Int J Clin Exp Med 2015;8(4):5485-5491
www.ijcem.com /ISSN:1940-5901/IJCEM0005013

Original Article

Prolonged action potential duration in cardiac ablation of *PDK1* mice

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Received December 18, 2014; Accepted February 25, 2015; Epub April 15, 2015; Published April 30, 2015

Abstract: The involvement of the AGC protein kinase family in regulating arrhythmia has drawn considerable attention, but the underlying mechanisms are still not clear. The aim of this study is to explore the role of 3-phosphoinositide-dependent protein kinase-1 (*PDK1*), one of upstream protein kinases of the AGC protein kinase family, in the pathogenesis of dysregulated electrophysiological basis. *PDK1*$^{F/F}$ αMHC-Cre mice and *PDK1*$^{F/F}$ mice were divided into experiment group and control group. Using patch clamping technology, we explored action potential duration in both groups, and investigated the functions of transient outward potassium channel and L-type Ca^{2+} channel to explain the abnormal action potential duration. Significant prolongation action potential duration was found in mice with *PDK1* deletion. Further, the peak current of transient outward potassium current and L-type Ca^{2+} current were decreased by 84% and 49% respectively. In addition, dysregulation of channel kinetics lead to action potential duration prolongation further. In conclusion, we have demonstrated that *PDK1* participates in action potential prolongation in cardiac ablation of *PDK1* mice. This effect is likely to be mediated largely through downregulation of transient outward potassium current. These findings indicate the modulation of the *PDK1* pathway could provide a new mechanism for abnormal electrophysiological basis.

Keywords: *PDK1*, action potential duration, transient outward potassium current, L-type Ca^{2+} current, patch clamp

Introductions

AGC (protein kinase A, protein kinase G and protein kinase C) protein kinase family has an established role in regulating physiological processes relevant to metabolism, growth, proliferation and survival [1]. 3-phosphoinositide-dependent protein kinase-1 (*PDK1*), one of upstream protein kinases of the AGC protein kinase family, phosphorylates and actives downstream factors, including protein kinase B (PKB)/Akt [2], p70 ribosomal S6 kinase (S6K) [3], serum and glucocorticoid-induced protein kinase (SGK) [4]. Recently, *PDK1* [5] and its upstream factor phosphatidyl inositol 3-kinase (PI3K) [6], are known to involved in cardiac remodeling and heart failure.

In the clinic, arrhythmia is one of the primary causes of sudden death in patients with heart failure [7]. Although the underlying mechanisms between heart failure and arrhythmia remain unclear, the most consistent electrophysiological changes in the ventricles are prolongation of the action potential (AP) [8]. And it is well accepted that dysregulated function of K^+ and Ca^{2+} current may disrupt AP conduction [9]. Especially, the involvement of the AGC protein kinase family in regulating arrhythmia has drawn considerable attention. Moreover, mice with *PDK1* deletion lead to heart failure with markedly reduced myocardium, smaller cardiomyocytes, thinner ventricles and enlarged atria [10]. And previous study suggested that drug-induced increases in action potential induce changes in multiple ion currents and were accompanied by decreased PI3K signaling [11]. Thus, the potential relationship between *PDK1* and the generation of arrhythmia warrants further investigation. To investigate new mechanism of action potential prolongation in the AGC protein kinase family, we aim at the effect of *PDK1* deletion on transient outward potassium current and L-type Ca^{2+} current with patch

clamp technique in a conditional deletion of *PDK1* mouse.

Materials and methods

Generation of PDK1 conditional deletion mice

Tissue-specific "knockout" approaches using the "Cre-Loxp" system were used to delete *PDK1* in the myocardium. *PDK1*-floxed mice were generated as previous described [12]. In brief, *PDK1*-floxed mice ($PDK1^{F/F}$) were crossed with αMHC (α-myosin heavy chain)-Cre mice to delete *PDK1* in cardiomyocyte [10]. Deletion of *PDK1* in the myocardium was confirmed and showed in **Figure 1** by Western blot analysis [13], and $PDK1^{F/F}$ littermates without the αMHC-Cre transgene were housed as control. This study was approved by the ethical committee of the First Affiliated Hospital of Nanjing Medical University, and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institute of Health (No. 85-23, revised in 1996).

Mouse cardiomyocytes isolation

Single ventricular myocytes were obtained by traditional enzymatic dissociation. Mice (8 weeks) were sacrificed by cervical dislocation. The hearts were perfused with Tyrode's solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 5 mM HEPES, 20 mM taurine, 10 mM glucose, and 1.8 mM $CaCl_2$ (pH 7.3) at 36°C for 1 min, followed by Ca^{2+} free Tyrode's solution at a reduced rate of 3 ml/min. After 5 minutes, Ca^{2+} free Tyrode's solution containing 150 U/ml Collagenase Type II (Worthington, Lakewood, USA) was applied for another 12 minutes until the hearts were palpably flaccid. Next, the ventricles were minced and gently agitated, and the cardiomyocytes were placed in KB solution containing 70 mM L-glutamic acid, 50 mM KOH, 40 mM KCl, 20 mM taurine, 20 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 10 mM D-glucose, and 10 mM HEPES (pH 7.3) at room temperature for patch clamp experiments.

Soultions

Cells were transferred to a chamber (Warner Instrument Co, USA) and perfused with bath solution at a constant rate of 1 ml/min. For the recording of transient outward potassium current, the bath solution contained 135 mM Choline chloride, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, 10 mM Glucose and 3 mM $CoCl_2$. The pipette solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 10 mM EGTA and 5 mM Na_2ATP. For the recording of L-type Ca^{2+} currents, the bath solution contained 120 mM Choline chloride, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 20 mM CsCl and 10 mM Glucose. The pipette solution contained 130 mM CsCl, 20 mM TEA-Cl, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM EGTA and 5 mM Na_2ATP. For AP recording, cells were perfused with Tyrode's solution and pipette solution contained: 120 mM Aspirate K, 25 mM KCl, 5 mM Na_2ATP, 1 mM $CaCl_2$, 10 mM EGTA and 5 mM HEPES. Pipettes (Sutter Instrument Co, USA) were pulled with a resistance of 1-2 MΩ. Current recording was performed with an Axopatch 200B amplifier (Molecular Devices, Union City, CA) and a Digidata 1440A (Molecular Devices, Union City, CA). All chemicals were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Data expressing large series resistance (> 10 MΩ) were rejected.

Voltage recording

Biophysical characteristics of the transient outward potassium current and L-type Ca^{2+} current were recorded using modified voltage-clamp protocols [14]. To explore transient outward potassium current, the cell was held at a holding potential of -40 mV, depolarized to a test potential of +70 mV (step width 10 mV, step duration 500 ms). Steady-state voltage dependence of inactivation was characterized using a 1000 ms prepulse between -100 mV and 0 mV (in 10 mV intervals), followed by depolarization to +50 mV. To explore the activation of the L-type Ca^{2+} current, the cell was held at a holding potential of -60 mV, depolarized to a test potential of +70 mV (step width 10 mV, step duration 500 ms). Steady-state voltage dependence of inactivation was characterized using a 1000 ms prepulse from -100 mV to +20 mV (in 10 mV intervals), followed by depolarization to 0 mV.

Current recording

Action potential duration (APD) recording were studied in current-clamp mode by threshold current pulse of 5 ms duration at the frequency. APD_{90} was measure at 90% repolarization.

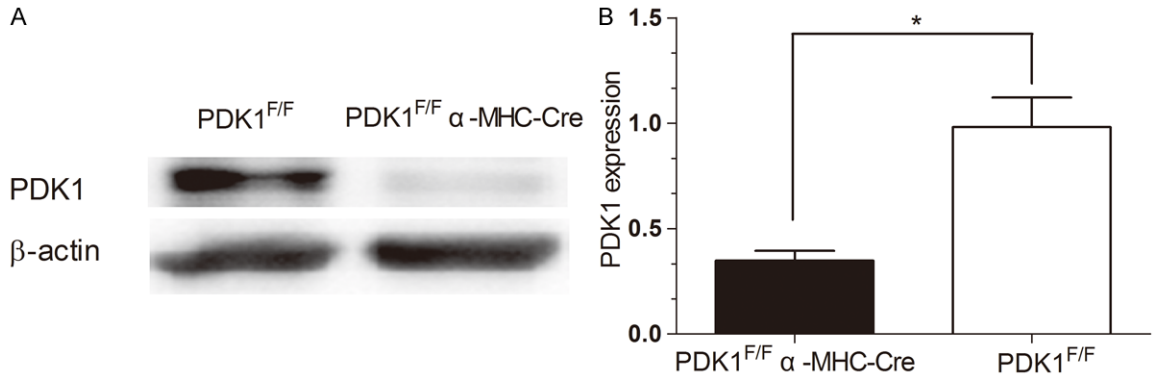

Figure 1. Generation of cardiomyocyte-specific *PDK1* deletion mice. (A) Western blot analysis of PDK1 expression in hearts from 3 *PDK1*$^{F/F}$ mice and 3 representative *PDK1*$^{F/F}$ αMHC-Cre knockout mice. β-actin was tested as a loading control. (B) Summarized data are expressed as +SEM values. **P* < 0.05.

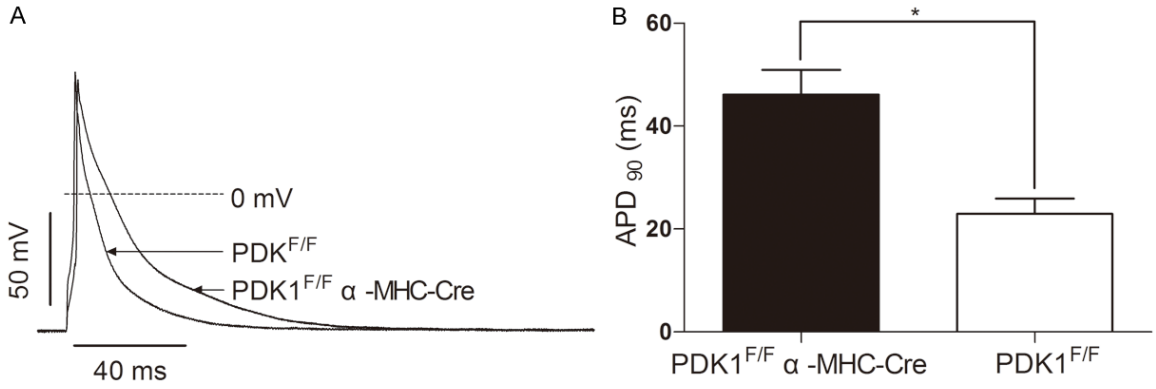

Figure 2. Effects of PDK1 deletion on action potential duration (APD). A. APD curves in control and PDK1 deletion group; B. Significantly prolonged ADP_{90} in *PDK1*$^{F/F}$ αMHC-Cre cells. **P* < 0.05.

Data analysis

All data were analyzed using pCLAMP v10.3 (Molecular Devices, Union City, CA). Steady state activation and inactivation were fit with the Boltzmann equation, G/G_{max} or I/I_{max} = [1+exp ((V-$V_{1/2}$)/k)]-1, where $V_{1/2}$ is potential for half-maximal (in)activation, and k is the slope factor. Data are expressed as means ± SEM. For statistical comparisons, the Student's t t-test was applied. Differences were considered statistically significant if $P < 0.05$.

Results

Action potential duration prolonged in PDK1 deletion mice

The prolongation of APD is established to play a role in the development of cardiac arrhythmia. And APD is dependent on the balance between depolarizing inward and repolarizing outward current. We therefore examined the effects of *PDK1* deletion on APD in ventricular myocytes. **Figure 2A** shows the traces of AP in *PDK1*-deletion and control group. APD at 90% repolarization was significantly longer in the *PDK1*-deletion cells than the control cells (**Figure 2B**, 46.08 ± 4.81, n = 10 vs. 22.95 ± 2.95 ms, n = 11; $P < 0.05$).

PDK1 deletion reduces the transient outward potassium current density and dysregulates channel kinetics

The transient outward potassium current is the major repolarizing outward current in mice. The suppression of outward potassium currents lead to the prolongation of APD. In **Figure 3C**, peak value of transient outward potassium cur-

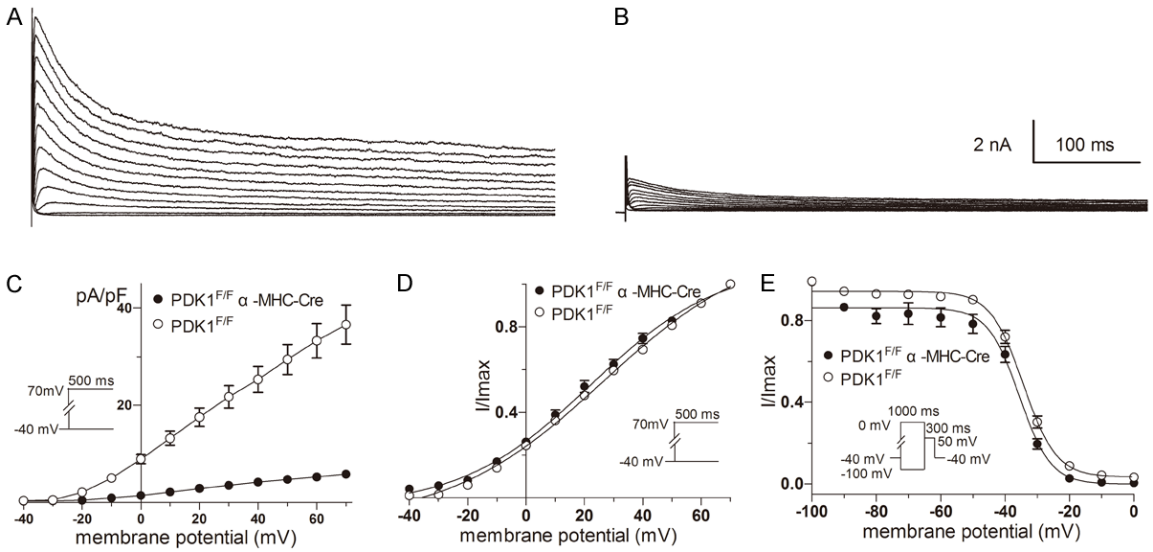

Figure 3. Dysregulation of transient outward potassium channel in *PDK1*$^{F/F}$ αMHC-Cre cells. A, B. Transient outward potassium current curves in control and *PDK1* deletion group; C. Deacreased transient outward potassium current in *PDK1* deletion cells; D, E. Voltage dependence of activation and inactivation of curves in control and *PDK1* deletion group.

Table 1. Biophysical parameters for transient outward potassium current

	Voltage dependence of activation			Voltage dependence of inactivation		
	$V_{1/2}$ (mV)	K	n	$V_{1/2}$ (mV)	K	n
PDK1$^{F/F}$	27.30 ± 2.41	26.44 ± 1.09	10	34.43 ± 0.82	-5.10 ± 0.32	15
PDK1$^{F/F}$ αMHC-Cre	27.17 ± 2.70	22.09 ± 1.38*	12	36.16 ± 0.95	-6.87 ± 1.83	7

*$P < 0.05$, compared with *PDK1*$^{F/F}$ group.

rents at +70 mV was 5.77 ± 0.68 pA/pF in the *PDK1* deletion cells (n = 12) and 36.60 ± 4.02 pA/pF in normal cells (n = 10). Thus the current amplitude was decreased by about 84% in *PDK1* deletion mice. To further investigate the gating characteristics, voltage-dependent activation/inactivation were examined. As shown in **Figure 3D**, fitting curve of steady-state activation is left-shifted for *PDK1*-deletion mice. For the *PDK1* knockout (n = 12) vs. control groups (n = 10), the $V_{1/2}$ for activation was 23.17 ± 2.70 mV vs. -27.30 ± 2.41 mV and the slope was 22.09 ± 1.38 vs. 26.44 ± 1.09 ($P < 0.05$, **Table 1**). Significant difference was observed in the change of slope, but the $V_{1/2}$ of the Boltzmann curves did not change significantly. For voltage-dependent inactivation (**Figure 3E**), the same trend of variation between the *PDK1* deletion (n = 7) and control (n = 15) groups (**Table 1**) was detected, and the $V_{1/2}$ for inactivation was -36.16 ± 0.95 mV vs. -34.43 ± 0.82 mV and the slope was -6.87 ± 1.83 vs. -5.10 ± 0.32. Though slight change was in the change of slope, these results suggested that the transient outward potassium was significantly inhibited by *PDK1* deletion.

PDK1 deletion reduces the L-type Ca^{2+} current density and dysregulates channel kinetics

L-type Ca^{2+} current is responsible for generation of the plateau of the action potential. Moreover, voltage-gated L-type Ca^{2+} channels have also been implicated in the development of arrhythmia and cardiac pathological remodeling. In **Figure 4C**, peak value of inward Ca^{2+} currents at +10 mV was 4.56 ± 0.38 pA/pF in the *PDK1* deletion cells (n = 10) and 8.91 ± 0.58 pA/pF in normal cells (n = 13). Compared to control cells, the L-type Ca^{2+} current is decreased by about 49% ($P < 0.05$) after *PDK1* deletion at 8 weeks. The dysregulated function of channel kinetics has also been implicated in Ca^{2+} influx. Next, we investigated the effect of *PDK1* on voltage-dependent activation/inactivation of L-type Ca^{2+} channels. As shown in **Figure 4E**, *PDK1* deletion induced hyperpolar-

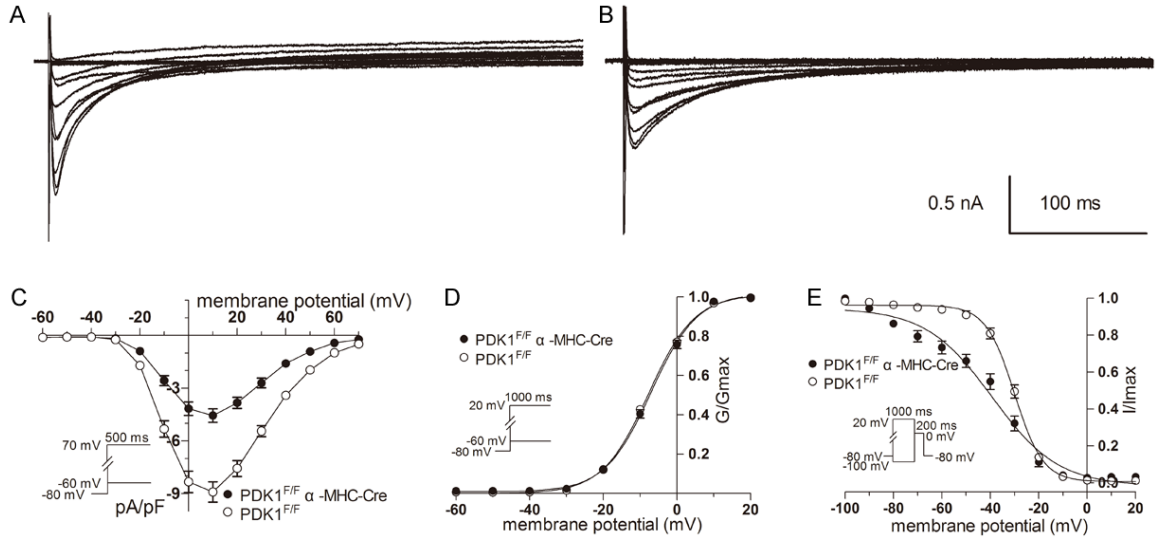

Figure 4. Downregulation of L-type Ca^{2+} Current channel in *PDK1*$^{F/F}$ αMHC-Cre cells. A, B. L-Ca^{2+} type current curves in control and PDK1 deletion group; C. Deacreased L-Ca^{2+} type current in PDK1 deletion cells; D, E. Voltage dependence of activation and inactivation of curves in control and PDK1 deletion group.

Table 2. Biophysical parameters for L-type Ca^{2+} current

	Voltage dependence of activation			Voltage dependence of inactivation		
	$V_{1/2}$ (mV)	K	n	$V_{1/2}$ (mV)	K	n
PDK1$^{F/F}$	7.01 ± 0.65	6.23 ± 0.16	9	-30.33 ± 0.82	-5.87 ± 0.28	11
PDK1$^{F/F}$ αMHC-Cre	7.70 ± 0.57	6.21 ± 0.11	12	-40.90 ± 3.06*	-13.67 ± 1.73	8

*$P < 0.05$, compared with *PDK1*$^{F/F}$ group.

izing shifts in the voltage-dependent channel inactivation. Exactly, compared to control group, the $V_{1/2}$ of inactivation was -40.90 ± 3.06 mV (n = 8) vs. -30.33 ± 0.82 mV (n = 11, *P < 0.05*) and the slope was -13.68 ± 1.73 vs. -5.87 ± 0.28 (*P* < 0.05, **Table 2**). On the contrary, there were no obvious changes in the biophysical parameters of L-type Ca^{2+} currents for activation (**Table 2**). In detail, the $V_{1/2}$ was -7.01 ± 0.65 mV (n = 9) vs. -7.70 ± 0.57 mV (n = 12) and the slope was -6.23 ± 0.16 vs. -6.21 ± 0.11 (**Table 2**). These result suggested that *PDK1* deletion caused down-regulating of L-type Ca^{2+} channels.

Discussion

Previous studies have found tamoxifen-inducible and heart-specific disruption of *PDK1* in adult mice lead to severe heart failure and sudden death in 11 weeks [5, 10, 15]. In the clinic, arrhythmia is one of the primary cardiovascular events in patients with heart failure [7]. Furthermore, in animal experiment group, the prolongation of APD and QTc interval is one of the most characteristic electrophysiological remodeling in failing heart, which is believed to mainly result from the down regulation of several outward potassium, including transient outward potassium current, I_{Ks} and I_{Kr} [16]. Lu's research reported a mechanism for drug-induced QT prolongation that involved changes multiple potassium channel and significant prolongation of APD [11]. Consistently, *PDK1* is the major downstream regulator of PI3K, we observed prolonged APD in *PDK1*$^{F/F}$ αMHC-Cre mice. The result indicated that deletion of *PDK1* affected certain ion channels, and lead to APD prolongation.

Transient outward potassium current is the major repolarizing outward current in mice and takes part in phase 1 to 3 of APD. In addition, dysregualtion of gating kinetics of channel affect the amount of K^+ outflux. In patch clamp analysis, we found the the current amplitude is decreased by about 84% at +70 mV, which indicated a serious fall in K^+ outflux. Next, we investigated the kinetics of steady-state voltage dependence of activation and inactivation. In

general, compared to control, no statistically significant difference was found in $V_{1/2}$, *PDK1* deletion slowed down the activation procession, but changes in slope which may lead to decreased K^+ outflux and prolonged APD in further. Thus, the downregulation of outward potassium currents observed in our *PDK1*$^{F/F}$ αMHC-Cre mice at least partly contribute to APD prolongation in these mice.

It is well accepted that plateau phase of cardiac action potential is sustained by an inward movement of Ca^{2+}. 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 [17]. In our research, we found peak L-type Ca^{2+} current was decreased by about 49% at 10 mV. Furthermore, compared to control group, the $V_{1/2}$ for inactivation was -40.90 ± 3.06 mV vs. -30.33 ± 0.82 mV, but no obvious changes was found in voltage-dependent activation. The results indicated a loss of function of L-type Ca^{2+} channel in *PDK1*$^{F/F}$ αMHC-Cre mice. For another, voltage-gated L-type Ca^{2+} channels are the primary sources of Ca^{2+} influx to initiate cardiac excitation-contraction coupling and the reduced excitation-contraction coupling in heart failure [17]. In theory, reduced Ca^{2+} influx can lead to shorten plateau phase, but we found the inhibited amplitude of outward potassium current (84%) was larger than that of inward L-type Ca^{2+} current (49%). Beside, L-Ca^{2+} channels are closed during phase 3, thus the downregulation of L-type Ca^{2+} current could not counteract the effects of seriously reduced outward potassium current on APD prolongation.

PDK1 activates a number of downstream AGC kinases, including Akt [2], p70 ribosomal S6 kinase (p70S6K) [3], and SGK1 [4]. It is unclear if results in our research are related to the reduction in *PDK1* or other changes associated with heart failure or other type outward potassium channels. Previous study suggested that SGK1 stimulates a variety of ion channels, but its effect was modest [18]. In addition, small G proteins are known to regulate a wide variety of ion channels. For example, K-Ras and RhoA increase the activity of the epithelial sodium channel (ENaC) via PI3K and PI(4)P5-kinase signaling pathways [19], and our previous work found sodium currents are decreased in Rheb1 cKO mice, which might be responsible for the phenotype of arrhythmia in Rheb1 deletion mice [20]. In conclusion, we have demonstrated for the first time that PDK1 participates in action potential prolongation in cardiac ablation of *PDK1* mice. Though a loss of function of L-type Ca^{2+} channel is found, this effect is likely to be mediated largely through downregulation of transient outward potassium current. These findings indicate the modulation of the *PDK1* pathway could provide a new mechanism for abnormal electrophysiological basis.

Acknowledgements

This work was supported by the following Grants: National Key Basic Research Program of China (973 Program), No. 2013CB531105, National Natural Science Foundation of China, Project No. 81170159, and The Six Peak Talents Foundation of Jiangsu Province, No. 2012-WS-018 to DWW.

Disclosure of conflict of interest

None.

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