

# Exchange-protein activated by cAMP (EPAC) regulates L-type calcium channel in atrial fibrillation of heart failure model

M.-X. ZHANG, J.-K. ZHENG, W.-W. WANG, F.-O. KONG, X.-X. WU, J.-K. JIANG, J.-X. PAN

Department of Cardiovascular Medicine, The Third Affiliated Hospital of Wenzhou Medical University, Ruian, China.

**Abstract. – OBJECTIVE:** Both atrial fibrillation (AF) and heart failure (HF) are increasingly prevalent and related to high hospitalization rate and mortality. AF is a cause as well as a consequence of HF, with complicated interactions resulting in impairment of cardiac systolic and diastolic function. Conversely, the complex structural and neurohormonal alterations in HF contribute to the occurrence and development of AF. However, the molecular mechanism remains unclear. This study aims to explore the effect of Exchange-protein activated by cAMP 1 (EPAC1) on AF in isoproterenol (ISO)-induced HF and the potential molecular mechanism.

**MATERIALS AND METHODS:** Mice and cultured isolated adult cardiomyocytes were treated with ISO and or not EPAC1 inhibitor CE3F4. Programmed electrical stimulation (PES) was performed to induce AF. EPAC1 expression was determined by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Western blot. Cellular electrophysiology was examined by whole cell patch clamp.

**RESULTS:** Both mRNA and protein levels of EPAC1 were upregulated in HF mice. ISO increased the AF susceptibility, and the negative effect was deteriorated by CE3F4. ISO mediated high AF susceptibility of HF via prolonging action potential and exciting L-type calcium channel (LTCC). These could also be reversed by CE3F4 treatment.

**CONCLUSIONS:** EPAC1 increased the AF susceptibility in ISO-induced HF mouse model via alternating LTCC.

*Key Words:*

EPAC1, Atrial fibrillation, L-type calcium, Heart failure.

## Introduction

Atrial fibrillation (AF) is the most encountered cardiac arrhythmia with an increased risk of

stroke and death<sup>1</sup>. Heart failure (HF) is common in AF patients; meanwhile, AF is also common in HF patients. AF is associated with a three-fold risk for heart failure (HF)<sup>2</sup>. Conversely, the complex structural and neurohormonal alterations in HF contribute to the occurrence and development of AF<sup>3</sup>. Both AF and HF are gradual prevalent and related to high hospitalization rate and mortality<sup>4,5</sup>. HF and AF share common risk factors and pathophysiologic processes, such as smoking, diabetes, hypertension, sleep apnoea, and coronary artery disease<sup>6</sup>. HF patients are in the state of neurohormonal imbalance with excitation of the renin-angiotensin-aldosterone system (RAAS), resulting in pathological changes involving an increase of filling pressures and afterload. These changes cause increased stretch and fibrosis in atrial, contributing to the development and maintenance of AF<sup>7-9</sup>. However, there are still some unsolved mechanisms about why HF patients are more susceptible to AF.

Sustained adrenergic overstimulation contributes to the pathogenesis of cardiac remodeling in HF. 3',5'-cyclic adenosine monophosphate (cAMP) is a famous secondary messenger in  $\beta$ -AR signaling. The cAMP was believed to be mediated only by protein kinase A (PKA) before. However, scientists detected another important PKA-independent effector of cAMP – the exchange protein directly activated by cAMP (EPAC) several years ago<sup>10,11</sup>. EPAC isoforms are very important in various human diseases, especially in HF. The EPAC protein family consists of two members: EPAC1 and EPAC2<sup>12,13</sup>. EPAC1 expresses nearly ubiquitously in the whole body including the cardiovascular system<sup>10,11</sup>. In the heart, EPAC1 is the major isoform. Dysfunction of L-type calcium channel (LTCC/ I<sub>Ca-L</sub>) is a critical mechanism in different kinds of cardiac

arrhythmias. The aim of our study was to investigate whether EPAC1 regulates LTCC and its role in AF generation in the mouse model of isoproterenol (ISO)-induced HF.

## Materials and Methods

### *Mice*

Mice used in the investigation were at the age of 8 weeks. All studies were approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center. To induce HF models, 8-week C57BL6 male mice were treated with ISO (30 mg/kg/day) for continuous 3 weeks with subcutaneous osmotic minipumps (Alzet, 1004, Cupertino, CA, USA) as previously described<sup>14</sup>. Controls were treated with saline using the same protocols.

### *Echocardiography*

Cardiac function was examined by transthoracic echocardiography in both groups at baseline and post 3 weeks of ISO/saline treatment. Mice were anesthetized by inhaling 2% isoflurane, and then, echocardiography was carried out with an ultrasound machine (Vevo 3100, Fujifilm Visual-Sonics, Toronto, ON, USA).

### *Langendorff Perfused Heart and Programmed Electrical Stimulation*

Mice were heparinized (30000 IU/ml) and then anesthetized by intraperitoneal injection with pentobarbital sodium (80 mg/kg). Hearts were perfused on the Langendorff system retrogradely with a Tyrode solution [(in mM): CaCl<sub>2</sub> 1.8; NaCl 126; Na<sub>2</sub>HPO<sub>4</sub> 0.3; KCl 5.4; MgCl<sub>2</sub> 1; glucose 10; HEPES 10; pH adjusted to 7.35 with NaOH] at 37°C and bubbled with 5% CO<sub>2</sub>. The system was delivered under a constant flow at a rate of 3 mL/min via a peristaltic pump.

Unipolar electrocardiogram (ECG) was monitored using two electrodes: negative one was above the aorta and the positive one was at the apex of the heart; another electrode was placed at right atrium to carry out epicardial pacing. Hearts were perfused with a Tyrode solution containing (or not) CE3F4 (1 mM) for 30 min at first. Then, programmed electrical stimulation (PES) was performed to induce AF and ECG was achieved. Inducibility of AF was tested by utilizing 5s bursts through electrodes with an automated stimulator (GY6328B; HeNan HuaNan Medical Science and Technology, Ltd. Zhengzhou, China) as previously

described<sup>11</sup>. Each heart was stimulated for five times, and the number of PES-induced AF was recorded.

### *Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western Blot*

Quantitative mRNA expression in mouse atrial samples was performed with protocols described previously<sup>15</sup>. We used primer sequences supplied on the PrimerBank (<https://pga.mgh.harvard.edu/primerbank/>) as following: EPAC1 forward: TCTTAC-CAGCTAGTGTTCGAGC; EPAC1 reverse: AAT-GCCGATATAGTCGCAGATG; GAPDH forward: AGGTCGGTGTGAACGGATTTG; GAPDH reverse: TGTAGACCATGTAGTTGAGGTCA.

Quantitative normalization was performed on EPAC1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA detection. The relative expression of EPAC1 was determined using the 2<sup>-ΔΔCT</sup> method.

Protein extraction and immunoblots were performed as the previous approaches<sup>16</sup>. Briefly, mouse atria were homogenized, and then, proteins were separated by polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride (PVDF) membrane, and afterwards immunoblotted against the antibodies: anti-EPAC1 (1:1000; Abcam, Cambridge, MA, USA) and anti-GAPDH (1:1000; Abcam, Cambridge, MA, USA) antibodies. After that, blots were exposed to secondary antibodies. The band intensity was quantified and is expressed as a percentage of GAPDH.

### *Isolation of Atrial Cardiomyocytes*

Hypertensive and control mice were heparinized 15 min before anesthesia using sodium pentobarbital (80 mg/kg). Hearts were quickly removed, and the aortas were rapidly attached to a Langendorff device. The apparatus was retrogradely perfused with a flow at a rate of 3 mL/min at constant 37°C. Hearts were firstly perfused with a Tyrode solution for 2 min to pump out the blood [(in mM): CaCl<sub>2</sub> 1.8; NaCl 126; Na<sub>2</sub>HPO<sub>4</sub> 0.3; KCl 5.4; MgCl<sub>2</sub> 1; glucose 10; HEPES 10; pH adjusted to 7.35 with NaOH]<sup>10</sup>. Then, the hearts were cleared with Ca<sup>2+</sup>-free Tyrode solution. Afterwards, hearts were further digested with the same Ca<sup>2+</sup>-free solution containing collagenase type II (0.6 mg/mL, 296 u/mg, Worthington) and 0.1% bovine serum albumin for another 8 to 12 min. The atrium was blew into atrial cardiomyocytes and placed in Krebs-Henseleit (KH) buffer solution (in mM) (KOH 85; KCl 30; MgCl<sub>2</sub> 1; glutamate

50; HEPES 10; EGTA 0.5; taurine 20; glucose 10; pH adjusted to 7.35 with KOH) at 4°C for at least 1 h and prepared for patch clamp. Cells cultured were reintroduced to Ca<sup>2+</sup> step by step (Ca<sup>2+</sup> concentration (in mM): 0.1, 0.3, 0.6, 0.9, and 1.2) and then added to M199 medium (Hyclone, USA) with or without EPAC1 inhibitor CE3F4 (10 μM). Cardiomyocytes were plated and maintained at 37°C in 5% CO<sub>2</sub>-enriched atmosphere. After 24 h of exposure to different treatments, cells were washed with a Tyrode solution before patch clamp.

### Patch Clamp

The digested myocytes were utilized to record action potentials (APs) at 37°C and I<sub>Ca-L</sub> at room temperature (21-25°C) using the whole cell patch-clamp technique by a patch clamp amplifier (Axopatch 200B). Pipettes were filled with the following internal solution (in mM): aspartic acid 100, CsOH 70, CsCl 40, MgATP 4, MgCl<sub>2</sub> 2, EGTA 10, HEPES 10 (pH adjusted to 7.3 with CsOH). Pipettes were tested before used to ensure a resistance of 2-4 MΩ. The bath was perfused with the external solution (in mM) when recording I<sub>L-Ca</sub>: TEA-Cl 145, CsCl 10, 2 CaCl<sub>2</sub>, MgCl<sub>2</sub> 0.5, HEPES 5, glucose 5.5 (pH adjusted to 7.4 with CsOH). Atrial myocytes were held at -60 mV, and I<sub>L-Ca</sub> was recorded by voltage steps from -60 to +60 mV, with 250 ms and 0.1 Hz. The bath was perfused with a Tyrode solution when recording APs. APs were elicited at 1 Hz by 4-ms current pulses at 120% threshold level.

### Statistical Analysis

All data were analyzed using GraphPad Prism 5 (La Jolla, CA, USA). Data were presented as means ± SD (standard deviation) and analyzed using a paired Student's *t*-test. *p*<0.05 was considered significant.

## Results

### Inducible AF was Increased in ISO-Infused HF

To investigate whether the HF model is successful, echocardiography was performed in each mouse. Table I showed that after 3-week ISO admission, hearts were enlarged and ejection fraction was significantly reduced. This suggested the HF model was feasible. To detect whether AF inducibility was increased in HF model, PES was performed in Langendorff perfused hearts. After 3-week ISO admission, PES-induced AF was significantly increased (Figure 1 A, 1B). The mean rate of AF inducibility was 80% after ISO infusion while the rate was only 12.5% in the control group.

### EPAC1 Expression was Upregulated in HF

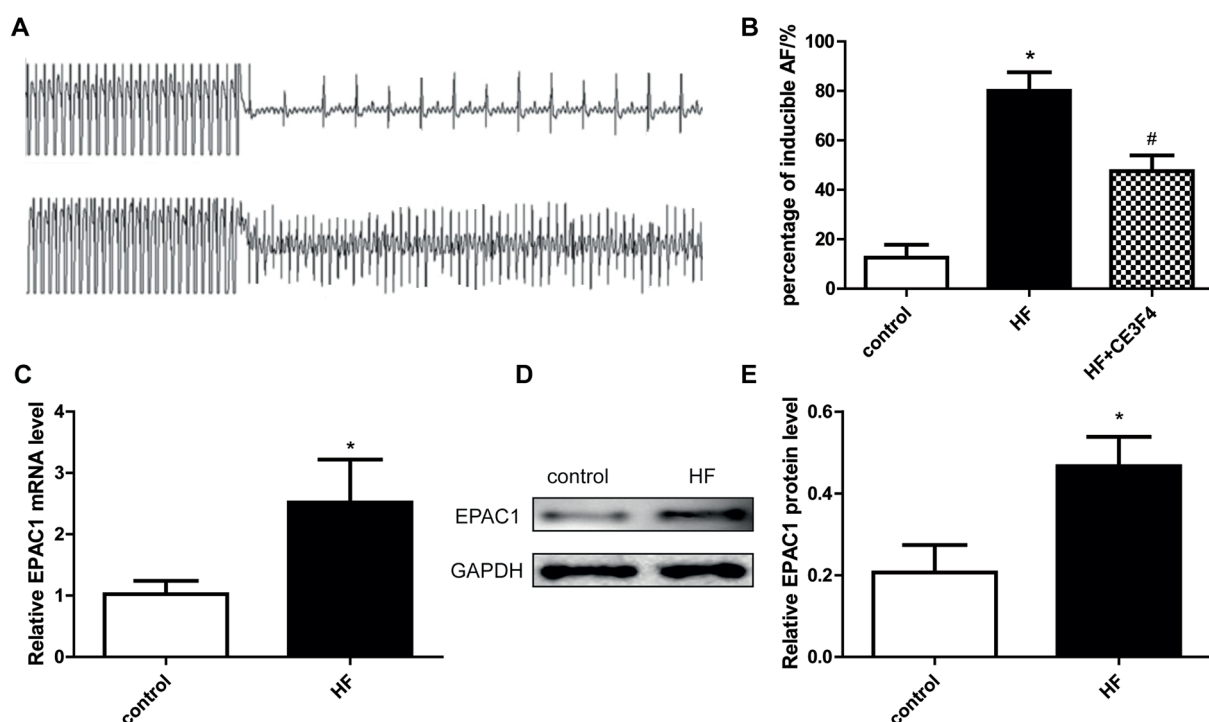
Next, we tested the mRNA level of EPAC1. The expression of EPAC 1 was increased in atrial tissues from HF mice compared with control (Figure 1C). Also, the EPAC1 protein was upregulated in atrial tissues from HF compared with control (Figure 1D, 1E).

### Effect of ISO on AP Duration and I<sub>Ca-L</sub> in Atrial Myocytes

We next examined the cellular electrophysiology in atrial myocytes of HF mice. Representative recordings (Figure 2A) and summary data illustrated that ISO increased APD at 50% (APD<sub>50</sub>, Figure 2B), 70% (APD<sub>70</sub>, Figure 2C), and 90% (APD<sub>90</sub>, Figure 2D) repolarization. APD<sub>50</sub> was increased by 32.77% (10.62 ± 0.5328 ms and 14.10 ± 0.5118 ms, *p*<0.05). APD<sub>70</sub> was increased by 36.36% (21.37 ± 0.7492 ms and 29.14 ± 1.291 ms, *p*<0.05). Also, APD<sub>90</sub> was increased by 30.43% (47.88 ± 1.704 ms and 62.45 ± 2.195 ms, *p*<0.05).

**Table I.** Echocardiography of hearts in mice after ISO admission and control groups.

|                | Control       | HF            | <i>p</i> |
|----------------|---------------|---------------|----------|
| LV ID; d (mm)  | 9.40 ± 0.28   | 11.15 ± 0.23  | < 0.001  |
| LV ID; s (mm)  | 5.99 ± 0.51   | 8.48 ± 0.22   | < 0.001  |
| LV PW; d (mm)  | 1.69 ± 0.04   | 2.06 ± 0.06   | 0.0036   |
| EF (%)         | 61.35 ± 1.01  | 42.54 ± 1.28  | < 0.001  |
| FS (%)         | 36.59 ± 1.58  | 25.15 ± 0.53  | < 0.001  |
| LV Vol; d (μL) | 497.1 ± 32.06 | 719.0 ± 32.88 | < 0.001  |
| LV Vol; s (μL) | 187.2 ± 14.79 | 406.4 ± 23.92 | < 0.001  |



**Figure 1.** AF inducibility and EPAC1 expression in ISO-induced HF. **A**, representative recordings of PES induced arrhythmia, the upper one represents no arrhythmia after PES, the below one means irregular atrial arrhythmia (AF) after PES. **B**, PES-induced AF in Langendorff perfused hearts of control, HF and HF+CE3F4 groups (n=10 in each group). **C**, mRNA and **(D)** **(E)** protein expression of atrial tissues (n=6 in each group). HF: heart failure; \* $p < 0.05$  vs. control; # $p < 0.05$  vs. HF.

We then measured the effects of ISO on ICa-L in atrial myocytes. In this experiment, we found that ISO had an active effect on atrial ICa-L density in basal conditions, as showed in representative recordings (Figure 3A) and summary current-voltage relationship data (Figure 3B).

#### ***Inhibition of EPAC1 Reduced AF in HF***

To access whether EPAC1 regulated AF formation, HF hearts were perfused by EPAC1 inhibitor-CE3F4 via the Langendorff system. The inducibility of AF was markedly decreased in CE3F4-treated HF mice compared with no CE3F4 infusion group (47.5% vs. 80%) (Figure 1B).

#### ***Inhibition of EPAC1 Reversed APD and ICa-L after ISO Treatment***

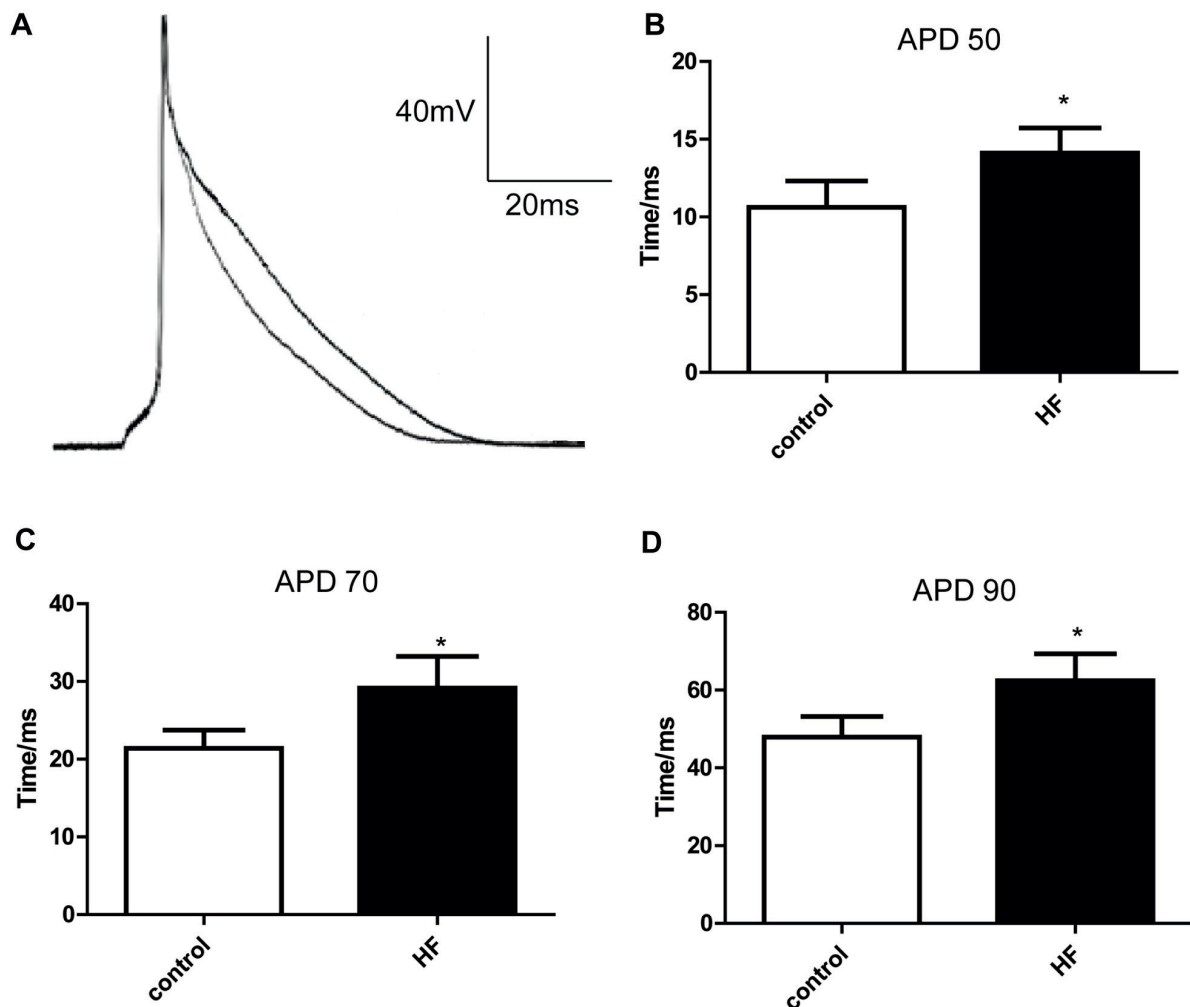
In the next part, we determined whether inhibition of EPAC1 affects APD and ICa-L with ISO treatment. Adult cardiomyocytes were cultured and dealt with vehicle, ISO or ISO + CE3F4 for 24 h and prepared for whole cell patch clamp. *In vitro*, APD was similarly increased after ISO treatment. APD50, APD70, and

APD 90 were respectively prolonged by 39.94%, 37.54%, and 35.07% (Figure 4 A, 4B). Administration of CE3F4 reversed the APD compared with ISO perfusion only. APD50, APD70, and APD 90 were reduced in ISO + CE3F4 group by 13.22%, 14.39%, and 14.22% compared to ISO group (Figure 4A, 4B).

Consistent with the effect on APD, CE3F4 also reversed the ICa-L excitation after ISO treatment. Representative recordings (Figure 5A) and summary current-voltage relationship data (Figure 5B) illustrated that, in isolated atrial cells, 24h-activation by ISO could increase ICa-L density, and this effect was weakened by CE3F4.

## **Discussion**

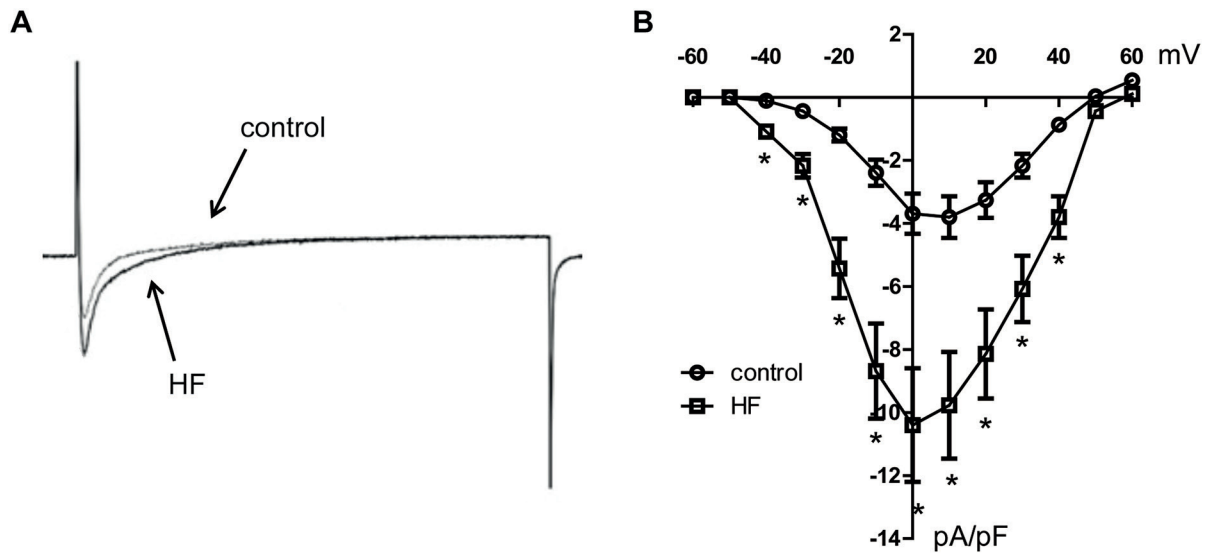
In the present work, we used an EPAC1 inhibitor to show that EPAC1 suppression inhibited AF in ISO-induced HF. Mechanistically, ISO infusion increased EPAC1 expression to promote L-type calcium channel opening, which prolongs AP. Thus, EPAC1 is essential for AF in HF.



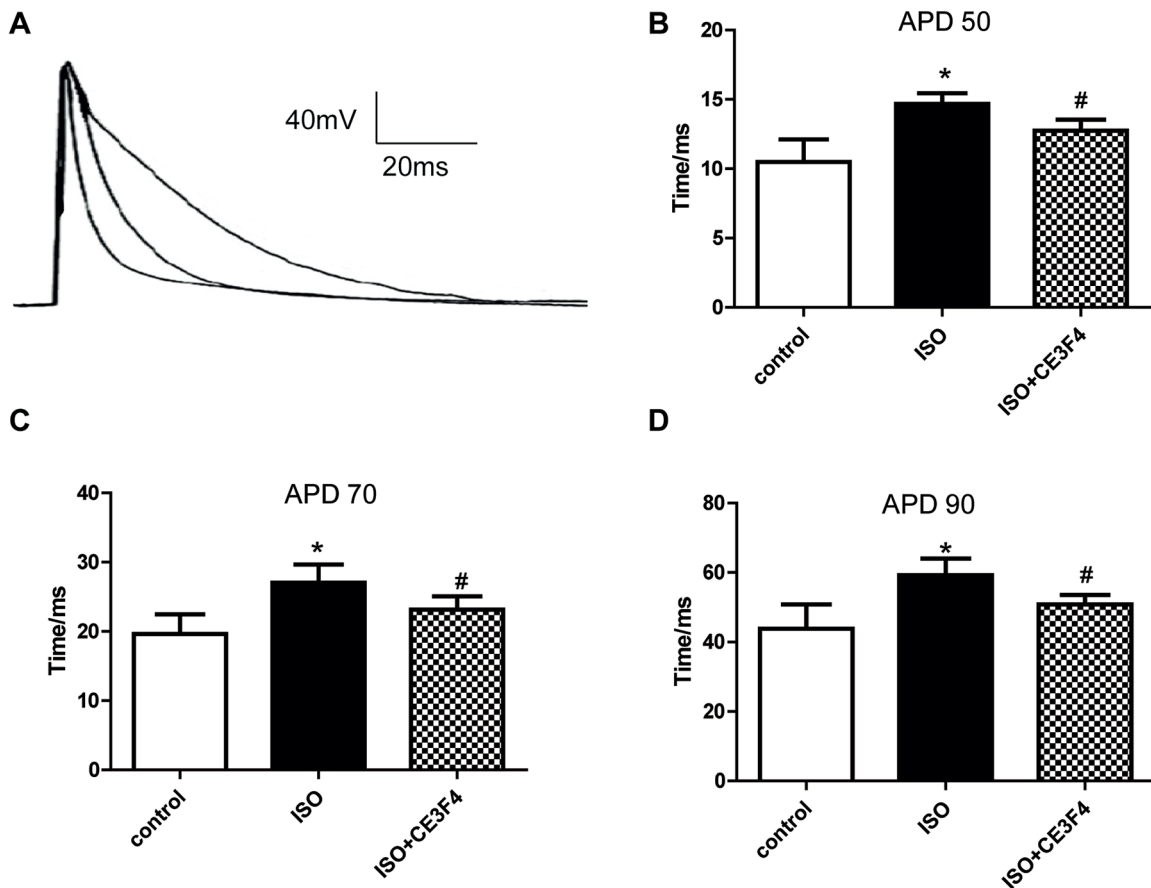
**Figure 2.** Prolonged AP in atrial myocytes of ISO-induced HF. *A*, Representative recordings of AP in atrial myocytes; APD 50 (*B*), APD 70 (*C*) and APD 90 (*D*) in adult isolated myocytes from control and HF mice. APD: action potential duration; HF: heart failure; \* $p < 0.05$  vs. control.

Previous researches have showed diverse effects of EPAC signaling on various cardiovascular functions such as proliferation and migration of vascular smooth muscle cells, vascular endothelial barrier function, regulation of vascular inflammation, cardiac hypertrophy, cardiomyocyte apoptosis, and electric remodeling<sup>17</sup>. Evidence indicates that EPAC is related to  $Ca^{2+}$  cycling,  $K^+$  and  $Na^+$  channel capability<sup>17,18</sup>. EPAC activation promotes sarcoplasmic reticulum (SR)  $Ca^{2+}$  leak through  $Ca^{2+}$ /calmodulin kinase-II (CaMKII), enhancing susceptibility to delayed after depolarizations<sup>18</sup>. In another study<sup>19</sup>, EPAC affects SR  $Ca^{2+}$  handling by inhibition of hyperphosphorylation of ryanodine receptor (RyR) on

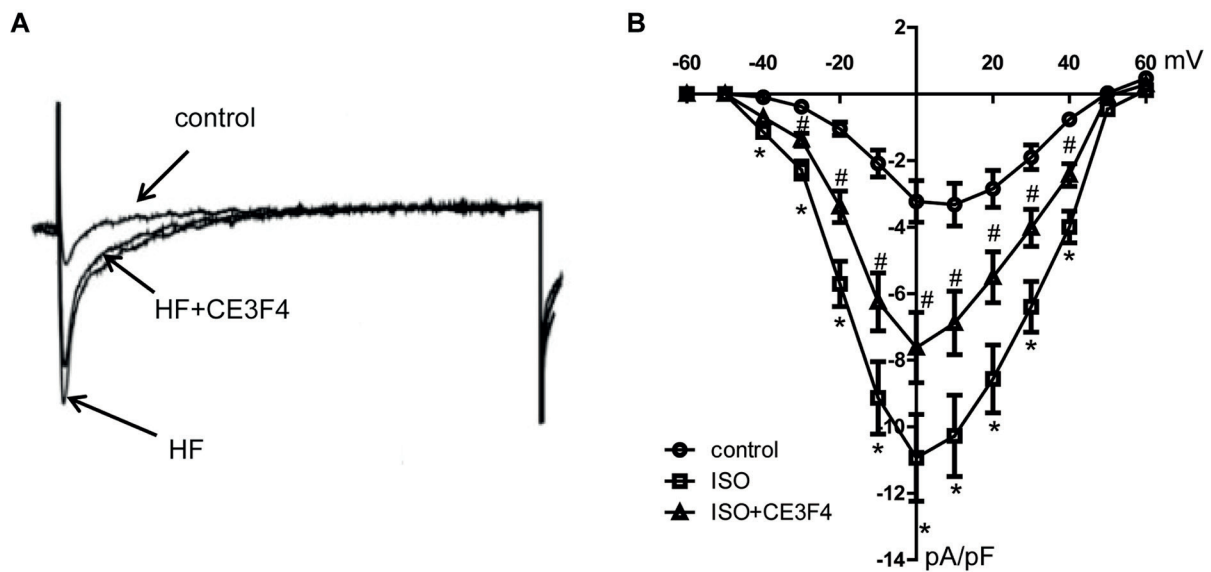
serine 2814/2815 and phospholamban on serine<sup>16</sup>. EPAC is also reported to increase the expression of transient receptor potential canonical 3 and 4 channels in isolated ventricular cardiomyocytes.  $Ca^{2+}$  influx via these two transient receptor potential canonical channels may lead to the development of arrhythmias<sup>20</sup>. The increase of late  $Na^+$ -current by CAMKII is important as well in the proarrhythmic effect of EPAC<sup>21</sup>. In addition, EPAC 1 activation decreases IKs and prolongs APD, which then increases susceptibility to early after depolarizations<sup>22</sup>. Our work is the first insight into the negative effect of EPAC1 on ICa-L on the cellular membrane. Due to the importance of ICa-L in calcium-induced calcium release,



**Figure 3.** Enhanced ICa-L in HF atrial cells. *A*, Representative recordings of AP in atrial myocytes; *B*, Atrial ICa,L I-V relationships in control and HF hearts (n=8 in each group). \**p*<0.05 vs. control.



**Figure 4.** Reversed AP in atrial myocytes after CE3F4 treatment. *A*, Representative recordings of AP in atrial myocytes; APD 50 (*B*), APD 70 (*C*) and APD 90 (*D*) in adult isolated myocytes from control, ISO, ISO+CE3F4 groups (n=8 in each group). APD: action potential duration; ISO: isoproterenol; \**p*<0.05 vs. control. #*p*<0.05 vs. ISO.



**Figure 5.** Decreased  $I_{Ca-L}$  after CE3F4 treatment in ISO-perfused atrial cells. **A**, Representative recordings of AP in atrial myocytes; **B**, Atrial  $I_{Ca,L}$  I-V relationships in control, in the presence of ISO, and after application of CE3F4 in the presence of ISO. (n=8 in each group). \* $p < 0.05$  vs. control. # $p < 0.05$  vs. ISO.

we observed clear effects of EPAC1 inhibition on reducing inducible AF in HF mouse hearts.

## Conclusions

In summary, we identified that EPAC1 could inhibit AF occurrence in ISO-induced HF. The molecular mechanism involved in the anti-arrhythmia effect of EPAC1 was associated with the inhibition of  $I_{Ca-L}$ . Our results suggest that EPAC1 inhibitor CE3F4 can be applied as a potential treatment strategy for the inhibition of AF in HF by shortening atrial APD and decreasing  $I_{Ca-L}$ .

## Conflict of Interest

The Authors declare that they have no conflict of interest.

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