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Research article

ER stress modulates Kv1.5 channels via PERK branch in HL-1 atrial myocytes: Relevance to atrial arrhythmogenesis and the effect of tetramethylpyrazine

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ABSTRACT

Endoplasmic reticulum (ER) stress is implicated in cardiac arrhythmia whereas the associated mechanisms remain inadequately understood. Kv1.5 channels are essential for atrial repolarization. Whether ER stress affects Kv1.5 channels is unknown. This study aimed to elucidate the response of Kv1.5 channels to ER stress by clarifying the unfolded protein response (UPR) branch responsible for the channel modulation. In addition, the effect of tetramethylpyrazine (TMP) on Kv1.5 channels was studied. Patch clamp and western-blot results revealed that exposure of HL-1 atrial myocytes to ER stress inducer tunicamycin upregulates Kv1.5 expression, increases Kv1.5 channel current (I_{Kur}) (14.91 ± 1.11 *vs.* 6.11 ± 1.31 pA/pF, $P < 0.001$), and shortened action potential duration (APD) (APD_{90}: 82.79 ± 5.25 *vs.*121.11 ± 6.72 ms, $P < 0.01$), which could be reverted by ER stress inhibitors. Blockade of the PERK branch while not IRE1 and ATF6 branches of UPR downregulated Kv1.5 expression, accompanied by a decreased I_{Kur} (9.03 ± 0.99 pA/pF) and a prolonged APD_{90} (113.69 ± 4.41 ms) ($P < 0.01$). PERK-mediated increases of Kv1.5 expression and I_{Kur} were also observed in HL-1 cells incubated with thapsigargin. TMP suppressed the enhancement of I_{Kur} (10.52 ± 0.97 *vs.* 17.52 ± 2.25 pA/pF, $P < 0.05$), prevented the shortening of APD (APD_{90}: 110.16 ± 5.36 *vs.* 84.84 ± 4.58 ms, $P < 0.05$), and inhibited the upregulation of Kv1.5 triggered by ER stress. Our study suggests that ER stress induces upregulation and activation of Kv1.5 channels in atrial myocytes through the PERK branch of UPR. TMP prevents Kv1.5 upregulation/activation and the resultant APD shortening by inhibiting ER stress. These results may shed light on the mechanisms of atrial arrhythmogenesis and the antiarrhythmic effect of the traditional Chinese herb TMP.

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Abbreviations

Action potential	AP
Action potential duration	APD
Activating transcription factor 6	ATF6
Atrial fibrillation	AF
Endoplasmic reticulum stress	ER stress
Glucose-regulated protein 78	GRP78
Inositol-requiring enzyme 1	IRE1
Protein kinase R-like ER kinase	PERK
Tetramethylpyrazine	TMP
Trimethylapigenin	TMA
Tunicamycin	TM
Unfolded protein response	UPR

1. Introduction

The generation of cardiac action potential (AP) is tightly controlled by a precisely timed series of activation and inactivation of ion channels [1,2]. Aberrant cell membrane depolarization resulting from prolongation or shortening of AP duration (APD) has been associated with arrhythmia. For example, inhibition of human ether-à-go-go-related gene (hERG) and aberrant activation of Nav1.5 channels prolongs APD and causes long-QT syndrome [3,4]. Enhancement of late sodium current increases APD and propensity for pro-arrhythmic events including early and delayed afterdepolarizations [5]. Shortening of APD due to decrease in inward Ca^{2+} or increase in outward K^+ currents increases atrial fibrillation (AF) susceptibility by promoting re-entry [6–8].

The ultrarapid outward current I_{Kur} is carried by Kv1.5, which is a voltage-gated K^+ channel specifically expressed in the atrium. Kv1.5 channel is encoded by *KCNA5* and plays a predominant role in atrial AP repolarization [9,10]. Previous studies have suggested the role of Kv1.5 in atrial arrhythmias. It was reported that the expression of Kv1.5 channels is reduced by more than 50 % in both the left and the right atrial appendages of AF patients [11,12]. E375X mutation in *KCNA5* causes functional loss of Kv1.5 channels in human atrial myocytes, leading to APD prolongation and early after-depolarization (EAD), which renders atrial myocytes susceptible to pathological excitability [13]. There were also reports relating gain-of-function mutations in *KCNA5* to arrhythmogenesis. SNX17 deficiency increases the membrane expression of Kv1.5 channels and I_{Kur} density, resulting in a shortened APD and consequently increases susceptibility to AF [14]. The enhancement of I_{Kur} resulted from gain-of-function mutations in KCNA5 was observed in early-onset lone AF patients [15].

Endoplasmic reticulum (ER) stress has been implicated in cardiac arrhythmia [16,17]. Disassociation of the ER chaperone glucose-regulated protein 78 (GRP78) from protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) initiates the activation of these proximal effectors of the unfolded protein response (UPR), and UPR triggered by ER stress may affect membrane expression of cardiac ion channels. In human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) activation of PERK branch of UPR was found to specifically downregulate Kv4.3 while activation of the IRE1 branch downregulate Cav1.2, and both branches downregulate Nav1.5, hERG, and KvLQT1 channels. Regulation of these major ion channels by UPR resulted in a significant prolongation of APD in hiPSC-CMs, which contributes to electric remodeling [18]. UPR activation also caused a loss of the α subunit of Kv4.3, which consequently reduced I_{to}, leading to APD shortening and phase 2 reentry [19]. These results suggest that ion channel dysfunction is a fundamental mechanism underlying electrical remodeling triggered by ER stress and targeting UPR might exert anti-arrhythmic action via regulating ion channels.

Tetramethylpyrazine (TMP) is the main bioactive alkaloid components extracted from the Chinese medical herb Ligusticum Chuanxiong, which has been clinically used for the treatment of cardiovascular diseases such as myocardial ischemia, hypertension, and atherosclerosis [20]. In addition to the well-known antioxidant and anti-inflammatory benefits, evidence has been accumulating in recent years showing the anti-ER stress property of TMP [21–23]. We previously demonstrated that TMP may protect coronary artery from homocysteine-induced functional impairment by restoring BK_{Ca} channel activity, and suppression of ER stress-mediated downregulation of the β1 subunit of BK_{Ca} channel is the underlying mechanism [22]. TMP was known to be able to reduce the incidence of ventricular tachycardia and fibrillation in rats subjected to ischemia/reperfusion [24,25], however, the antiarrhythmic mechanisms of TMP remain poorly understood. Whether TMP may prevent Kv1.5 channel malfunction by antagonizing ER stress is yet to be investigated.

Herein, this study aimed to understand the response of Kv1.5 channels to ER stress and the effect of TMP in atrial myocytes, with further clarification of the UPR branch responsible for Kv1.5 channel modulation. Our findings concerning the role of PERK-mediated Kv1.5 upregulation in APD shortening, and the anti-ER stress-dependent restoration of APD and Kv1.5 function by TMP provided new insights into atrial arrhythmogenesis and the antiarrhythmic mechanisms of TMP.

2. Materials and methods

2.1. Chemicals and reagents

Amphotericin B and trimethylapigenin (TMA) purchased from Sigma-Aldrich (St. Louis, MO, USA) were first dissolved in dimethylsulfoxide (DMSO) and then diluted to the final concentrations in the standard external or internal solution just before use.

Tunicamycin, Tetramethylpyrazine (TMP), 4-PBA, TUDCA, GSK2606414, GSK2850163, and Ceapin A7 were purchased from MedChemExpress (MCE, Shanghai, China), which were also prepared as stock solutions in DMSO and stored at −80 °C. The final concentration of DMSO was less than 0.01 %. RIPA lysis buffer was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

2.2. Culture and treatment of HL-1 atrial myocytes

HL-1 atrial myocytes were purchased from ATCC and cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific, USA). When grown to 75 % confluence, the cells were treated for 24 h with the ER stress inducer tunicamycin (5 μg/mL) in the presence or absence of one of the following: 4-PBA (2 mmol/L) or TUDCA (200 μmol/L) (ER stress inhibitors), GSK2606414 (0.5 μmol/L, PERK specific inhibitor), GSK2850163 (10 μmol/L, IRE1 specific inhibitor), Ceapin A7 (500 nmol/L, ATF6 specific inhibitor), or TMP (100 μmol/L). Another ER stress inducer thapsigargin (400 nmol/L) [26] was also used to treat the cells for 24 h in the presence or absence of GSK2606414. HL-1 cells exposed to the vehicle served as the control. Cells were then collected for Western blot analysis of ER stress molecules and Kv1.5 protein. For electrophysiological experiments, the cells were detached with 0.25 % trypsin-EDTA (Beyotime, Shanghai, China) at 37 °C for 3 min, resuspended in fresh culture medium and then seeded on glass cover slip. The cells were then subjected to different treatments as above before patch clamp recording of AP and Kv1.5 channel current.

2.3. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)

Cell apoptosis was detected by TUNEL using One-step TUNEL in situ apoptosis assay kit (Elabscience, China) as described elsewhere [27]. The HL-1 cells were seeded on glass coverslips and fixed with 4 % paraformaldehyde, followed by permeabilization with 0.1 % Triton X-100. Nuclei of the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and the cells were observed under a fluorescence microscope (DP73, Olympus). The number of TUNEL-positive cells and the total number of cells in 16–18 randomly selected microscopic fields were counted for each group. The ratio of apoptotic HL-1 cells was calculated by dividing the number of TUNEL-positive cells by the total number of cells.

2.4. Patch-clamp recording

Patch pipettes were fabricated from glass capillaries (OD, 1.5 mm; ID, 0.86 mm, Sutter Instrument, USA) using a Sutter P-1000 microelectrode puller (Sutter Instrument, Novato, USA). When filled with standard pipette solution, the pipette had a resistance of 2–4 MΩ. All electrophysiological recordings were performed using EPC10 patch-clamp (HEKA, Lambrecht, Germany) at room temperature (22–24 °C).

2.4.1. Recording of Kv1.5 channel current

Patch-clamp recording of Kv1.5 channel current was performed according to the previously described method with some modifications [9,10,15]. Patch pipettes were filled with a solution containing (mmol/L): KCl 140, EGTA 10, $MgCl_2$ 4, HEPES 10, $CaCl_2$ 1.8, Na_2ATP 4 (pH 7.2 adjusted with KOH). The bath solution contained (mmol/L): KCl 5.4, NaCl 150, HEPES 10, $MgCl_2$ 2, $CaCl_2$ 2, Glucose 11.1 (pH 7.4 adjusted with NaOH). HL-1 atrial myocytes were held at −40 mV and voltage steps ranging from −60 to +80 mV were applied for 300 ms in 10-mV step increments. K^+ currents were recorded in whole-cell mode with further application of the Kv1.5 channel blocker trimethylapigenin (TMA, 30 μmol/L) to differentiate the Kv1.5 current component [28]. Data were analyzed with PulseFit software (HEKA). The current was normalized by cell capacitance into current densities (pA/pF).

2.4.2. Recording of AP

Perforated patch-clamp technique using amphotericin B (250 μg/mL) was employed to record AP in the current-clamp mode [10,29]. Pipette solution contained (mmol/L) NaCl 5, KCl 20, Glutamate 120, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, Mg-ATP 2.5 (pH 7.2 adjusted with KOH). The external solution contained (mmol/L) NaCl 135, KCl 4, $MgCl_2$ 1, $CaCl_2$ 2, NaH_2PO_4 0.33, HEPES 10, Glucose 10 (pH 7.4 adjusted with NaOH). APs were evoked with a supra-threshold (1500 pA, 5 ms duration) current pulse at a rate of 1 Hz. The AP parameters, including APD at 30 %, 50 %, and 90 % repolarization (APD_{30}, APD_{50}, and APD_{90}) [10,15,29] were analyzed and compared among different treatment groups.

2.5. Western blot analysis

Whole cell protein was extracted with RIPA lysis buffer containing protease and phosphatase inhibitor cocktail (Thermo Fisher Scienitific, USA). The protein extraction was centrifuged at 4 °C for 20 min at 12,000 g and mixed with loading buffer, heated up to 95 °C for 10 min, then fractioned by a denaturing 10 % sodium-dodecyl-sulfate poplyacrylamide gel electrophoresis (40 μg per lane) for 120 min at 90V and electro-transferred to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, USA). The membrane was blocked with 5 % non-fat milk/TBS for 90 min at room temperature and incubated with the primary antibody in TBS overnight at 4 °C, followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG or horse anti-mouse IgG secondary antibodies (1:3000, CST, Massachusetts, USA) for 1 h at room temperature. Primary antibodies used include Kv1.5 (1:200, Alomone Labs, Israel), GRP78 (1:1000, Abcam, Cambridge, UK), PERK (1:1000, CST, Massachusetts, USA), phosphorylated (Thr980) PERK (1:500, Bioss, Massachusetts, USA), eIF2α (1:1000, Abcam, Cambridge, UK), phosphorylated (Ser51) eIF2α (1:500, Abcam,

Cambridge, UK), IRE1 (1:1000, MilliporeSigma), phosphorylated (Ser724) IRE1 (1:1000, Abcam Cambridge, UK), XBP1 (1:800, Abcam, Cambridge, UK), ATF6 (1:500, Proteintech, Wuhan, China), GAPDH (1:2000, Proteintech, Wuhan, China). The blots were visualized by enhanced chemiluminescence. The images were captured by Kodak Image Station 4000 R and quantified using Kodak MI SE software. GAPDH was used as internal control.

2.6. Statistical analysis

All values are presented as mean ± SEM. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Scheffe post hoc test (SPSS, version 20.0). Student *t*-test was used for comparisons between two groups. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. ER stress shortens APD and upregulates Kv1.5 channel expression in HL-1 atrial myocytes

Exposure of HL-1 atrial myocytes to the ER stress inducer tunicamycin resulted in shortening of APD. As compared to control cells, APD_{90} decreased from 121.11 ± 6.72 to 82.79 ± 5.25 ms ($P < 0.01$).

APD_{30} and APD_{50} showed a slight decrease, though not statistically significant (APD_{30}: 15.92 ± 0.88 *vs.* 18.61 ± 1.44 ms in control; APD_{50}: 28.64 ± 0.94 *vs.* 34.18 ± 3.69 ms in control, $P > 0.05$) (Fig. 1A). Western blot analysis showed that tunicamycin exposure caused a significant upregulation of Kv1.5 protein (Fig. 1B), which provided a molecular explanation for the shortening of APD. At the indicated concentration and exposure time (5 μg/ml, 24h), tunicamycin did not induce appreciable apoptosis of HL-1 cells (Supplementary Fig. 1).

3.2. Inhibition of ER stress prevents APD shortening and Kv1.5 channel upregulation

Induction of ER stress by tunicamycin in HL-1 atrial myocytes was evidenced by the increased expression of ER chaperone GRP78 and the activation of UPR branches. All three branches of UPR including PERK, IRE1, and ATF6 were activated, which could be antagonized by ER stress inhibitors. As shown in Fig. 2, in tunicamycin-exposed cells, both 4-PBA (Fig. 2A) and TUDCA (Fig. 2B) downregulated the expression of GRP78 and ATF6, suppressed the phosphorylation of PERK and IRE1, and inhibited eIF2α phosphorylation and XBP1 induction, which are the signaling downstream of PERK and IRE1 respectively.

Inhibition of ER stress by 4-PBA and TUDCA reversed the APD shortening. In comparison to the HL-1 atrial myocytes treated with tunicamycin alone, cells co-treated with 4-PBA or TUDCA showed remarkably restored APD_{90} (109.31 ± 3.69 ms in 4-PBA + tunicamycin; 106.97 ± 4.39 ms in TUDCA + tunicamycin, *vs.* 80.93 ± 5.39 ms in tunicamycin, $P < 0.001$), which did not differ from the value of control cells (137.17 ± 5.45 ms, $P > 0.05$) (Fig. 2C). The restoration of APD_{90} was associated with a decreased expression level of Kv1.5 channels (Fig. 2D).

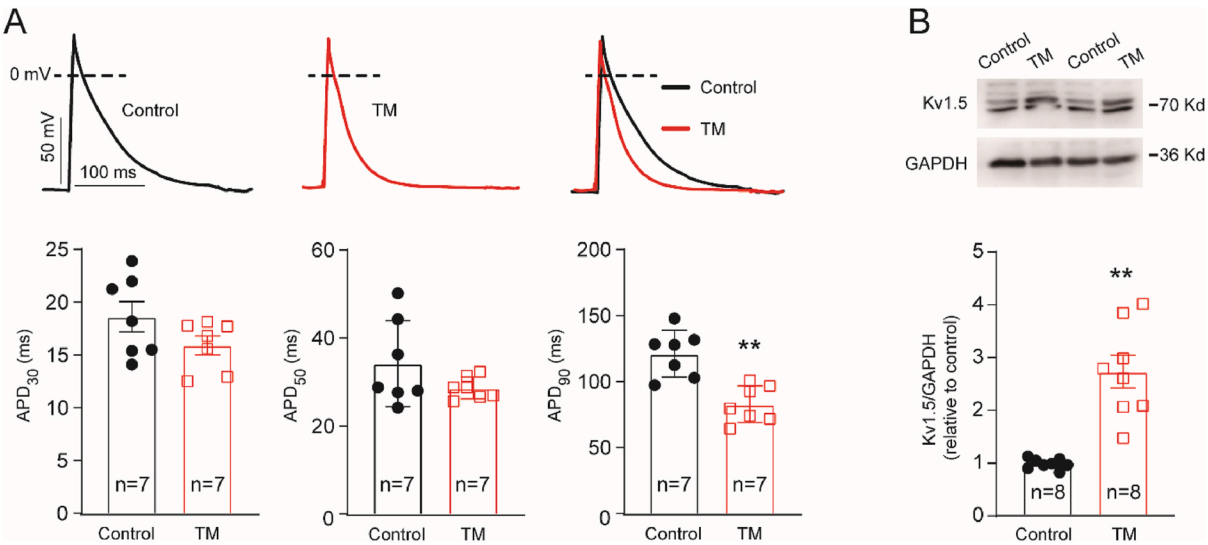

Fig. 1. ER stress shortens action potential duration (APD) and upregulates Kv1.5 channel expression in HL-1 atrial myocytes. (A) Representative recording traces of action potential and grouped data of APD in HL-1 atrial myocytes treated or not treated with tunicamycin (TM). **(B)** Representative blots and grouped data of the expression level of Kv1.5 channels in atrial myocytes treated with or without TM. Full blots are shown in Supplementary Figs. 3–1B. $^{**}P < 0.01$ *vs.* control. n indicates the number of independent experiments performed with cells from different culture batches.

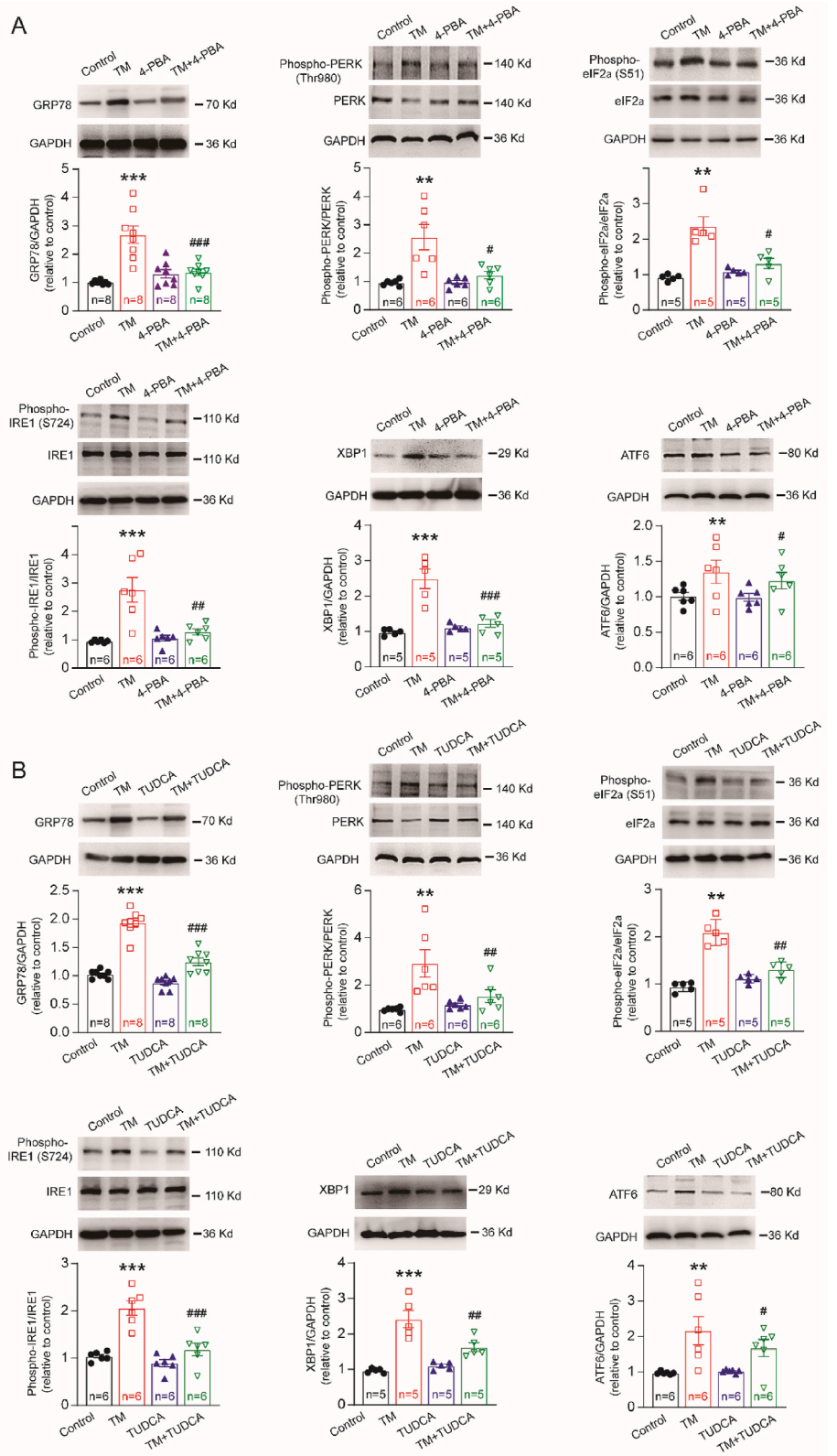

Fig. 2. Inhibition of ER stress prevents action potential duration (APD) shortening and Kv1.5 channel upregulation in HL-1 atrial myocytes. (A&B) Representative blots and expression levels of ER stress molecules in cells from different treatment groups. **TM:** tunicamycin, ER stress inducer; **4-PBA and TUDCA:** ER stress inhibitors. Full blots are shown in Supplementary Figs. 3–2A&B. (C) Representative recording traces of action potential

and grouped data of APD, showing TM-induced shortening of APD and the restoration of APD_{90} by 4-PBA and TUDCA. **(D)** Inhibition of ER stress by 4-PBA or TUDCA prevents TM-induced upregulation of Kv1.5 channels. Full blots are shown in Supplementary Figs. 3–2D. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.*TM. n indicates the number of independent experiments performed with cells from different culture batches.

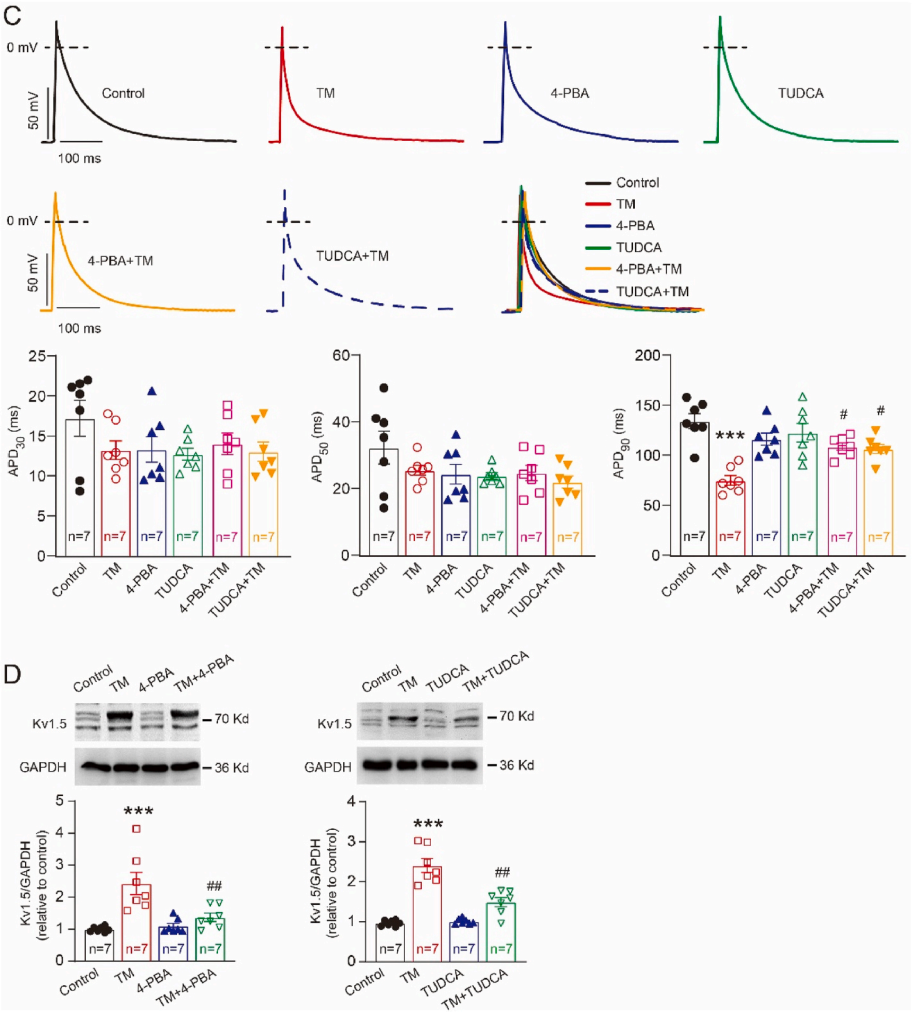

Fig. 2. (*continued*).

3.3. Inhibition of ER stress suppresses enhancement of Kv1.5 channel current

Patch clamp recording showed that inhibition of ER stress with 4-PBA or TUDCA prevents Kv1.5 channel from tunicamycin-induced activation in HL-1 atrial myocytes. The whole-cell K^+ current was significantly increased in HL-1 atrial myocytes subjected to tunicamycin exposure (35.43 ± 2.19 *vs.* 23.22 ± 2.63 pA/pF in control, $P < 0.01$) (Fig. 3A), which was proved to be largely attributed to the enhancement of Kv1.5 channel current. The Kv1.5 current component (I_{Kur}) was differentiated by using the Kv1.5 channel blocker TMA. After tunicamycin exposure, I_{Kur} increased from 6.11 ± 1.31 to 14.91 ± 1.11 pA/pF ($P < 0.001$) (Fig. 3A). Compared with tunicamycin-exposed HL-1 atrial myocytes, cells exposed to tunicamycin while co-treated with 4-PBA exhibited significantly smaller K^+ current density (21.39 ± 1.37 pA/pF, $P < 0.001$) and the current density of I_{Kur} (8.37 ± 0.87 pA/pF, $P < 0.01$) (Fig. 3B). TUDCA had a similar inhibitory effect as 4-PBA on Kv1.5 channel activation (Fig. 3B). The enhancement of I_{Kur} caused by tunicamycin was suppressed (8.87 ± 0.85 pA/pF *vs.* 16.83 ± 2.35 pA/pF, $P < 0.01$), resulting in a significant decrease of whole-cell K^+ current (25.17 ± 2.21 *vs.* 36.38 ± 2.08 pA/pF, $P < 0.05$). The decrease of I_{Kur} by 4-PBA and TUDCA in tunicamycin-exposed HL-1 atrial myocytes were associated with a decreased expression of Kv1.5 channels (Fig. 2D).

3.4. PERK branch of UPR mediates ER stress-induced Kv1.5 channel upregulation

In order to elucidate through which UPR branch ER stress induces Kv1.5 channel expression, the specific inhibitors of PERK, IRE1

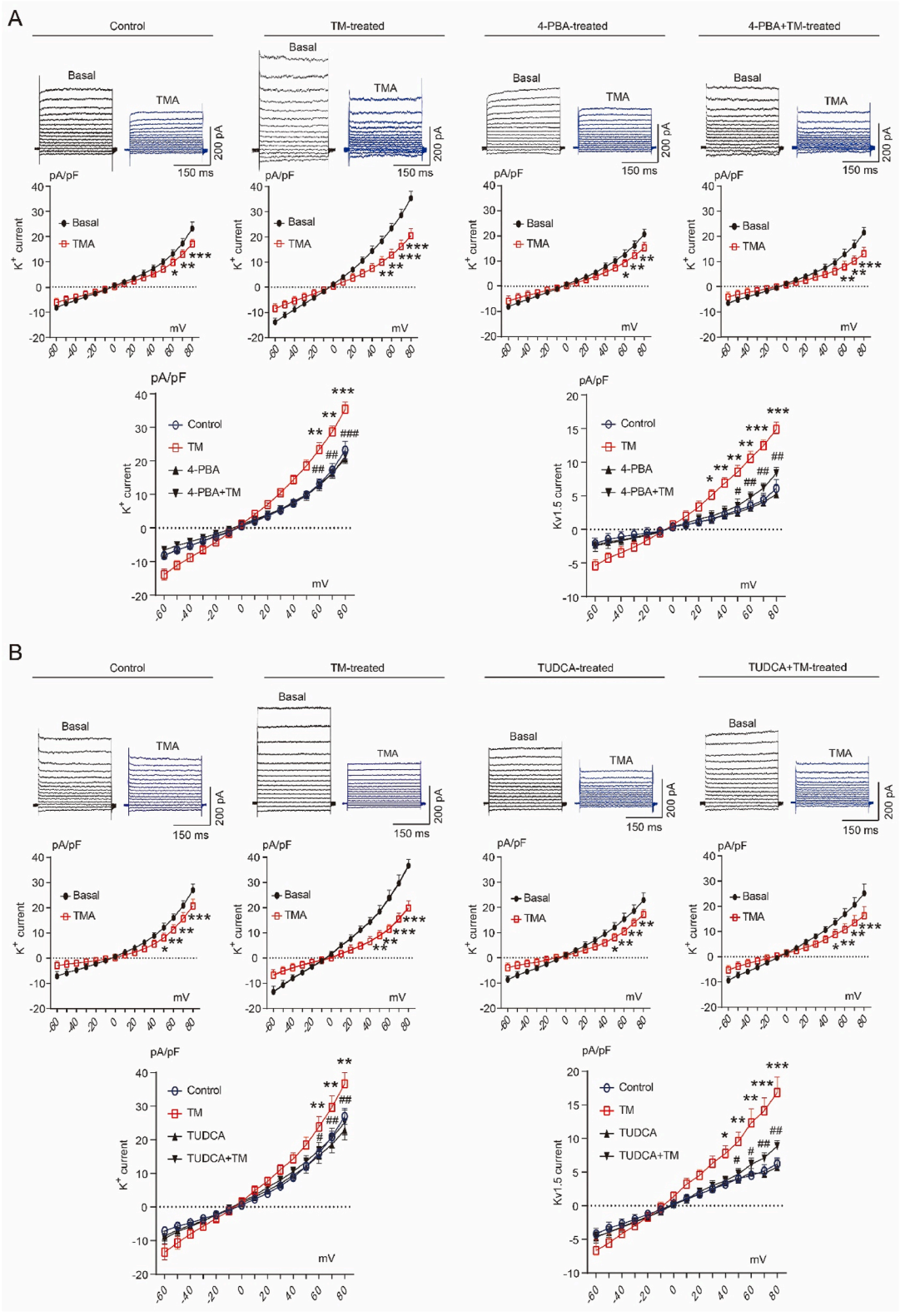

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Fig. 3. Inhibition of ER stress suppresses the enhancement of Kv1.5 channel current in HL-1 atrial myocytes. Treatment of HL-1 cells with ER stress inhibitor 4-PBA **(A)** or TUDCA **(B)** prevents Kv1.5 channels from tunicamycin (TM)-induced activation. Representative traces and I-V curves of whole-cell K^+ currents of cells from different treatment groups before and after application of Kv1.5 channel blocker trimethylapigenin (TMA) **(A&B, *upper panel*)**. Summarized data of whole-cell K^+ current **(A&B, *lower panel-left*)** and Kv1.5 channel current ($I_{Kv1.5} = I_{Basal}$-I_{TMA}) **(A&B, *lower panel-right*)** from different treatment groups (n = 9). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.*TM. n indicates the number of independent experiments performed with cells from different culture batches.

and ATF6 were employed to treat tunicamycin-exposed HL-1 atrial myocytes. The efficacy of each inhibitor was demonstrated by the downregulation of its target molecule, saying, PERK inhibition by GSK2606414, IRE1 inhibition by GSK2850163, and ATF6 inhibition by Ceapin A7 (Fig. 4A). Inhibition of PERK, as evidenced by decreased phosphorylation, attenuated the upregulation of Kv1.5 protein expression, while inhibition of IRE1 and ATF6 showed no reversal effect on tunicamycin-induced Kv1.5 upregulation (Fig. 4B). These results suggested that activation of the PERK branch of UPR leads to Kv1.5 channel upregulation in HL-1 atrial myocytes.

3.5. Inhibition of PERK branch of ER stress normalizes Kv1.5 channel current and APD

In HL-1 atrial myocytes subjected to tunicamycin exposure, co-treatment with the PERK inhibitor GSK2606414 significantly decreased the whole-cell K^+ current (25.44 ± 1.41 *vs.* 35.39 ± 2.06 pA/pF, $P < 0.01$) and the I_{Kur} current carried by Kv1.5 channels (9.03 ± 0.99 vs. 16.38 ± 2.37 pA/pF, $P < 0.05$) (Fig. 5A). The decrease of I_{Kur} current could be explained by the downregulation of Kv1.5 channel expression, as shown in Fig. 4B.

Inhibition of PERK with GSK2606414 attenuated tunicamycin-induced APD shortening. The APD_{90} was restored by GSK2606414 from 82.54 ± 5.58 to 113.69 ± 4.41 ms ($P < 0.01$) (Fig. 5B). Collectively, these results indicated that ER stress induces Kv1.5 channel dysfunction with a resultant APD shortening through activation of the PERK branch of UPR.

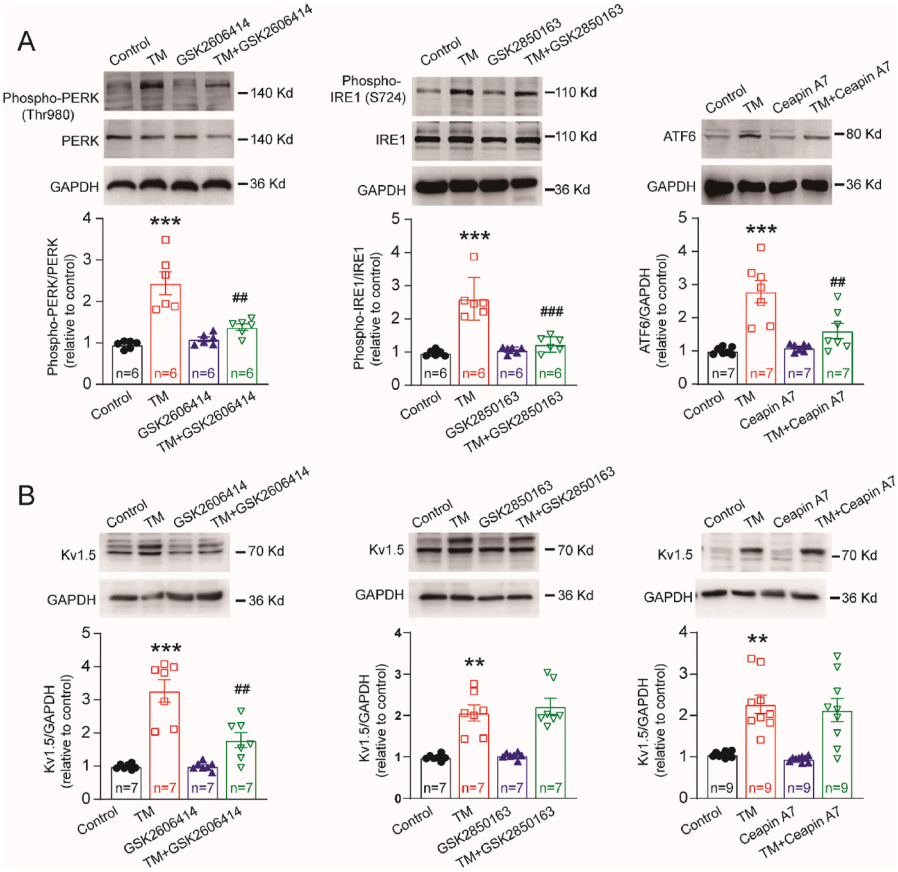

Fig. 4. PERK branch of unfolded protein response mediates ER stress-induced upregulation of Kv1.5 channels in HL-1 atrial myocytes. (A) The PERK inhibitor GSK2606414 and the IRE1 inhibitor GSK2850163 respectively suppressed the phosphorylation of PERK and IRE1, and the ATF6 inhibitor Ceapin A7 lowered ATF6 protein level, showing the effectiveness of each inhibitor. **(B)** Inhibition of PERK with GSK2606414 antagonized tunicamycin (TM)-induced Kv1.5 upregulation, but inhibition of IRE1 and ATF6 showed no reversal effect. Corresponding full blots are shown in Supplementary Figs. 3–4A&B. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.*TM. n indicates the number of independent experiments performed with cells from different culture batches.

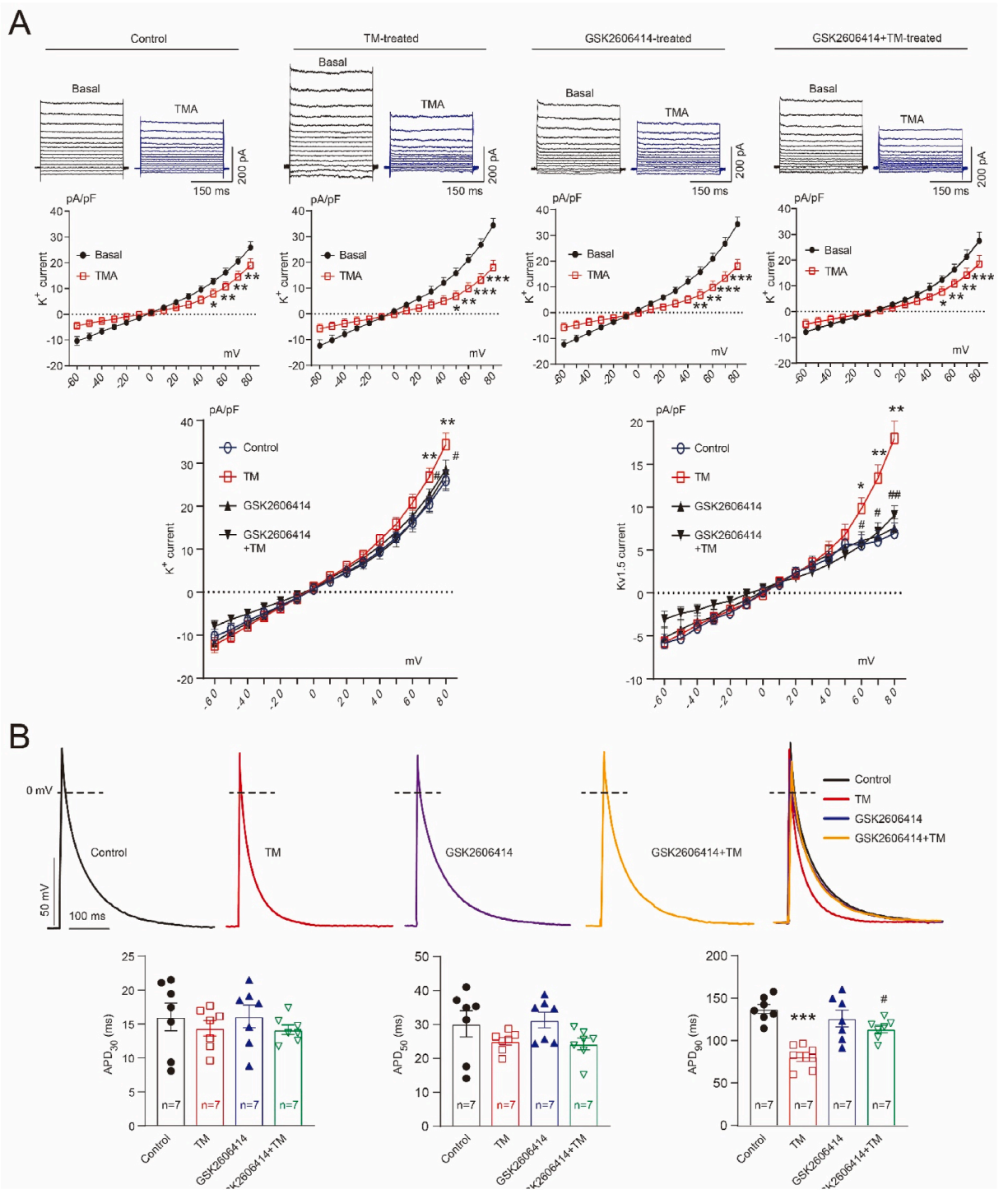

Fig. 5. Inhibition of the PERK branch of ER stress normalizes Kv1.5 channel current and action potential duration (APD). (A) Representative traces and I-V curves of whole-cell K^+ currents of cells from different treatment groups before and after application of Kv1.5 channel blocker trimethylapigenin (TMA) **(A, *upper panel*)**. Summarized data of whole-cell K^+ current **(A, *lower panel-left*)** and the Kv1.5 channel current ($I_{Kv1.5} = I_{Basal}$-I_{TMA}) **(A, *lower panel-right*)** of cells from different treatment groups (n = 9). **(B)** Representative traces of action potential and grouped data of APD (n = 7), showing the restoration of APD_{90} by the PERK inhibitor GSK2606414 in tunicamycin (TM)-exposed HL-1 atrial myocytes. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*.TM. n indicates the number of independent experiments performed with cells from different culture batches.

Further experiments with thapsigargin, which triggers ER stress by a mechanism different from tunicamycin, also proved the mediatory role of PERK in Kv1.5 channel activation. As shown in Fig. 6, HL-1 atrial myocytes incubated with thapsigargin showed an increased expression of ER chaperone GRP78 and activation of PERK branch, meanwhile, increased expression of Kv1.5 channels and enhancement of Kv1.5 channel current were observed (15.16 ± 0.82 *vs.* 8.94 ± 0.73 pA/pF in control, $P < 0.01$). Inhibition of PERK activation with GSK2606414 during thapsigargin incubation significantly lowered the expression level of Kv1.5, which was accompanied by a decrease in Kv1.5 channel current (10.01 ± 0.75 pA/pF, $P < 0.01$ *vs.* thapsigargin) (Fig. 6).

3.6. TMP prevents Kv1.5-associated APD shortening through antagonizing ER stress

TMP inhibits the expression of GRP78 and ATF6 as well as the phosphorylation of PERK and IRE1 in HL-1 atrial myocytes exposed to tunicamycin, indicating the anti-ER stress effect of TMP (Fig. 7A). Treating the tunicamycin-exposed HL-1 atrial myocytes with TMP significantly suppressed the enhancement of I_{Kur} (10.52 ± 0.97 *vs.* 17.52 ± 2.25 pA/pF, $P < 0.05$) and such inhibition resulted in a decrease in whole-cell K^+ current (26.49 ± 1.95 *vs.* 37.96 ± 2.84 pA/pF, $P < 0.01$) (Fig. 7B). The decrease of I_{Kur} was associated with a downregulation of Kv1.5 channel expression (Fig. 7C). AP recording showed that TMP treatment prevents APD shortening induced by ER stress. The APD_{90} was increased to 110.16 ± 5.36 ms in cells co-incubated with tunicamycin and TMP, comparing to 84.84 ± 4.58 ms in cells incubated with tunicamycin alone ($P < 0.05$). (Fig. 7D).

4. Discussion

The present study demonstrated that 1) Kv1.5 upregulation/activation contributes to ER-stress induced APD shortening in atrial myocytes; 2) The PERK branch of UPR mediates ER stress-induced upregulation of Kv1.5 channels; 3) TMP prevents Kv1.5 upregulation/activation and resultant APD shortening by inhibiting ER stress.

HL-1 atrial myocytes derived from the AT-1 mouse atrial cardiomyocyte tumor lineage maintain a cardiac-specific phenotype and display a prominent delayed rectifier K^+ current [30], which makes it a good experimental system for studying arrhythmia, *e.g.* atrial fibrillation, and ion channels bearing delayed rectifier K^+ currents including Kv1.5 [31,32]. TMA was proved to be an effective blocker for Kv1.5 channels, both in HEK 293 cells overexpressed with hKv1.5 (IC_{50} = 6.4 μmol/L) and in human atrial myocytes (IC_{50} = 8.0 μmol/L) [28]. In this study, with the use of TMA (30 μmol/L), we identified the ultrarapid delayed rectifier K^+ current I_{Kur} carried by Kv1.5 channels, which forms an important component of the K^+ current in HL-1 cells. Supplementary Fig. 2 illustrated the concentration-response relationship of the blocking effect of TMA (1, 3, 10, 30, and 100 μmol/L) on I_{Kur} in HL-1 cells, showing IC_{50} at 6.37 ± 1.36 μmol/L and near-maximal inhibition at 30 μmol/L.

Re-entry is a major mechanism underlying *arrhythmogenesis* [33,34]. APD shortening caused by ion channel dysfunction results in a short effective refractory period (ERP), and ERP shortening promotes re-entry [35]. Studies in animal models and in patients with chronic AF demonstrated the contributing role of decreased L-type Ca^{2+} current ($I_{Ca,L}$), increased inward rectifier K^+ current (I_{K1}), and increased constitutively-active acetylcholine-independent K^+ current ($I_{K,ACh}$) in APD shortening [8,36,37]. I_{Kur} plays an important role in AP repolarization and it is affected by AP morphology. For example, short, triangular AP could enhance I_{Kur}, which increases the contribution of I_{Kur} to atrial repolarization [38]. Van Wagoner et al. showed that in atrial myocytes isolated from patients with chronic AF, reduction of I_{Kur} density is associated with a downregulation of Kv1.5 protein [11]. Christophersen and colleagues found that patients with early-onset lone AF have high prevalence of variants in *KCNA5*. Both loss-of-function and gain-of-function of Kv1.5 mutations enhance AF susceptibility, resulting from prolongation or shorting of the atrial AP and the ERP [15]. Studies in SNX17 knockout rats and in rats subjected to rapid pacing revealed an association between AF susceptibility and APD shortening due to increased expression of Kv1.5 [14]. In this study, we demonstrated that Kv1.5 channel expresses in response to ER stress, leading to an enhancement of I_{Kur} and resulting in APD shortening in HL-1 atrial myocytes. This is to our knowledge the first evidence showing the regulatory effect of ER stress on the expression and function of Kv1.5 channels.

Previous studies have suggested the potential of ER stress inhibitor in the treatment of AF. Intraperitoneal injection of 4-PBA lowered AF incidence and shortened AF duration in mice fed a high-fat diet [39]. In tachypaced HL-1 myocytes, 4-PBA prevented autophagy activation and Ca^{2+}transient loss [40]. In vivo treatment with sodium salt of PBA (Na-PBA) attenuated AF progression in atrial-tachypaced dogs and further experiments using isolated atrial cardiomyocytes from the dog showed Na-PBA protects against tachypacing-induced electrical remodeling, including shortening of APD and reductions in L-type Ca^{2+} channel current [36]. Our finding regarding the reversal effect of 4-PBA and TUDCA on APD shortening in ER stressed-HL-1 myocytes is consistent with previous reports. These data in conjunction with the results showing the antagonizing effect of 4-PBA and TUDCA on I_{Kur} enhancement provided new mechanistic insight into the role of ER stress in atrial electrical remodeling.

Further experiments using specific inhibitors targeting the three main ER stress sensors, saying, PERK, IRE1 and ATF6, showed that only the PERK inhibitor could attenuate the upregulation of Kv1.5 channel expression, along with a significant decrease of I_{Kur} and reversal of APD shortening. This indicated that ER stress modulates Kv1.5 channels through PERK-mediated UPR pathway, implicating a potential of targeting PERK in atrial electrical remodeling. Future studies are warranted to clarify how PERK induces Kv1.5 protein expression, which may help identify more targetable molecules to prevent Kv1.5 channel dysfunction and adverse electrical remodeling. It is known that PERK activation abates general mRNA translation but selectively promotes the translation of the transcription factor ATF4 [41]. There are studies suggesting ATF4-dependent activation of Akt including ChIP assay showing the binding of ATF4 to the promoter sequence of Akt1 [42,43]. In conjunction with evidence showing Akt-mediated upregulation of Kv1.5 channels [44,45], we speculate that PERK activation may induce Kv1.5 channel expression through ATF4/Akt-dependent mechanism, which however needs to be confirmed in future studies.

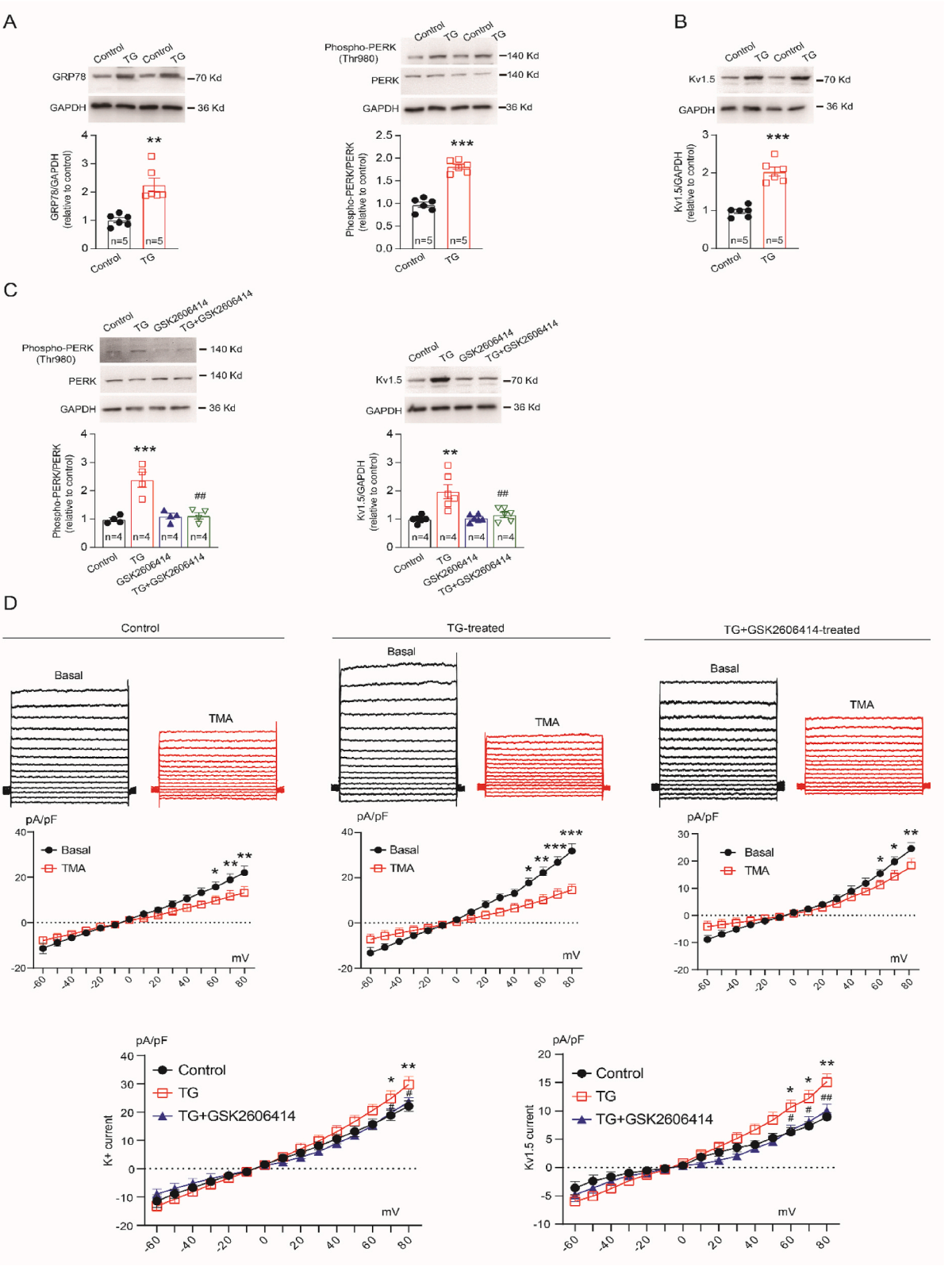

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Fig. 6. Inhibition of PERK activation triggered by thapsigargin (TG) prevents the increase of Kv1.5 protein and Kv1.5 channel current. (A) TG induced ER stress in HL-1 cells, involving activation of the PERK branch. **(B)** TG upregulated the protein expression of Kv1.5 channels. **(C)** Inhibition of PERK by GSK2606414 prevented the upregulation of Kv1.5 protein induced by TG. Corresponding full blots are shown in Supplementary Fig.3-6A, 6B and 6C. (D) Inhibition of PERK by GSK2606414 prevented TG-induced enhancement of Kv1.5 channel current. Representative traces and I-V curves of whole-cell K^+ currents of cells from different treatment groups before and after application of Kv1.5 channel blocker trimethylapigenin (TMA) **(D, *upper panel*)**. Summarized data of whole-cell K^+ current **(D, *lower panel-left*)** and the Kv1.5 channel current ($I_{Kv1.5} = I_{Basal}$-I_{TMA}) **(D, *lower panel-right*)** of cells from different treatment groups (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* control; $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 *vs.*TG. n indicates the number of independent experiments performed with cells from different culture batches.

Another important finding of this study is that the traditional Chinese medical herb TMP may protect Kv1.5 channels from ER stress-induced dysfunction. The increase of Kv1.5 protein expression and the enhancement of I_{Kur} were significantly downregulated by TMP in HL-1 myocytes subjected to tunicamycin, and such downregulation led to restoration of APD. Determination of ER stress molecules confirmed the involvement of anti-ER stress mechanism in TMP-conferred protection on Kv1.5 channels. Activation of all the three UPR branches was inhibited by TMP, evidenced as downregulation of ATF6 and decrease of PERK and IRE1 phosphorylation. These data were consistent with our previous work in vasculature showing the potent anti-ER stress capacity of TMP. Through inhibiting ER stress, TMP alleviates angiotensin II- and homocysteine-induced coronary dilator dysfunction, and antagonizing ER stress-mediated loss of BK_{Ca} β1 subunits underlies the protection against homocysteine conferred by TMP on BK_{Ca} channel function [22,23]. The present study added another piece of evidence indicating the anti-ER stress property of TMP and furthered our understanding of the antiarrhythmic mechanisms of TMP.

This study has several limitations. One is that the patch clamp recording was conducted at room temperature. Though such condition is widely adopted for patch clamp experiments, we are aware of the influence of temperature on APD values [29,46]. Although changes in the absolute value of APDs will not affect the data interpretation and conclusion of this study, future studies conducted at physiological temperature may yield data with more clinical relevance. Another limitation is that all the experiments were performed in vitro, further in vivo studies are warranted to validate the link between ER stress and Kv1.5 channel dysfunction in cardiac arrhythmia. In addition, there is one issue worthy of further discussion and exploration. Immunoblot detection of Kv1.5 protein shows two bands with molecular masses of ~75 kDa and ~68 kDa, which are known to represent the mature fully glycosylated form and the immature core-glycosylated form of the channel respectively. As a glycosylation inhibitor, tunicamycin is likely to decrease the density of the higher molecular weight band. However, our immunobloting data does not support this notion. In most experiments, the density of both higher and lower molecular weight bands was observed to be significantly enhanced. We assume this may be due to a significant increase in the overall protein level of Kv1.5 resulted from PERK activation, which surpasses (or masks) the glycosylation-related impact on the visualization of Kv1.5 immunoblots, at least in the experimental setting of the present study. Besides, as to the Kv1.5 polyclonal antibody used, we in fact do not know to what extent it is glycosyl epitope-specific.

In conclusion, the present study demonstrated that ER stress upregulates and activates Kv1.5 channels in HL-1 atrial myocytes via the PERK branch of UPR, and TMP prevents Kv1.5 upregulation/activation and resultant APD shortening through inhibiting ER stress. These results may shed light on the mechanisms of atrial arrhythmogenesis and the antiarrhythmic effect of the traditional Chinese herb TMP.

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Data availability statement

Data associated with this study is included in article/supp. material/referenced in the article.

CRediT authorship contribution statement

Xiang-Chong Wang: Writing – original draft, Visualization, Investigation, Formal analysis. **Yang Zhou:** Investigation. **Huan-Xin Chen:** Investigation. **Hai-Tao Hou:** Investigation. **Guo-Wei He:** Supervision, Resources. **Qin Yang:** Writing – review & editing, Visualization, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

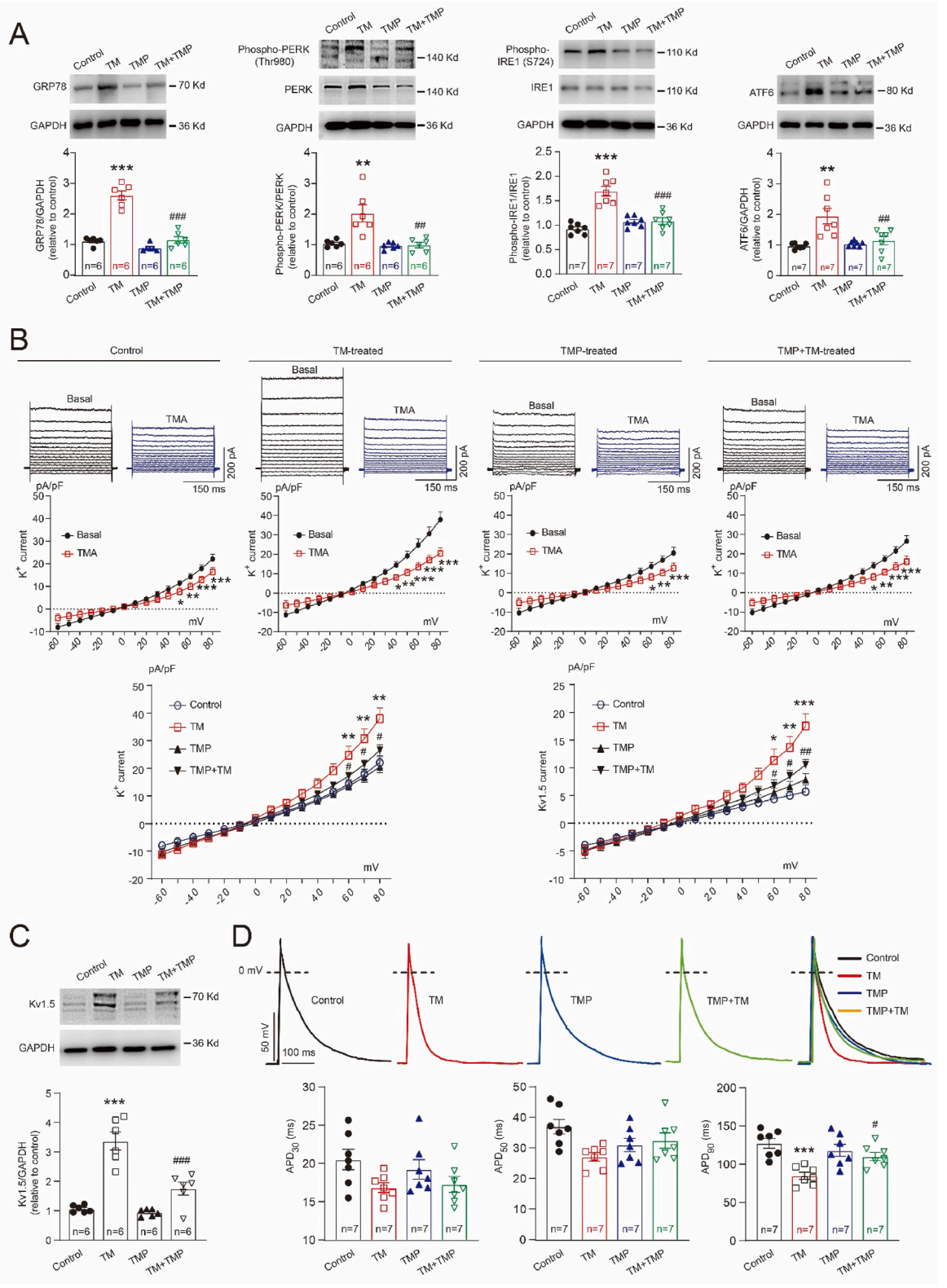

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Fig. 7. Tetramethylpyrazine (TMP) prevents Kv1.5-associated APD shortening through antagonizing ER stress in HL-1 atrial myocytes. (A) TMP exhibits strong anti-ER stress activity against tunicamycin (TM) in HL-1 atrial myocytes. Full blots are shown in Supplementary Figs. 3–7A. (B) TMP suppresses Kv1.5 channel current in HL-1 atrial myocytes exposed to TM. Representative traces and I-V curves of whole-cell K^+ current from different treatment groups before and after application of Kv1.5 channel blocker trimethylapigenin (TMA) **(B, *upper panel*)**. Summarized data of whole-cell K^+ current **(B, *lower panel-left*)** and the Kv1.5 channel current ($I_{Kv1.5} = I_{Basal}\text{-}I_{TMA}$) **(B, *lower panel-right*)** of cells from different treatment groups (n = 9). **(C)** TMP inhibits TM-induced expression of Kv1.5 channels (n = 6). Full blots are shown in Supplementary Figs. 3–7C. (D) TMP prevents TM-induced APD shortening. Representative action potential traces from different treatment groups **(D, *upper panel*)**. Summarized data showing the restoration of APD_{90} by TMP **(D, *lower panel*,** n = 7). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs*. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs*.TM. n indicates the number of independent experiments performed with cells from different culture batches.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37767.

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