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Myricetin inhibits $K_v1.5$ channels in HEK293 cells

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Abstract. Myricetin (Myr) is a flavonoid that exerts anti-arrhythmic effects. However, its potential effects on ion channels have remained elusive. The aim of the present study was to investigate the effects of Myr on $K_v1.5$ channels in HEK293 cells. The current of $K_v1.5$ channels (I_{kur}) in HEK293 cells was recorded using the whole-cell patch-clamp technique and the expression of the $K_v1.5$ protein was measured using western blot analysis 24 h after treatment with Myr. The results showed that 5 μM Myr significantly reduced I_{kur} from 215.04±40.59 to 77.72±17.94 pA/pF ($P<0.05$; n=5). Myr increased the current suppression from 0 to 0.31±0.12 and 0.55±0.11 over 5 or 20 min, respectively. In addition, I_{kur} decreased from 376.23±1.30 to 270.19±4.28 pA/pF when the frequency was increased from 0.5 to 4 Hz in HEK293 cells treated with 10 μM Myr for 5 min. Furthermore, Myr reduced $hK_v1.5$ protein expression in a dose-dependent manner. These results demonstrated that Myr inhibited I_{kur} and the expression of $hK_v1.5$ in HEK293 cells in a dose-, time- and frequency-dependent manner. These observations partly explained the mechanisms by which Myr exerts anti-arrhythmic effect.

Introduction

Atrial fibrillation (AF) is a common type of tachyarrhythmia (1), and has drawn the attention of researchers due to its high incidence and serious complications (2,3). However, the optimal method of treating AF has remained controversial (4,5). Therefore, it is crucial to identify more effective and safer treatments, particularly those using atrial-selective blockers.

The current of $K_v1.5$ channels (I_{kur}) is the main outward ion flow during atrial action potential re-polarization. The *KCNA5* gene encodes the $K_v1.5$ sub-unit, which is the main molecular component of I_{kur}. I_{kur} is present in the atrium (6), but not in ventricles (7); therefore, I_{kur} is a potential target for atrium-specific arrhythmia therapy.

Myricetin (Myr) is a flavonoid compound present in numerous plants, including *Myrica rubra* (Lour.) Zucc. Its chemical structure is shown in Fig. 1A. Myr has a variety of pharmacological effects, including anti-bacterial (8), analgesic (8), anti-tumor (9,10), anti-allergic (11-13), anti-oxidant (14,15), blood sugar-lowering (16) and hepatoprotective actions (17). It also exerts protective effects against cardiovascular diseases, including atherosclerosis (18), ischemia-reperfusion injury (19), myocardial infarction (20) and hypertension (21,22). However, the mechanisms by which Myr exerts anti-arrhythmic effects have remained elusive. It has been indicated that a Myr derivative, hexamethyl Myr, is able to inhibit I_{kur} (23); therefore, the present study examined whether Myr also inhibits I_{kur} and investigated the underlying mechanisms.

Materials and methods

Reagents. Myr (98% purity) was provided by Dr Yong Ye (College of Pharmacy, Guangxi Medical University, Nanning, China). It was prepared as a 10-mM stock solution in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored in aliquots at 4˚C prior to use. For use in the subsequent experiments, it was diluted to 0.5, 3 or 10 μM with extracellular fluid, as described below.

Solutions. The I_{kur} recording solution (extracellular solution) consisted of 130 mM NaCl and 0.33 mM Na_2HPO_4 $x12H_2O$ (both from KeLong Chemical, Chengdu, China) as well as 5.4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (all from Sangon Biotech Co. Ltd., Shanghai, China), 5.5 mM glucose (Bio Basic Inc, Markham, OT, Canada) and NaOH (Jinshan Chemical Reagent Co. Ltd, Chengdu, China) to adjust the pH to 7.4. The electrode solution contained 110 mM L-aspartate (Sigma-Aldrich), 110 mM KOH (Sangon Biotech Co. Ltd.),

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20 mM KCl, 8 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM ethylene glycol tetraacetic acid (all from Sigma-Aldrich), 10 mM HEPES (Sangon Biotech Co. Ltd.) and KOH to adjust the pH to 7.2; 5 mM Mg-adenosine triphosphate (Sigma-Aldrich) was added immediately prior to use, and the solution was filtered through 0.22-μm microporous membranes (Merck Millipore, Carrigtwohill, Republic of Ireland). The lysis buffer consisted of 250 mM glucose, 20 mM 3-(*N*-morpholino)propanesulfonic acid and l mM dithiothreitol (Sigma-Aldrich); 1% (v/v) protease inhibitor (Calbiochem; EMD Millipore, Billerica, MA, USA) was added prior to use.

Cell culture and transfection. HEK293 cells (American Type Tissue Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and penicillin/streptomycin (Sigma-Aldrich). Cells were transiently transfected with $hK_v1.5$-overexpression vector in six-well plates (Corning-Costar, Corning, NY, USA) using 3 μl Entranster^{TM}-H transfection reagent (Engreen Biosystem Co. Ltd, Beijing, China) and 3 μg of $hK_v1.5$ cDNA in pEGFP-N1 vector (Sangon Biotech Co. Ltd.). A pEGFP-N1 was used as a negative control. Subsequent assays were performed at 24 h subsequent to transfection. Cells used for electrophysiology experiments were seeded at a density of $1x10^4$ cells/35-mm dish with glass cover slips.

MTT assay. MTT assays were used to assess the toxicity of Myr. Logarithmically growing cells were seeded at a density of $1\text{-}5x10^4$ cells/well in 96-well plates (Corning-Costar) and were allowed to adhere for 24 h at 37˚C. The medium was then replaced with fresh medium containing various concentrations of Myr. The cells were maintained for two days and the toxicity of Myr was determined using MTT (Amresco LLC, Solon, OH, USA). Ten microliters of MTT (5 mg/ml stock) was added to each well followed by incubation for 4 h. The seeding medium was then discarded and 0.15 ml DMSO was added to each well. The absorbance (A) was read using a multimode microplate reader (Infinite M200; Tecan Group, Ltd., Männedorf, Switzerland) at 570 nm and the cell viability was determined as follows: $(1\text{-}A_{\text{treated group}}/A_{\text{control group}}$ x100.

Western blot analysis. Cells ($4x10^5$/ml) were incubated with or without Myr (0-100 μM) for 24 h in six-well plates. Cells were collected and lysed in lysis buffer. After sonication in ice water, crude lysates were cleared by centrifugation at 12,000 xg for 5 min at 4˚C. The total protein concentration of the lysates was measured using a bicinchoninic acid protein assay kit (Solarbio, Beijing, China). Lysates were diluted at a 4:1 ratio with 5X loading buffer (Beyotime Institute of Biotechnology, Haimen, China) and boiled for 5 min. Equal amounts of protein (10 μg) were loaded onto 10% SDS-polyacrylamide gels and separated by electrophoresis using an electrophoresis apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The separated proteins were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Immun-Blot® PVDF membrane; Bio-Rad Laboratories, Inc.). Following electrotransfer, the membranes were blocked with 5% non-fat milk (Yili Industrial Group Co. Ltd, Inner Mongolia, China) in Tris-buffered saline with Tween 20 for 60 min on an orbital shaker (Qilinbeier Instrument and Manufacture Co. Ltd, Haimen, China) and then incubated with rabbit polyclonal primary antibodies against $K_v1.5$ (A03K0017; 1:200 dilution; BlueGene Biotech Co., Ltd., Shanghai, China) overnight at 4˚C followed by biotin (AP132B; 1:10,000; EMD Millipore) for 1 h and goat anti-rabbit horseradish peroxidase-conjugated antibodies (sc-2004; 1:50,000; Santa Cruz Biotechnology, Dallas, TX, USA) for 30 min at room temperature. Rabbit polyclonal antibody of the endogenous protein GAPDH (Santa Cruz Biotechnology) was used as a loading control. Protein bands were detected using enhanced chemiluminescence western blotting substrate (Pierce Biotechnology Inc, Rockford, IL, USA), imaged using the Universal Hood II system (Bio-Rad Laboratories, Inc.) and quantified using Quantity one software v4.6.2 (Bio-Rad Laboratories, Inc.).

Patch-clamp recordings. Currents were recorded using the whole-cell patch-clamp technique at room temperature (~22˚C). The protocol used 200-msec voltage steps between -50 mV and +60 mV from a holding potential of -80 mV, followed by a return to -40 mV (Fig. 2A). The current amplitude was measured from 150 msec to the end of the depolarization step. Borosilicate glass electrodes (1.2 mm optical density) were pulled using a Brown-Flaming puller (model P-97; Sutter Instrument Co, Novato, CA, USA); they had a tip resistance of 5-7 MΩ when filled with the electrode solution. The membrane current was recorded in voltage-clamp mode using an EPC-10 amplifier and Pulse software (Heka Elektronik, Lambrecht, Germany). A 3 M NaCl-agar salt bridge was used as the reference electrode. The tip potential was set to zero before the patch pipette touched the cell. After a GΩ seal was obtained, the cell membrane was ruptured using gentle suction to establish the whole-cell configuration. The series resistance (Rs) and membrane capacitance were compensated prior to the onset of recordings. The membrane capacity (Cm) was recorded using 10-msec voltage steps to -65 mV from a holding potential of -60 mV, followed by a return to -60 mV (Fig. 2B). The formula used to calculate the membrane capacity was as follows: Cm=0.98x A x τ/5, with amp as the current amplitude and τ as the time constant. I_{kur} was recorded at various time-points in the presence of various concentrations of Myr, and Myr-untreated cells were used as a control. Prior to measurements, cells were perfused with extracellular solution containing various concentrations of Myr at a rate of 3 ml/min. The perfusion volume was ~15 ml per experiment so that the original solution was replaced completely. The Myr incubation time-dependency was analyzed using the mean ± standard error of the current suppression ratio $(I_C\text{-}I_A)/I_C$, where I_C represents the I_{kur} in the control group and I_A stands for I_{kur} in the presence of Myr.

Statistical analysis. Electrophysiological data were analyzed using Patchmaster (Heka Elektronik), Clampfit 10.0 (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA), and Origin 9 software (OriginLab, Northampton, MA, USA). The results were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA), and values are expressed as the mean ± standard error. Statistical comparisons were made using Student's *t*-tests for two groups of data, or analysis of variance for multiple groups of data. $P<0.05$ was considered to indicate a statistically significant difference.

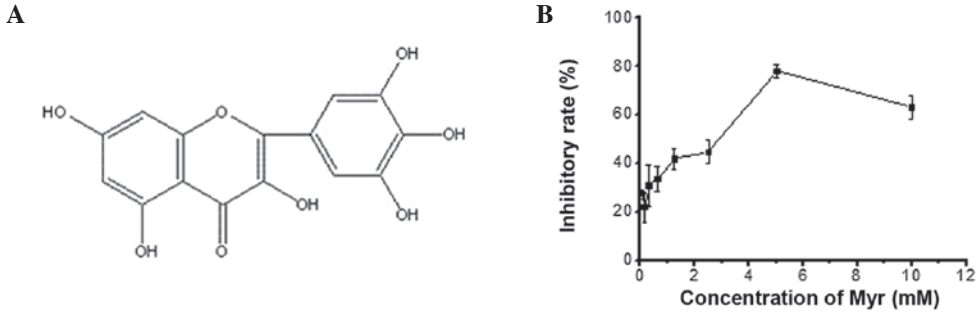

Figure 1. Toxicity of Myr. (A) Chemical structure of Myr. (B) Dose-dependent effects of Myr on the viability of HEK293 cells. Myr was non-toxic to HEK293 cells at doses of 0-100 μM, which rendered it suitable for use in patch-clamp experiments at these concentrations. Results are representative of a minimum of three independent experiments. Myr, myricetin.

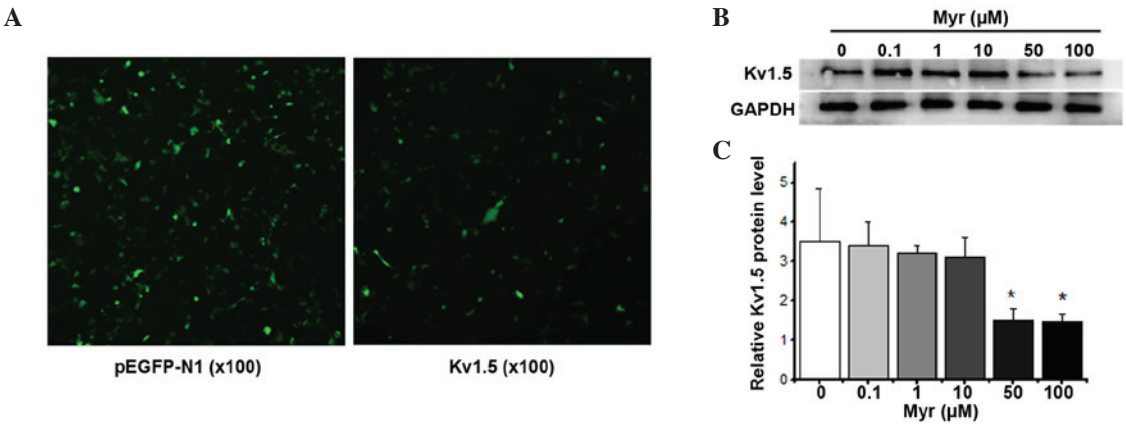

Figure 2. Myr inhibits hK$_v$1.5 protein expression. (A) Images of HEK293 cells subsequent to transient transfection with the human K$_v$1.5 wild type plasmid (hK$_v$1.5) using Entranster™-H transfection reagent for 24 h. A pEGFP-N1 plasmid was used as a negative control. (B) Effect of Myr on K$_v$1.5 expression. The levels of K$_v$1.5 protein were assessed using western blot analysis following treatment with Myr (0-100 μM) for 24 h. Myr induced a dose-dependent decrease in the expression of K$_v$1.5 after 24 h. (C) Quantitative analysis of K$_v$1.5 protein expression. The data are expressed as the mean ± standard deviation. *P<0.05 vs. control group (0 μM Myr). The data were calculated from three independent experiments. Myr, myricetin.

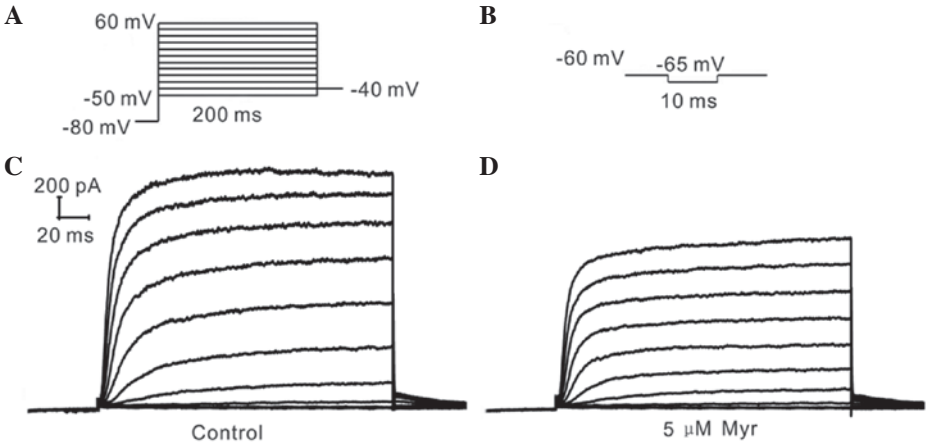

Figure 3. Myr inhibits I_{kur}. (A and B) Schematics representing the protocols used to record I_{kur} and membrane capacitance. (C and D) I_{kur} in a typical HEK293 cell transiently expressing the *hK$_v$1.5* gene in (C) the absence (control) and (D) in the presence of 5 μM. I_{kur}, current of K$_v$1.5 channels; Myr. Myr, myricetin.

Results

Toxicity of Myr. To determine an effective, non-toxic concentration of Myr for use in the patch-clamp assay, MTT assays were performed following treatment of HEK293 cells with Myr at a range of doses. Myr caused a dose-dependent decrease in cell growth between 2 and 6 mM with an IC_{50} of 4.66 mM (Fig. 1B). However, no cell growth inhibition was observed at 0-100 μM Myr, which was therefore used in the subsequent experiments.

Myr inhibits hK$_v$1.5 protein expression in a dose-dependent manner. A K$_v$1.5 wild-type overexpression plasmid was transiently transfected into HEK293 cells using Entranster™-H

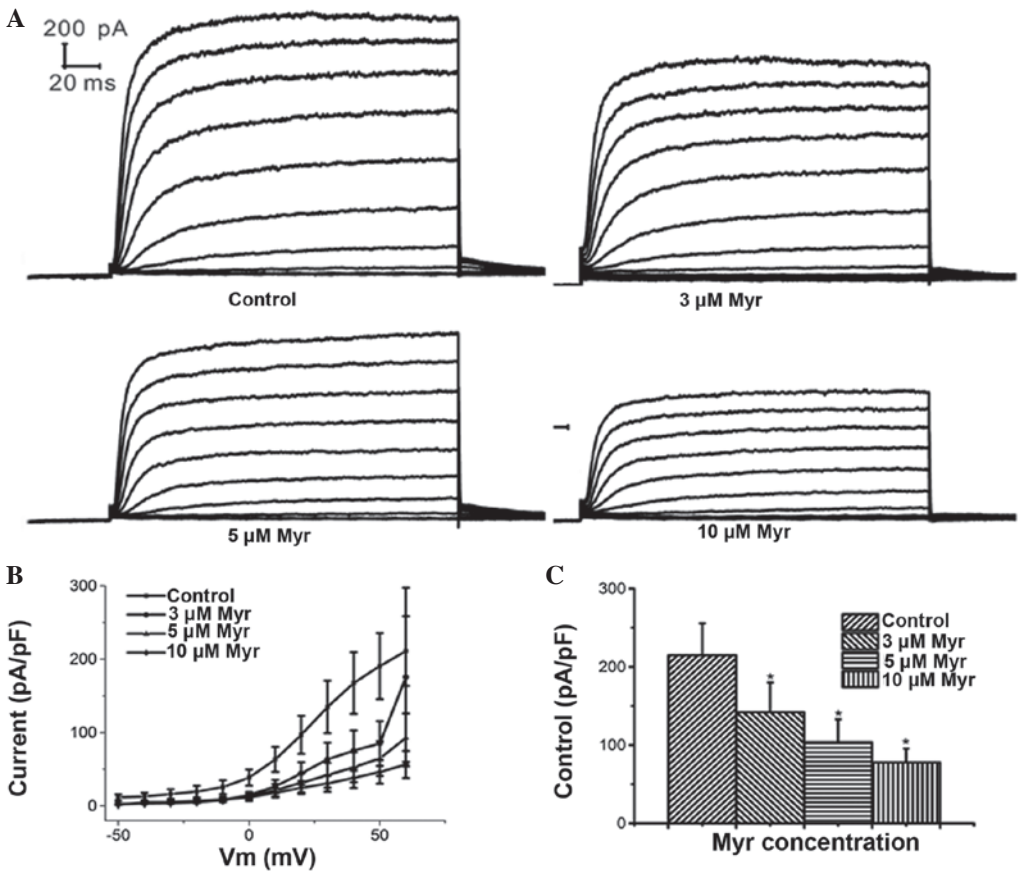

Figure 4. Myr inhibits I_{kur} in a dose-dependent manner. (A) The I_{kur} amplitude in the absence (control) and presence of various concentrations of Myr. (B) Current-voltage associations of I_{kur} in control cells and in the presence of 3, 5 or 10 μM Myr. (C) Values are expressed as I_{kur}, at 60 mV, Myr significantly reduced I_{kur} in a dose-dependent manner. Values are expressed as the mean ± standard error. *P<0.05 vs. control group; n=5. Myr, myricetin; I_{kur}, current of K_v1.5 channels.

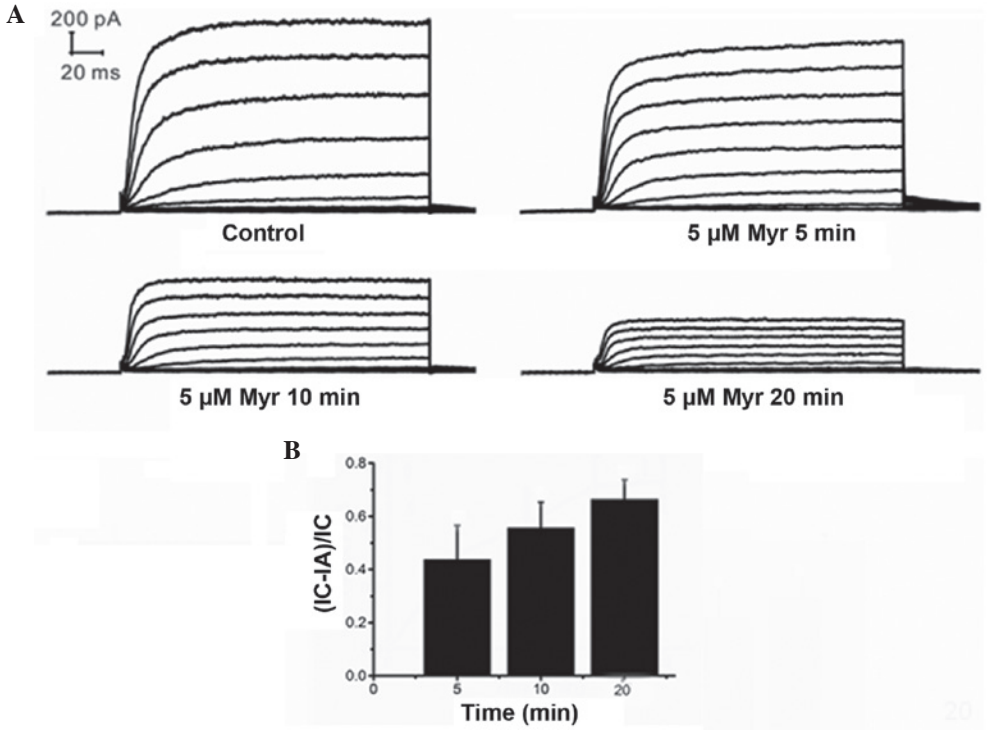

Figure 5. Myr inhibits I_{kur} in a time-dependent manner. (A) Time course of Ikur recorded in typical HEK293 cell transiently expressing hK_v1.5 gene in the absence and presence of 5 μM Myr with a 200-ms test pulse from -80 to +60 mV then back to -40 mV. The inhibitory effect of Myr on I_{kur} is time-dependent. (B) Drug-sensitive current expressed as a proportion of the current in the absence (IC) or presence of Myr (IA). Time-dependency of (IC-IA)/IC. Values are expressed as the mean ± standard error (n=7). Myr, myricetin; I_{kur}, current of K_v1.5 channels; IC, I_{kur} in control group; IA, I_{kur} in the presence of Myr; (IC-IA)/IC, current suppression ratio.

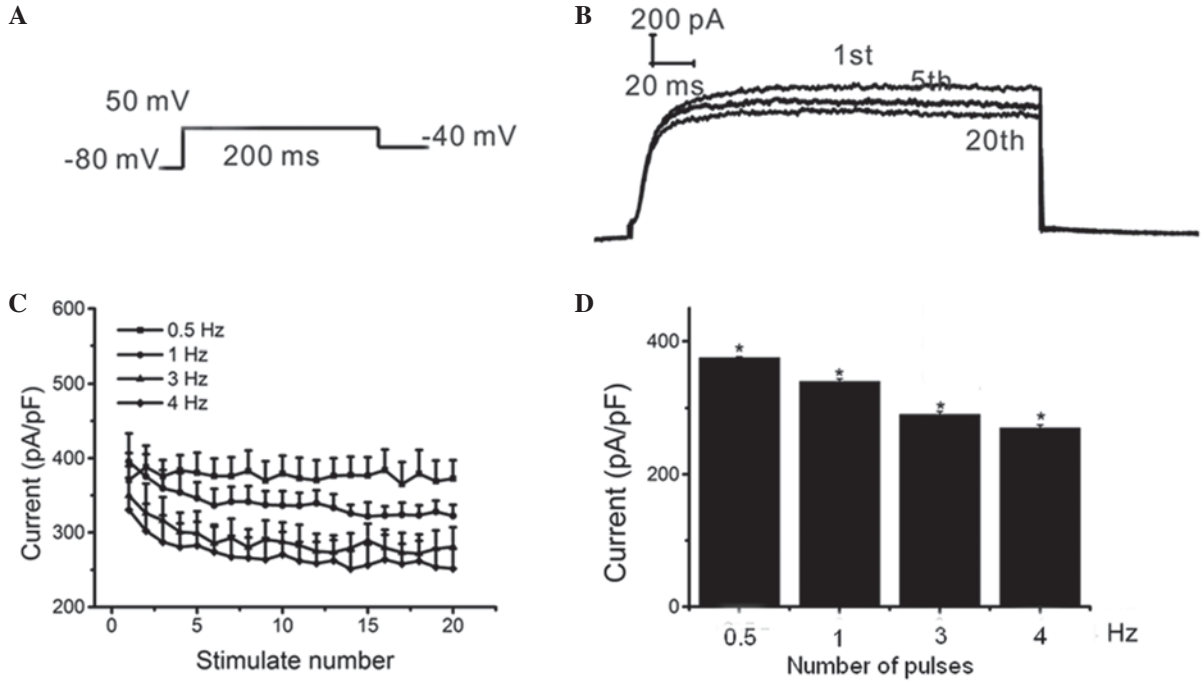

Figure 6. Myr inhibits I_{kur} in a frequency-dependent manner. (A) Schematic depicting the protocol used to record I_{kur}. (B) I_{kur} traces recorded with 200-msec single voltage steps from a holding potential of -80 mV to +50 mV using a train of 20 pulses at 3 Hz in cells exposed to 10 μM Myr for 5 min. The inhibition was stronger at pulse 20 compared with that at the earlier pulses, suggesting use-dependent inhibitory effects. (C) Inhibition of I_{kur} at various frequencies (0.5, 1, 3 and 4 Hz). The use-dependent effects increased as the depolarization frequency was raised. (D) I_{kur} at various pulse frequencies (0.5, 1, 3 and 4 Hz). Values are expressed as the mean ± standard error (n=5). *P<0.05 between each set of two groups. Myr, myricetin; I_{kur}, current of $K_v1.5$ channels.

transfection reagent (Engreen Biosystem Co., Ltd., Beijing, China) for 24 h (Fig. 2A), and the levels of $K_v1.5$ protein were assessed using western blot analysis after treatment with Myr (0-100 μM) for 24 h (Fig. 2B). Myr induced a dose-dependent decrease in the expression of $K_v1.5$ after 24 h.

Myr inhibits I_{kur}. Patch clamp experiments on cells perfused with extracellular solution containing various concentrations of Myr showed that 5 μM Myr reduced the current amplitude from 215.01±40.59 to 77.72±17.94 pA/pF, suggesting that I_{kur} was significantly inhibited by Myr (P=0.011 vs. control; n=5) (Fig. 3).

Myr inhibits I_{kur} in a dose-dependent manner. At a clamping voltage of 60 mV, the current density (Im/Cm, pA/pF) was 142.15±37.80, 103.75±29.32 and 77.72±17.94 in the presence of 3, 5 and 10 μM Myr, respectively (Fig. 4A-C), which was significantly different from that in the control group (215.01±40.59 pA/pF; all P<0.05; n=5). This suggested that Myr inhibited I_{kur} at concentrations of 3-10 μM in a concentration-dependent manner.

Myr inhibits I_{kur} in a time-dependent manner. I_{kur} was recorded from 5 to 30 min after the addition of Myr to the chamber to evaluate the time-dependent effects of Myr on I_{kur}. The results indicated that the effects of Myr were time-dependent (Fig. 5A and B). The effects of time were analyzed using the mean ± standard error of the current suppression ratio (IC-IA)/IC, where IC represents the I_{kur} in the control group and IA stands for I_{kur} in the presence of Myr. The (IC-IA)/IC was 0.3101±0.1234 at 5 min (n=7; P=0.046 vs. control), 0.4091±0.1180 at 10 min (n=7; P=0.066 vs. 5 min; P=0.013 vs. control), 0.05352±0.0978 at 15 min (n=7, P=0.004 vs. 10 min; P=0.009 vs. 5 min; P=0.002 vs. control), and 0.5497±0.1060 at 20 min (n=7; P=0.453 vs. 15 min; P=0.023 vs. 10 min; P=0.004 vs. control). These results demonstrated that the I_{kur} amplitude decreased with time and that the inhibitory effects of Myr increased over time.

Myr inhibits I_{kur} in a frequency-dependent manner. A train of 20 pulses, each with a 200-msec single voltage step, were used from a holding potential of -80 mV to +50 mV at frequencies of 0.5, 1, 3 and 4 Hz (Fig. 6A). The inhibitory effects of Myr on I_{kur} increased with the continuous depolarization voltage pulses. In addition, the inhibition of I_{kur} by Myr was dependent on the number of pulses (Fig. 6B and C), which suggested that Myr inhibited I_{kur} in a rate- or frequency-dependent manner. Specifically, I_{kur} was 376.23±1.30, 340.01±4.25, 290.59±4.44 and 270.193±4.28 pA/pF at 0.5, 1, 3 and 4 Hz, respectively, and changes between each set of two groups were significant (n=5, P<0.001). Furthermore, the inhibition of I_{kur} significantly increased when the frequency was increased from 0.5-4 Hz (P<0.05) (Fig. 6D). These results suggested that Myr inhibited I_{kur} in HEK293 cells in a frequency-dependent manner.

Discussion

I_{kur} participates in phases I and II of atrial re-polarization, and affects atrial rhythm and frequency by changing the action potential duration and effective refractory period. Therefore, abnormal electrical activity is closely associated with the occurrence and maintenance of AF. Furthermore, I_{kur} is present

in the atria (6), but not in the ventricles of the human heart (11). Human atrial I_{kur} is encoded by the *hK$_v$1.5* (or *hKCNA5*) gene (7,11,24). Due to its atrial specificity, drugs that affect I_{kur} have atrium-specific effects without affecting ventricular function. Certain studies suggested that K_v1.5 channels may represent novel targets for the treatment of AF (25,26). The present study demonstrated that Myr was able to block I_{kur} in a dose-, time- and frequency-dependent manner.

The treatment of AF using drug- or non-drug-associated methods, including catheter-based or surgical interventions, has received significant attention in recent years (27). Catheter ablation has been proposed as an effective nonpharmacological alternative for AF that is often, however not always, the second-line treatment (28). In addition, surgical ablation of AF is commonly performed in cases with other indications for cardiac surgery and it is limited to cases with low surgical risk (29). However, non-drug treatments are limited by factors including their indications, medical conditions and costs. Therefore, drug-based therapies remain the primary method used to treat AF (30). Western medicine is widely applied for anti-arrhythmic treatment; however, its effectiveness is only 30-60%, and varying degrees of arrhythmogenic effects have been reported (31). By contrast, Traditional Chinese Medicine comprises effective anti-arrhythmic treatments, which may provide novel anti-arrhythmia therapies for clinical use. Flavonoids are a class of compounds with the structure of 2-phenyl chromone, observed to be ubiquitously observed in food, including fruits, vegetables, nuts and plant-derived beverages (tea and wine) (32,33). Flavonoids possess a variety of pharmacological activities, including against infection (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancer and additional age-associated diseases (34). Epidemiological studies suggest that the beneficial cardiovascular health effects of diets high in fruit and vegetables are associated with their flavonoid content (35). Flavonoids such as apigenin have been reported to reduce the occurrence of various cardiovascular diseases including coronary disease, arrhythmia, atherosclerosis, hypertension, ischemic stroke and peripheral arteriopathy, congestive heart failure (36). In addition, certain flavonoids such as luteolin-7-O-β-D-glucopyranoside (36), catechin (37), quercetin (38), hesperidin/hesperetin (39), silymarin (40) and genistein (41) exhibited cardioprotective effects. Acacetin, a natural flavone, has been reported to selectively inhibits human atrial repolarization potassium currents and prevents atrial fibrillation in dogs (42). Ampelopsin exerts anti-arrhythmic effects in an aconitine-induced rat arrhythmic model, and the underlying electrophysiological mechanism was demonstrated to be partly associated with the inhibition of I_{Na} and enhancement of I_{K1}, and prolongation of action potential duration (43). Myr is a natural flavonol identified to be present in onions, tea and other natural plants, is advantageous due to its cardioactive components (44). Its chemical structure is similar to that of ampelopsin, which exerts anti-arrhythmic effects (43). Previous studies have suggested that Myr possesses cardioprotective effects (19,20). The present study was designed to identify the possible anti-arrhythmic mechanism of action of Myr in order to provide a theoretical basis for anti-arrhythmic treatments using Myr and other Traditional Chinese Medicinal compounds.

The results of the present study revealed that Myr inhibited I_{kur} *in vitro*, which provided the basis for further *in vivo* experiments. However, the conclusions of the present study do not warrant effects on the human body due to the lack of knowledge of the complex regulatory mechanisms *in vivo*. Questions regarding the mechanisms of action of Myr, for example whether it functions by binding to binding sites, whether these include K_v1.5 channels and whether it affects other ion channels, remain to be investigated in further studies.

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