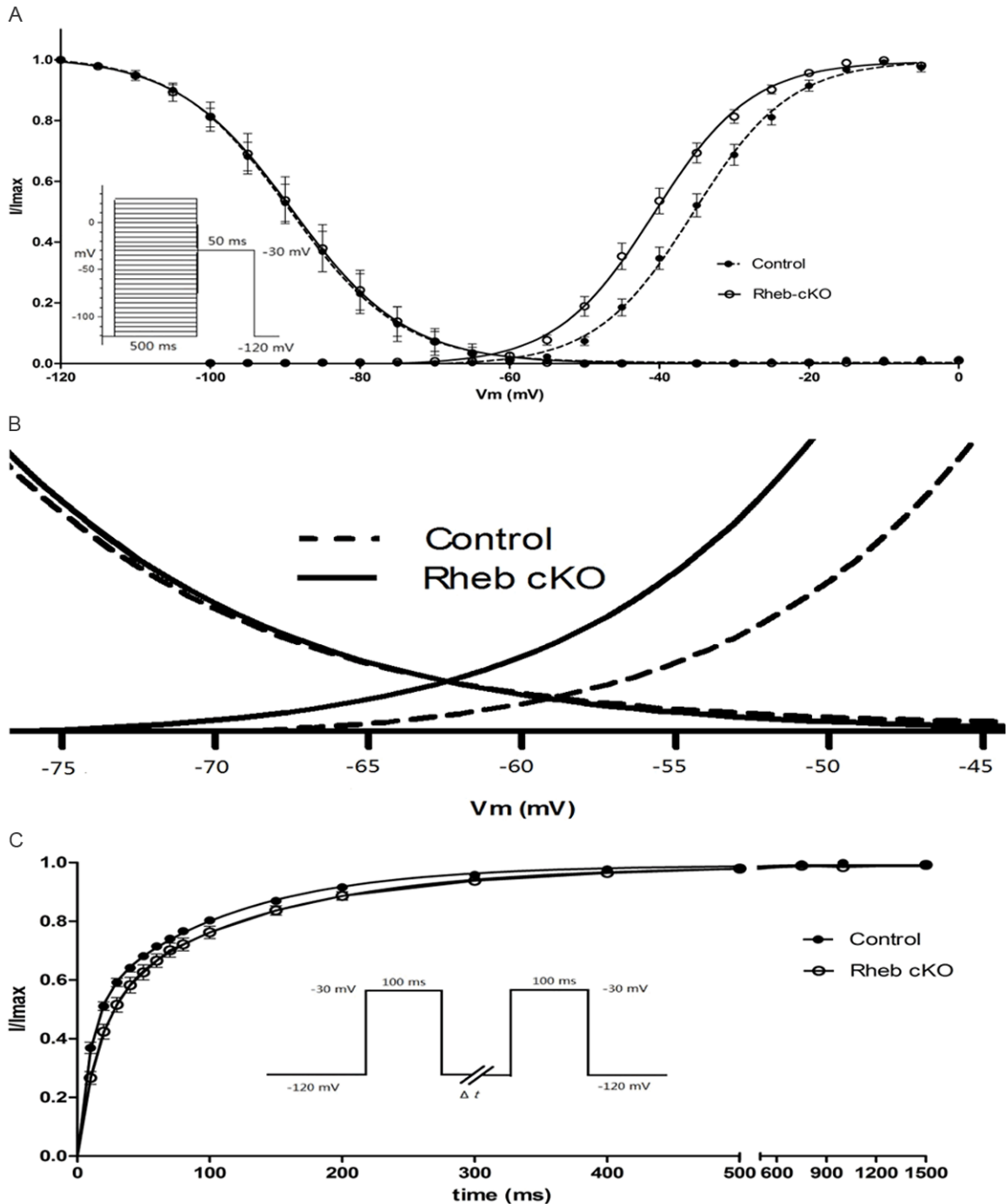


Figure 2. Different kinetics of sodium channels between controls and Rheb cKO mice. (A) Inactivation curves (B) Window currents (C) Time course of recovery for inactivation.

ing congenital Long QT syndrome type 3 (LQTS3), idiopathic ventricular fibrillation, Brugada's syndrome, sick sinus syndrome and progressive cardiac conduction defect [9, 12-14, 22]. Nav1.5, a canonical cardiac sodium channel, is encoded by SCN5A and its blockers can decrease sodium currents in cardiomyocytes

and thereafter decrease conduction velocity in the heart, which can be antiarrhythmic but can also engage reentrant circuits and provoke arrhythmia [9, 11-13, 20]. Interestingly, we found an increase in the window current in cardiomyocytes from Rheb1 cKO mice, which can increase sodium conductance over a wide

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Table 1. Sodium channel characteristics in Rheb-cKO and wild type mice

	Voltage dependence of activation		Voltage dependence of inactivation		Recovery from inactivation		n	N
	$V_{1/2}$ (mV)	K	$V_{1/2}$ (mV)	K	τ_1 (ms)	τ_2 (ms)		
Control	-40.72 ± 1.18	6.37 ± 0.12	-88.80 ± 2.4	6.90 ± 0.13	6.90 ± 0.49	73.02 ± 1.44	13	3
Rheb-cKO	$-35.35 \pm 1.12^{**}$	6.33 ± 0.23	-89.43 ± 0.64	6.77 ± 0.23	$11.59 \pm 1.00^{**}$	$86.46 \pm 4.04^*$	16	4

Values presented are mean \pm SEM *P < 0.05; **P < 0.01 (vs. control, student's t-test). N means the number of mice while n means the number of cells.

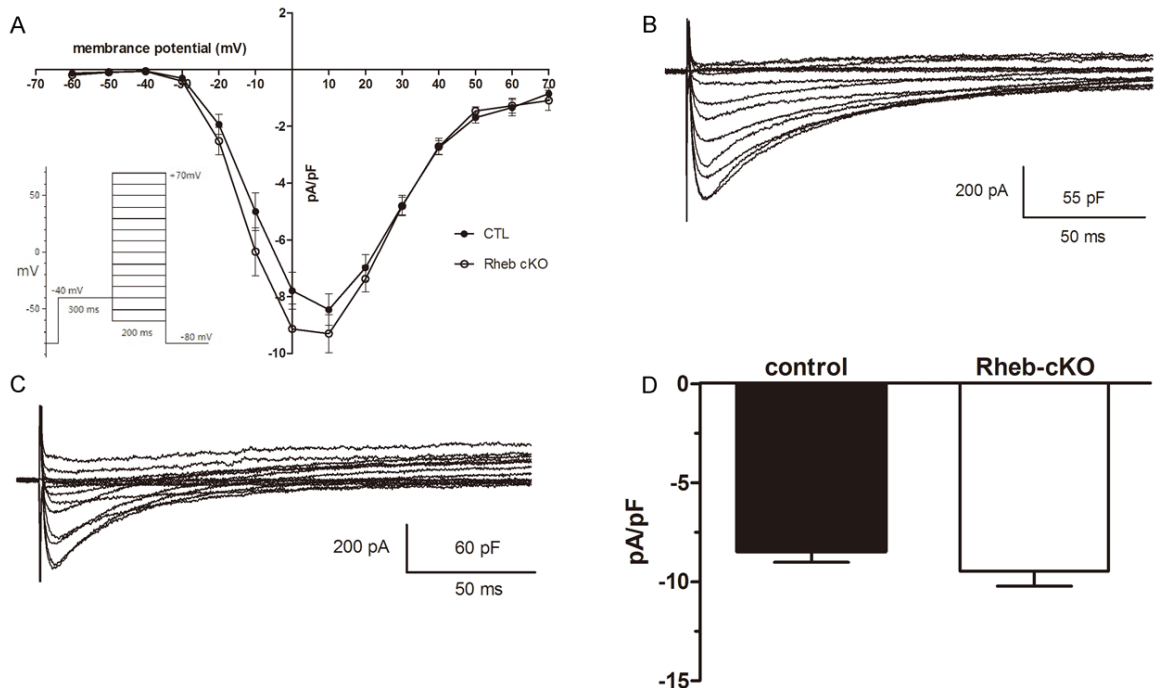


Figure 3. L-type calcium currents are preserved in Rheb1 cKO mice. (A) Peak current-voltage curves (B) and (C) L-type calcium current traces of the control and Rheb1 knockout cardiomyocytes, respectively (D) Current density. Currents were recorded from -60 to 70 mV in 10-mV increments after a pre-pulse (-40 mV, 300 ms). The holding potential was -80 mV.

range of cardiac membrane potentials [9, 12, 13]. Thus, the reduced sodium currents observed in our Rheb1 cKO mice at least partly contribute to the ventricular arrhythmia phenotype in these mice.

Increased Ca^{2+} influx via voltage-gated L-type Ca^{2+} channels has been implicated in the genesis of arrhythmia in cardiac pathological hypertrophy and heart failure [9, 15, 17, 23]. Voltage-gated L-type Ca^{2+} channels are the primary sources of Ca^{2+} influx to initiate cardiac excitation-contraction coupling. The reduced excitation-contraction coupling in heart failure is well-known [9, 17, 23]. Therefore, we determined if L-type calcium currents might also contribute to the genesis of arrhythmia observed in

our Rheb1 cKO mice. Interestingly, we found that L-type calcium currents were preserved in our Rheb1 cKO mice, indicating that L-type calcium currents might not contribute to the ventricular arrhythmia phenotype in these mice. In addition, the ventricular arrhythmia phenotypes in these Rheb1 cKO mice are unlikely to be solely the consequence of heart failure phenotype.

In conclusion, we have found that sodium currents are decreased in Rheb1 cKO mice, which might be responsible for the phenotype of arrhythmia in Rheb1 cKO mice. Understanding the molecular composition of sodium ion channel complexes in the heart of these Rheb1 cKO mice will be critical to develop innovative and

effective therapies for the treatment of cardiac arrhythmia.

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Disclosure of conflict of interest

None.

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