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Original Article

Blocking effect of methylflavonolamine on human $Na_V1.5$ channels expressed in *Xenopus laevis* oocytes and on sodium currents in rabbit ventricular myocytes

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Aim: To investigate the blocking effects of methylflavonolamine (MFA) on human $Na_V1.5$ channels expressed in *Xenopus laevis* oocytes and on sodium currents (I_{Na}) in rabbit ventricular myocytes.
Methods: Human $Na_V1.5$ channels were expressed in *Xenopus* oocytes and studied using the two-electrode voltage-clamp technique. I_{Na} and action potentials in rabbit ventricular myocytes were studied using the whole-cell recording.
Results: MFA and lidocaine inhibited human $Na_V1.5$ channels expressed in *Xenopus* oocytes in a positive rate-dependent and concentration-dependent manner, with IC_{50} values of 72.61 μmol/L and 145.62 μmol/L, respectively. Both of them markedly shifted the steady-state activation curve of I_{Na} toward more positive potentials, shifted the steady-state inactivation curve of I_{Na} toward more negative potentials and postponed the recovery of the I_{Na} inactivation state. In rabbit ventricular myocytes, MFA inhibited I_{Na} with a shift in the steady-state inactivation curve toward more negative potentials, thereby postponing the recovery of the I_{Na} inactivation state. This shift was in a positive rate-dependent manner. Under current-clamp mode, MAF significantly decreased action potential amplitude (APA) and maximal depolarization velocity (V_{max}) and shortened action potential duration (APD), but did not alter the resting membrane potential (RMP). The demonstrated that the kinetics of sodium channel blockage by MFA resemble those of class I antiarrhythmic agents such as lidocaine.
Conclusion: MFA protects the heart against arrhythmias by its blocking effect on sodium channels.

Keywords: methylflavonolamine; lidocaine; sodium channel; *Xenopus* oocytes; ventricular myocytes

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Introduction

Methylflavonolamine (MFA, Figure 1A) is a novel flavonoid compound that is synthesized by the Shanghai Institute of Pharmaceutical Industry. It can prevent heart arrhythmias during experiments[1–3], increase coronary blood flow, deter experimental myocardial infarction[4, 5], and protect the aorta from atherosclerosis in cholesterol-fed rabbits[6] and the myocardium from injury induced by adriamycin in mice[7]. In addition, MFA possesses weak β-receptor blocking action[8] and has been shown to block calcium channels, demonstrating calcium antagonistic properties in isolated aortic strips[9] and in the whole-cell patch-clamp technique[10, 11]. To date, however, there are no reports regarding the effect of MFA on cardiac sodium channels.

Cardiac voltage-gated sodium channels ($Na_V1.5$) respond to the depolarizing drive associated with the generation of action potentials (APs) in the myocardium. Thus, they play an essential role in the conduction of electrical impulses and contribute to the control of AP duration and effective refractory period (ERP). Today, many class I antiarrhythmic agents (eg, lidocaine) are effective in the treatment of arrhythmias, such as tachycardia and ventricular fibrillation, and their effectiveness is attributable to their ability to decrease the upstroke velocity of APs and sodium currents (I_{Na}) in cardiac myocytes[12]. As a result, suppression of cardiac sodium channels is always regarded as an important indicator for the need to develop agents to potentially eliminate cardiac arrhythmias under clinical conditions. It has been reported that MFA shows an antiarrhythmic effect on myocardiac ischemia-reperfusion, aconitine, ouabain and epinephrine-induced ventricular

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tachycardia[1, 3, 13, 14]. To further clarify the antiarrhythmic effect of MFA, this study aimed to investigate its blocking effects on human $Na_V1.5$ channels expressed in *Xenopus* oocytes compared to the blocking effects of lidocaine. Moreover, we evaluated MFA's blocking effects on sodium currents (I_{Na}) in rabbit ventricular myocytes to further determine the prospect of this drug for treatment in cardiovascular diseases.

Materials and methods

Animals

Female *Xenopus laevis* were purchased from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences (Beijing, China). These frogs were reared in three pools filled with chlorine-free water under suitable conditions, with the temperature controlled strictly at 18–20 °C. The use of healthy adult rabbits were approved by the Experimental Animal Center of Wuhan University of Science and Technology, Wuhan, China. Animals used in this investigation conformed to "the Guide for the Care and Use of Laboratory Animals Regulated by Administrative Regulation of Laboratory Animals of Hubei Province."

In vitro transcription and functional expression in *Xenopus* oocytes

The plasmid pcDNA3.1 contains the coding region for the human SCN5A ($Na_V1.5$) sodium channel α subunit, which was a gift from Professor BENNETT at Duke University Medical Center. Before this construct was used in these experiments, the positions of the electrophoresis bands were found to be consistent with the construction of the pcDNA 3.1 HA-SCN5A ($Na_V1.5$) plasmid vector (Figure 1B). Complementary RNAs (cRNAs) for injection into oocytes were prepared using the mMESSAGE mMACHINE ® T7 Kit (Ambion, Austin, TX, USA) after linearization of the expression construct with Csp45 I (TOYOBO, Japan). *Xenopus* frogs were anesthetized by cooling on crushed ice for 30−40 min. Ovarian lobes were digested with 1.5 mg/mL type IA collagenase (Sigma Chemical, St Louis, MO, USA) in a Ca^{2+}-free ND96 solution for 1 h to remove follicle cells. Stage IV and V *Xenopus* oocytes were injected with 45 nL (1 μg/μL) of $Na_V1.5$ cRNAs per oocyte using a Nanoject microdispenser (Drummond Scientific, Broomhall, PA, USA) and then cultured in ND96 solution supplemented with 100 units/mL penicillin, 100 units/mL streptomycin and 2.5 mmol/L pyruvate at 17 °C for 4−5 d before being used in voltage clamp experiments. The ND96 solution contained (in mmol/L): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$ and 5 HEPES (titrated to pH 7.5 with NaOH).

Cardiac ventricular cell isolation

Healthy adult rabbits (1.5−3 kg) of either sex, were anesthetized by intravenous injection of 25% urethane (5 mL/kg). The heart was excised and retrogradely perfused in a modified Langendorff apparatus with Ca^{2+}-free Tyrode's solutions bubbled with 100% O_2 and maintained at 37 °C. The Ca^{2+}-free Tyrode's solution contained (in mmol/L): 135 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 HEPES and 10 glucose (pH 7.4, adjusted with NaOH). The heart was then perfused with Ca^{2+}-free Tyrode's solution containing collagenase type I (0.3 mg/mL) and BSA (0.9 mg/mL) for 30 min, followed by KB solution for another 5 min. The KB solution contained (in mmol/L): 70 KOH, 40 KCl, 3 $MgCl_2$, 20 KH_2PO_4, 0.5 EGTA, 50 *L*-glutamic acid, 20 taurine, 10 HEPES and 10 glucose (pH 7.4, adjusted with KOH). The ventricles were cut into small chunks and gently agitated in KB solution. The cells were then filtered through nylon mesh and stored in KB solution at 4 °C.

Electrophysiological recording

Recordings were performed 4–12 days after oocyte injection. A standard two-microelectrode voltage-clamp technique was used to record currents at 21–23 °C. The glass microelectrodes were both filled with 3 mol/L KCl, and their tips were beveled using a 1300M Micropipette Beveler (World Precision Instruments, Sarasota, FL, USA) to obtain a resistance of 1–3 MΩ. Oocytes were clamped with a standard and advanced two-microelectrode voltage-clamp amplifier (Warner OC-725C, Warner Instruments, Hamden, CT, USA) using the pCLAMP software (Axon Instruments, Foster City, CA, USA). Oocytes were superfused with ND96 solution at a rate of 2.0 mL/min. Control currents were recorded repeatedly at 1 min intervals, with the drugs applied until the control peak currents achieved a stable level.

Using a patch-clamp amplifier (EPC-10 USB, HEKA Electronic, Lambrecht, Pfalz, Germany), the single-pipette whole-cell patch-clamp technique was used to record the I_{Na} and APs of single cardiac ventricular myocytes from healthy adult rabbits. For recording of I_{Na}, the pipette solution contained (in mmol/L): 120 $CsCl_2$, 1 $CaCl_2$, 5 $MgCl_2$, 5 Na_2ATP, 10 TEACl, 11 EGTA and 10 HEPES (pH 7.3, adjusted with CsOH). The bath solutions for recording of I_{Na} contained (in mmol/L): 30 NaCl, 105 $CsCl_2$, 1 $CaCl_2$, 1 $MgCl_2$, 0.05 $CdCl_2$ 10 HEPES and 10 glucose (pH 7.4, adjusted with CsOH). To record the APs, the pipette solution contained (in mmol/L): 120 KCl, 1 $CaCl_2$, 5 $MgCl_2$, 5 Na_2ATP, 11 EGTA, 10 HEPES and 10 glucose (pH 7.3, adjusted with KOH). The bath solution was the Tyrode's solution, described above, with 1.8 mmol/L $CaCl_2$ added.

Six different conditioning clamp protocols were used to record I_{Na}. The first one examined the concentration-dependent relationship of drugs on I_{Na}, which was determined by measuring the peak inward current for oocytes or myocytes depolarized from -120 mV to -40 mV in the absence and presence of MFA (5–250 μmol/L) or lidocaine (5–750 μmol/L).

The current-voltage (*I–V*) relationship was determined by 100 ms depolarizing pulses to potentials ranging from -100 mV to+30 mV in 5 mV increments from a holding potential of -120 mV.

The voltage-dependence of steady-state inactivation was determined using 100 ms conditioning prepulses from a holding potential of -100 mV to -40 mV in 5 mV increments, followed by a test pulse to -10 mV for 100 ms.

The time course of recovery of I_{Na} from steady-state inactivation was studied using a conventional double-pulse protocol.

A 50 ms prepulse to -40 mV from a holding potential of -120 mV was followed by a variable recovery period (from 2 ms to 80 ms) and then by a test pulse to -40 mV for 50 ms.

To measure the rate-dependent effect of MFA, a series of 30 depolarizing pulses with 30 ms duration from a holding potential of -120 mV to -40 mV at different stimulation frequencies (1, 2 and 4 Hz) were applied.

To clarify the time course of the development of I_{Na} blockage, the current amplitude was measured as a function of various durations of the conditioning pulse. Prepulses of various durations (1−100 ms) to -30 mV from a holding potential of -120 mV were first applied. Another pulse was given after a 250 ms recovery period at -120 mV, and finally a 20 ms test pulse was given to -30 mV.

Action potentials were elicited by depolarizing pulses delivered for a duration of 5 ms and at 1.5-fold above the threshold at a rate of 1 Hz. All currents were digitally sampled at 10 kHz, low pass filtered at 1 kHz and saved on a hard drive for *post hoc* measurement.

After the application of drugs, the data were collected and analyzed when blockage approached a stable state.

Data analysis

Clampfit 10.0 software (Axon Instruments, USA), Master+FitMaster (v2x32, HEKA), Origin 7.0 software (Origin Laboratory, Northampton, MA, USA) and Excel (Microsoft, Redmond, WA, USA) software were used for data acquisition and analysis. Fractional blockage was defined as $f=1-I_{drug}/I_{control}$, where $I_{control}$ and I_{drug} were the current amplitudes in the absence and presence of MFA, respectively. Concentration-response curves were fitted by the Hill equation: $(I_{control}-I_{drug})/I_{control}=B_{max}/[1+(IC_{50}/D)^n]$, where B_{max} was the maximum blockage of currents, IC_{50} was the concentration of MFA for half-maximum blockage, D was the concentration of MFA, and n was the Hill coefficient. The data regarding steady-state activation and inactivation relationships of I_{Na} were fitted to the Boltzmann equation: $Y=1/[1+\exp(V_m-V_{1/2})/k]$, where V_m was the membrane potential, $V_{1/2}$ was the half-activation or half-inactivation potential, and k was the slope factor. For the steady-state activation and inactivation curve, Y stood for the relative conductance (G/G_{max}) and relative current (I/I_{max}). The data of recovery from inactivation relationships of I_{Na} were fitted to a single exponential equation: $Y=A*\exp(-t/\tau)+C$, where A represented the proportion of channels recovering with time constant τ, and t was the recovery time interval. Data were presented as mean±SD. Student's t tests for paired and unpaired data were used to compare the control group to the treated group. A P-value< 0.05 was considered statistically significant.

Drugs

MFA [4'-methyl-7-(2-hydroxy-3-isopropylamino-propoxy)-flavone hydrochloride], a gift from the Shanghai Institute of Pharmaceutical Industry (Shanghai, China), was solubilized in distilled water as 10 mmol/L stock solutions prior to dilution to the final concentrations in the cell external solution. MFA was used at concentrations ranging from 5 to 250 μmol/L. Lidocaine hydrochloride was purchased from Shandong Hualu Pharmaceutical Co, Ltd (Shandong, China), solubilized in distilled water as 20 mmol/L stock solutions and used at final concentrations ranging from 5 to 750 μmol/L. All other chemicals were obtained from Sigma Chemical (Saint Louis, MO, USA). To keep the concentrations of the various types of ions constant, we strictly controlled the perfusion rate using the BPS-4 perfusion device (ALA Scientific Instruments, Inc, Westbury, NY, USA) and a constant-flow pump.

Results

Confirmation of I_{Na}

Currents were recorded first in the absence and then in the presence of 20 μmol/L TTX with 100 ms voltage steps from a holding potential of -120 mV to -40 mV. The current was blocked mainly in *Xenopus* oocytes (n=6, Figure 1C), indicating that these currents were attributed to the sodium currents.

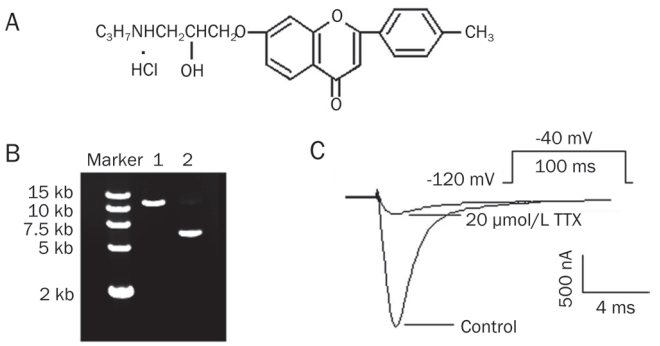

Figure 1. MFA, plasmid and sodium current. (A) Diagram of the molecular structure of MFA. (B) Electrophoresis identification of the pcDNA 3.1 HA-SCN5A ($Na_V1.5$) plasmid vector; Lane 1, the plasmid with Csp45 I restriction enzyme digestion; Lane 2, the pcDNA 3.1 HA-SCN5A ($Na_V1.5$) plasmid vector. (C) Confirmation of I_{Na}. Depolarizing pulses from -120 mV to -40 mV for 100 ms were applied. Current tracings of human $Na_V1.5$ channels expressed in *Xenopus* oocytes are superimposed before (control) and during superfusion of 20 μmol/L TTX.

Concentration-dependent blockage of MFA or lidocaine on I_{Na} in *Xenopus* oocytes

The relationship between the unblocked fraction of human $Na_V1.5$ channels expressed in *Xenopus* oocytes and the concentrations of MFA or lidocaine is clearly shown in Figure 2A and 2B. Because the blockage approached a stable state for 3−5 min after drug application, we collected data 5 min after adding the drugs. The suppressive effect of MFA on $Na_V1.5$ channels was concentration-dependent in the range of 5−250 μmol/L, with an average IC_{50} value of 72.61±3.27 μmol/L and the Hill coefficient of 1.79±0.21. The maximal suppression was up to 68.21%±5.54% by MFA 250 μmol/L (n=18). In comparison, the average IC_{50} value of lidocaine on I_{Na} was 145.62±4.43 μmol/L and the Hill coefficient was 1.42±0.21 (n=8). The results showed that MFA blocked cardiac sodium channels

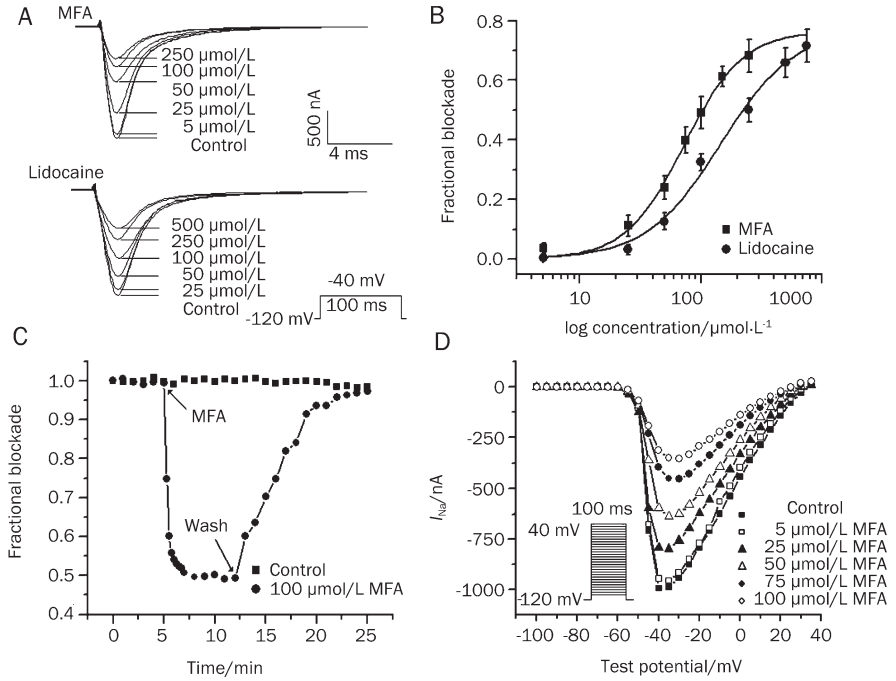

Figure 2. Concentration-dependent blockage of I_{Na} by MFA or lidocaine. Depolarizing pulses from -120 mV to -40 mV for 100 ms were applied. (A) Current tracings of human $Na_V1.5$ channels expressed in *Xenopus* oocytes are superimposed before (control) and during superfusion of MFA (5−250 μmol/L) or lidocaine (5−750 μmol/L). (B) The concentration-response curves are plotted based on data from panel A and fitted by the Hill equation. (C) Time-course of the effects of MFA on human $Na_V1.5$ channels expressed in *Xenopus* oocytes. Oocytes were perfused with ND96 solution for 5 min before application of 100 μmol/L MFA or without drug and again with ND96 solution (washout). The normalized currents are plotted during the recording course. (D) The effects of MFA on the current-voltage relationship of human $Na_V1.5$ channels expressed in *Xenopus* oocytes. Depolarizing pulses (100 ms duration) were applied from a holding potential of -120 mV to various potentials ranging from -100 mV to +40 mV with 5 mV increments.

with a potency that was 2-fold greater than that of lidocaine. In addition, the time-dependent effects of MFA on cardiac I_{Na} were assessed. Blockage of I_{Na} occurred rapidly in the presence of 100 μmol/L MFA and was almost completely reversed after washout for 10 min (n=6, Figure 2C) .

Effects of MFA on the *I–V* relationship of I_{Na} in *Xenopus* oocytes

The *I-V* relationship of human $Na_V1.5$ channels expressed in *Xenopus* oocytes is illustrated in Figure 2D. Under the control condition, I_{Na} was detected at the potentials positive to -60 mV, attaining the maximum value at -40 mV and reversing its polarity above 30 mV in *Xenopus* oocytes. In the presence of 100 μmol/L MFA, I_{Na} was suppressed, thereby shifting the maximum activation potential to a more positive value of 10 mV; however, the reversal potential was not modified (n=10).

Effects of MFA or lidocaine on activation and inactivation kinetics of I_{Na} in *Xenopus* oocytes

The effects of MFA or lidocaine on activation and inactivation kinetics of human $Na_V1.5$ channels expressed in *Xenopus* oocytes are shown in Figure 3. The half-activation potential ($V_{1/2}$) measured before and after the application of 100 μmol/L MFA was -47.9±2.3 mV and -41.0±3.7 mV, respectively (n= 8, P<0.05), with k values of 3.19±0.53 and 3.93±0.46 (n=8, P>0.05). Compared to the control, after the application of 250 μmol/L lidocaine, the $V_{1/2}$ was –44.5±2.6 mV (n=6, P<0.05) and the k value was 3.48±0.43 (n=6, P>0.05; Figure 3A and 3C).

By contrast, the effects of MFA and lidocaine on the inactivation kinetics of I_{Na} were congruent, and the steady-state inactivation curves of I_{Na} were clearly shifted toward more negative potentials in the presence of MFA and lidocaine. The $V_{1/2}$ and k values of human $Na_V1.5$ channels were -72.1±3.5 mV and 5.85±0.63 in controls and -78.8±2.2 mV (n=8, P<0.05) and

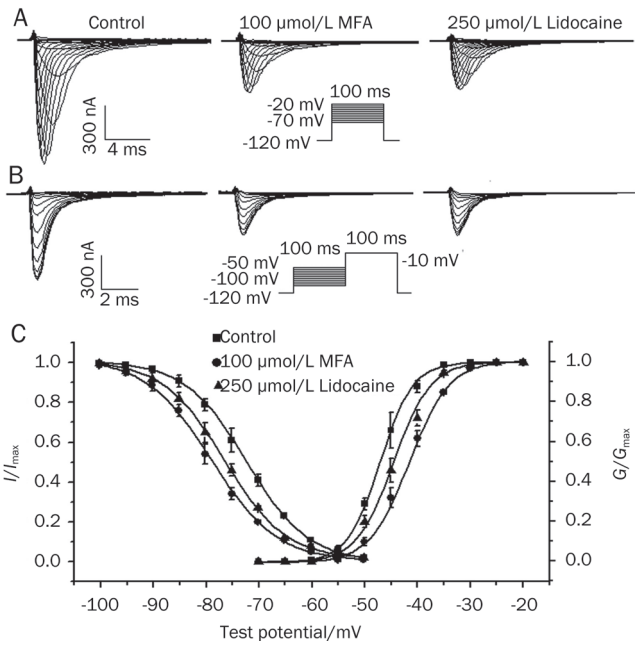

Figure 3. Effects of MFA or lidocaine on steady-state activation and inactivation of I_{Na} in *Xenopus* oocytes. The activation protocol was obtained from the current-voltage relationship (100-ms depolarizing pulses to potentials ranging from -70 mV to -20 mV in 5 mV increments), and the inactivation curve was obtained using of a double-pulse protocol (100-ms conditioning prepulses from a holding potential of -100 mV to -40 mV in 5 mV increments), followed by a test pulse to -10 mV for 100 ms. (A and B) The activation and inactivation currents of human $Na_V1.5$ channels expressed in *Xenopus* oocytes before and after superfusion of 100 μmol/L MFA or 250 μmol/L lidocaine, respectively. (C) The normalized steady-state activation and inactivation of human $Na_V1.5$ channels expressed in *Xenopus* oocytes plotted in the absence and presence of 100 μmol/L MFA or 250 μmol/L lidocaine.

6.25±0.51 (n=8, P>0.05) in the presence of 100 μmol/L MFA, respectively. Compared to controls, after the application of 250 μmol/L lidocaine, the $V_{1/2}$ increased to –76.4±2.0 mV (n=6, P<0.05), with a k value of 5.86±0.55 (n=6, P>0.05; Figure 3B and 3C).

Effects of MFA or lidocaine on recovery kinetics of I_{Na} in *Xenopus* oocytes

The effects of MFA and lidocaine on recovery kinetics of human $Na_V1.5$ channels expressed in *Xenopus* oocytes are presented in Figure 4. The time constants of recovery from the steady-state inactivation were 2.26±0.25 ms in controls and 3.13±0.25 ms in the 100 μmol/L MFA group (n=8, P<0.05; Figure 4B), respectively. The time constant in the presence of 250 μmol/L lidocaine was 3.05±0.23 ms (n=6, P<0.05; Figure 4B). These results confirmed that MFA delayed the recovery time of I_{Na} from the inactivation state.

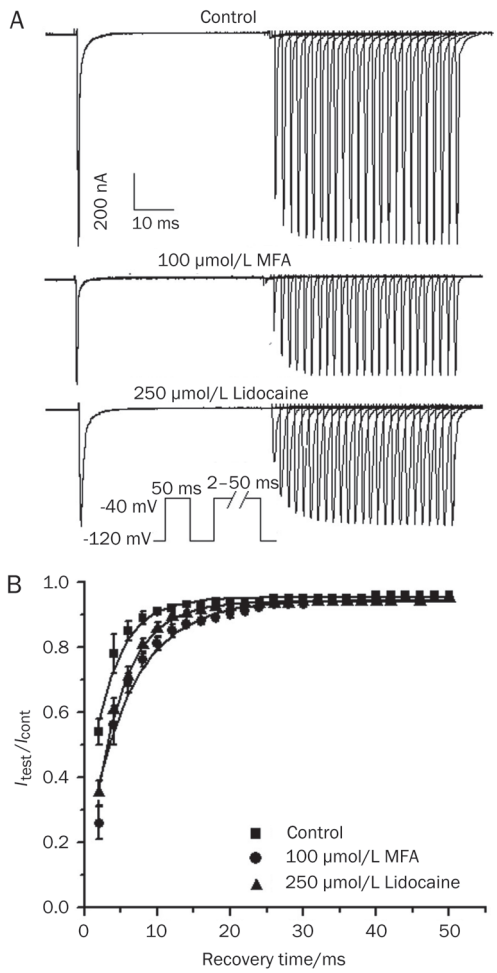

Figure 4. Effects of MFA or lidocaine on recovery from inactivation of I_{Na} in *Xenopus* oocytes. The recovery from inactivation was studied using a conventional double-pulse protocol (a 50-ms prepulse to -40 mV from a holding potential of -120 mV, followed by a variable recovery period from 2–80 ms and then by a test pulse to -40 mV for 50 ms). (A) Example tracing of the recovery from inactivation of human $Na_V1.5$ channels expressed in *Xenopus* oocytes before and after superfusion of 100 μmol/L MFA or 250 μmol/L lidocaine. (B) The normalized recovery from inactivation of human $Na_V1.5$ channels expressed in *Xenopus* oocytes plotted in the absence and presence of 100 μmol/L MFA or 250 μmol/L lidocaine.

Positive rate-dependent blockage of MFA or lidocaine on I_{Na} in *Xenopus* oocytes

The rate-dependent blockage of MFA and lidocaine on human $Na_V1.5$ channels expressed in *Xenopus* oocytes is illustrated in Figure 5. Under the control condition, the peak amplitude of I_{Na} did not change after 30 repetitive depolarizing pulses at 4 Hz. On the contrary, 100 μmol/L MFA or 250 μmol/L lidocaine produced a progressive decline of the I_{Na} amplitude by the 1−4 Hz depolarization (n=6). The blocking effect of MFA on I_{Na} was enhanced along with an increase in the frequency of stimuli, which showed a positive rate-dependence; however, it reached a plateau more slowly than that of lidocaine (Figure 5B and 5D).

Onset of I_{Na} blockage by MFA in *Xenopus* oocytes

Figure 6 shows the onset of I_{Na} blockage by MFA in *Xenopus* oocytes to clarify the time course of its development. In comparison to control cases in which current amplitude failed to reduce over time in the presence of 100 μmol/L MFA, the I_{Na} was reduced by 13.37%±2.18% after a prepulse of 2 ms (n=7). Because a 2 ms prepulse was considered long enough to activate sodium channels but too short to inactivate them, the blockage of I_{Na} observed here could be attributed to the I_{Na} blockage in the activated state. By contrast, when the prepulse duration was increased to 10 ms or longer, a much greater reduction (from 23.51%±3.68% to 51.9%±5.85%) in the test I_{Na} was observed (n=7, Figure 6B), indicating that MFA preferentially bound to the channel in the inactivated state.

Effects of MFA on I_{Na} in rabbit ventricular myocytes

To further evaluate the effect of MFA on I_{Na}, we recorded I_{Na} and channel kinetics in rabbit ventricular myocytes using the whole-cell patch-clamp technique. The results showed that 75 μmol/L MFA suppressed I_{Na} by 44.6%±9.7%. After washout of MFA, the cardiac I_{Na} recovered substantially, and the effect of MFA was reversed in drug-free solution (n=11, Figure 7A). By comparing the I-V relationship in the absence and presence of 100 μmol/L MFA, we found that I_{Na} was suppressed without significantly changing the contour of the I-V curves (n=8, Figure 7B and 7D). This was different from effects of MFA on the I–V relationship in *Xenopus* oocytes. In addition, there were also variances in activation kinetics between the two expression systems. In rabbit ventricular myocytes, the activation characteristics of cardiac I_{Na} was not clearly affected by MFA, as the $V_{1/2}$ measured before and after the application of 100 μmol/L MFA was -47.1±4.3 mV and -46.1±4.1 mV (n=7, P>0.05), respectively, with k values of 4.48±0.72 and 3.42±0.57 (n=7, P>0.05; Figure 7B and 7E). The effects of MFA on the inactivation and recovery kinetics of I_{Na}, however, were congruent with that of the currents observed in *Xenopus* oocytes. The steady-state inactivation curves of I_{Na} were clearly shifted

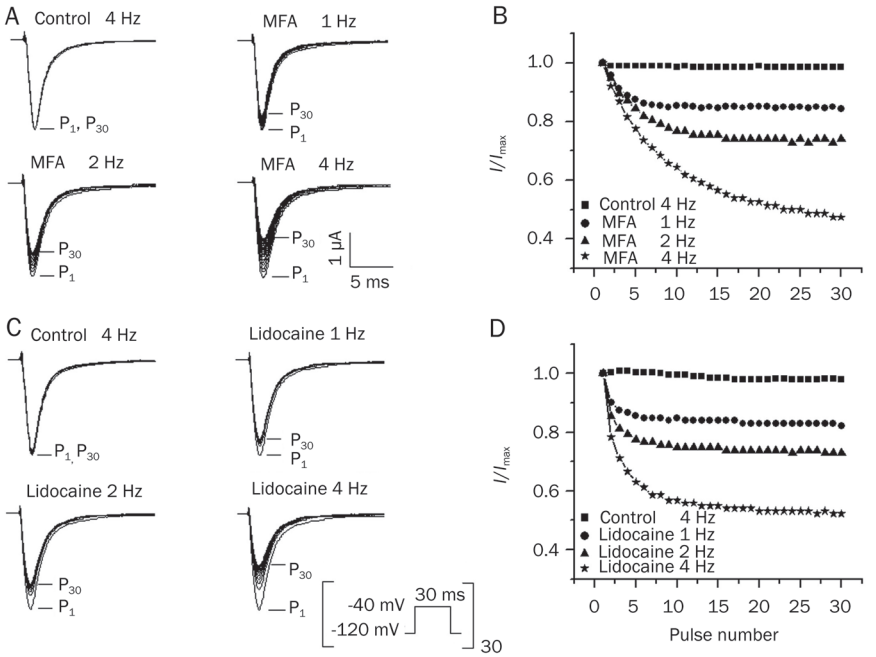

Figure 5. Rate-dependent blockage of I_{Na} by MFA or lidocaine in *Xenopus* oocytes. A series of 30 depolarizing pulses with 30 ms duration from a holding potential of -120 mV to -40 mV at different stimulation frequencies was applied to rabbit ventricular myocytes. After stimulating at each frequency, cells were perfused with normal solution, and I_{Na} recovered substantially. (A and C) Superimposed tracings of I_{Na} obtained from different experiments: in control at 4 Hz, and in the presence of 75 μmol/L MFA or 250 μmol/L lidocaine at 1 Hz, 2 Hz and 4 Hz. (B and D) Relative I_{Na} was plotted against each pulse number. Relative I_{Na} was defined as the ratio of I_{Na} at each pulse number/I_{Na} at the first pulse.

toward more negative potentials in the presence of MFA, with $V_{1/2}$ and k values of -65.6±4.2 mV and 9.39±0.88, respectively, in controls and -76.1±4.6 mV (n=8, P<0.05) and 10.26±0.95 (n=8, P>0.05) in the presence of 100 μmol/L MFA (Figure 7C and 7F). The time constants of the recovery from steady-state inactivation were 12.99±2.73 ms in controls and 26.55±3.31 ms in the 100 μmol/L MFA group (n=6, P<0.01; Figure 8A and B), which confirmed that MFA delayed the recovery time constant of I_{Na} from the inactivation state. Likewise, MFA blocked cardiac I_{Na} in a positive rate-dependent manner in rabbit ventricular myocytes. Compared to the controls, 75 μmol/L MFA produced a progressive decline in the I_{Na} amplitude with 1−4 Hz depolarization, with the most significant blockage at 4 Hz (Figure 8C and 8D).

Effects of MFA on APs in rabbit ventricular myocytes

The effects of MFA on the AP configuration are shown in Table 1 and Figure 9. Compared to the control condition, 75 μmol/L MFA caused a significant decrease in the maximum upstroke velocity (V_{max}) and amplitude (APA) of APs. Moreover, MFA shortened AP duration at 90% (APD_{90}), 50% (APD_{50}) and 30% (APD_{30}) of repolarization, but did not affect the resting membrane potential (RMP). After MFA washout, the AP configuration returned to that of the control.

Table 1. Effects of MFA on AP characteristics in rabbit ventricular myocytes. n=10. [a]P>0.05, [c]P<0.01 *vs* Control. [d]P>0.05, [f]P<0.01 *vs* MFA 75 μmol/L.

	RMP (mV)	APA (mV)	APD_{90} (ms)	APD_{50} (ms)	APD_{30} (ms)	V_{max} (V/s)
Control	80±1.6	125±2.5	233±9.7	202±8.7	119±7.1	221±14
75 μmol/L MFA	80±1.2[a]	89±3.7[c]	106±7.7[c]	92±7.3[c]	75±6.1[c]	100±8.6[c]
Washout	80±1.6[d]	119±3.9[f]	179±8.3[f]	163±7.1[f]	106±7.5[f]	193±17[f]

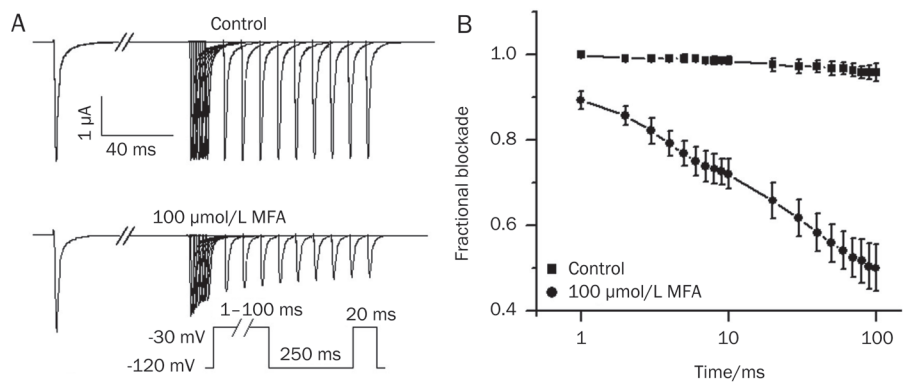

Figure 6. Onset of I_{Na} blockage by MFA in *Xenopus* oocytes. Prepulses of various durations (1−100 ms) were applied to -30 mV from a holding potential of -120 mV, after a 250 ms recovery period at -120 mV and after a 20 ms test pulse was applied again to -30 mV. (A) Current tracings of human $Na_V1.5$ channels expressed in *Xenopus* oocytes before and after superfusion of 100 μmol/L MFA. (B) The normalized onset blockage of I_{Na} in *Xenopus* oocytes plotted in the absence and presence of 100 μmol/L MFA.

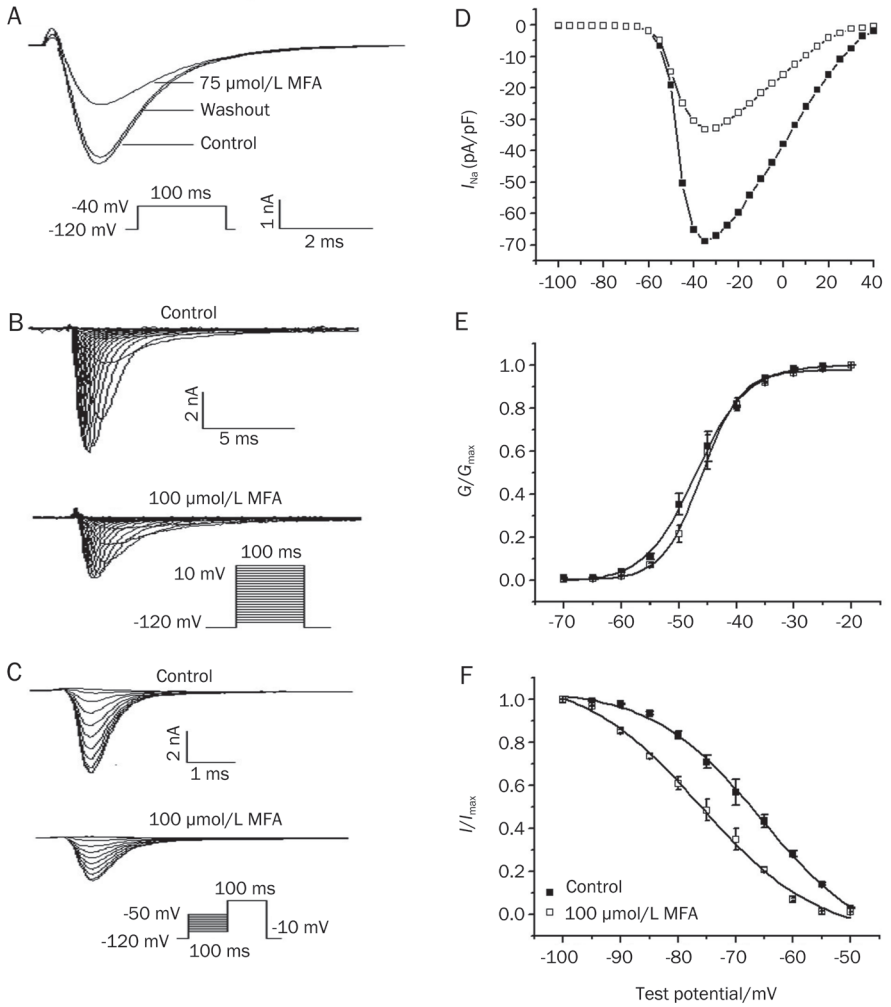

Figure 7. Effects of MFA on current-voltage relationship, steady state activation and inactivation of I_{Na} in rabbit ventricular myocytes. The pulse protocols were as described in Figures 2 and 3. (A) Sodium current tracings of rabbit ventricular myocytes superimposed before (control), during superfusion of MFA 75 μmol/L and after washout. (B and C) Activation and inactivation currents of rabbit ventricular myocytes before and after superfusion of 100 μmol/L MFA, respectively. (D–F) The effects of 100 μmol/L MFA on the current-voltage relationship, steady-state activation and inactivation curves of I_{Na} in rabbit ventricular myocytes, respectively.

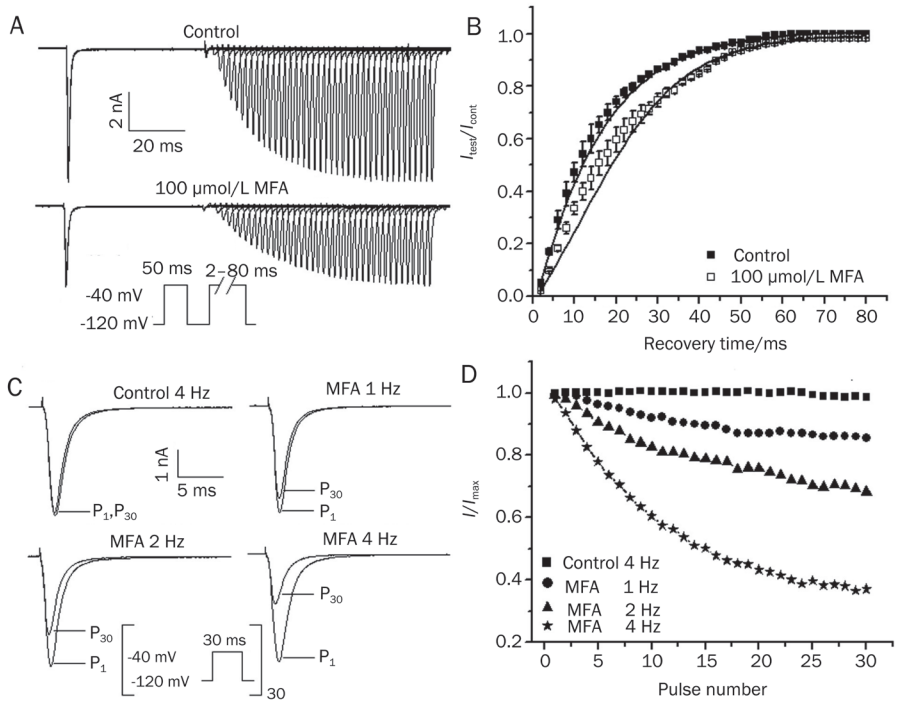

Figure 8. Effects of MFA on the recovery from inactivation and rate-dependent blockage of I_{Na} in rabbit ventricular myocytes. The clamp protocols were as described in Figures 4 and 5. (A and C) Current tracings of rabbit ventricular myocytes before and after superfusion of 100 or 75 μmol/L MFA, respectively. For reasons of clarity, only the first (P_1) and the last (P_{30}) currents are shown. (B and D) Normalized I_{Na} was plotted in the absence and presence of 100 or 75 μmol/L MFA and against the recovery time or each pulse number, respectively.

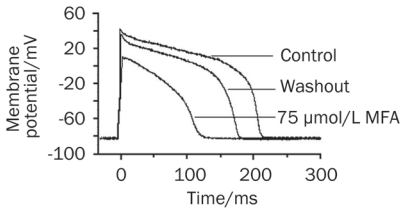

Figure 9. Effects of MFA on AP characteristics in rabbit ventricular myocytes. APs were elicited by depolarizing pulses delivered for 5 ms and at 1.5-fold above the threshold at a rate of 1 Hz. Representative recordings of APs are superimposed before (control), during superfusion of MFA 75 μmol/L and after washout.

Discussion

We compared the pharmacological actions of MFA on human $Na_V1.5$ channels expressed in *Xenopus* oocytes to those of lidocaine, a clinically used class I antiarrhythmic agent. In addition, the effects of MFA on human $Na_V1.5$ channels expressed in *Xenopus* oocytes and on I_{Na} in rabbit ventricular myocytes were confirmed. The main findings of this study are that MFA blocked I_{Na} in both expression systems, and its blocking properties are similar to those observed for lidocaine. This provides experimental evidence of the antiarrhythmic action of MFA.

The blocking effects of MFA on cardiac I_{Na} were compared to those of lidocaine using various conditioning clamp protocols, indicating that MFA is more potent than lidocaine in blocking cardiac sodium channels. We observed that MFA blocked cardiac sodium channels with a potency that was 2-fold greater than lidocaine, and it made the steady-inactivation curve shift toward more negative potentials (Figure 2). Yongfu *et al* showed that the characteristic blockage of sodium channels is associated with larger leftward shifts in the steady-state inactivation, a process necessary for the prevention of arrhythmias[15]. Thus, MFA has greater potency for cardiac sodium channels compared to lidocaine, suggesting that this drug may have better anti-arrhythmic efficacy.

We also compared the effects of MFA on two kinds of cardiac I_{Na} in two expression models. The results showed that the blocking effects of MFA on the sodium channels in both systems were quite similar. Besides its suppressive action on I_{Na}, this drug significantly shifted the steady-state inactivation curves toward more negative potentials and delayed the time of recovery from inactivation. Interestingly, we found that the effects of MFA on the electrophysiological characteristics of sodium channels were not all the same in the two expression systems. In *Xenopus* oocytes, MFA caused a significant shift to more positive potentials in the maximum activation potential of the *I-V* curve and the half-activation potential of the steady state activation curve; however, MFA failed to change these parameters in rabbit ventricular myocytes. We maintain that these inconsistent results were dependent on the distinctions existing between the two expression systems. Whereas human $Na_V1.5$ channels expressed in *Xenopus* oocytes contained only the α-subunits of sodium channels, intact sodium channels naturally expressed in myocardial cell membranes of mammals include not only functional α-subunits but also accessory β-subunits (including β1, β2 and β3). It has been repeatedly reported that β-subunits can modify channel properties and interact with cytoskeletal extracellular matrix properties. Thus, these accessory proteins can change the dynamic characteristics of sodium channels during activation, inactivation and recovery from inactivation[16–19]. These factors therefore likely play an important role in the mechanism of the different effects of MFA on I_{Na} in the two experimental expression systems.

Under voltage-clamp and whole-cell patch-clamp recording conditions, MFA caused significant decreases of the I_{Na} amplitude in *Xenopus* oocytes and rabbit ventricular myocytes. Under current-clamp recording conditions, MFA also produced marked decreases in the V_{max} and APA, indicating that experimental results were congruent. Meanwhile, this drug clearly shortened APD, which is consistent with previous experimental results showing that MFA blocked calcium channels in guinea pig and rat ventricular myocytes[10, 11].

In this study, findings of MFA acting on dynamic characteristics of sodium channels showed that this drug not only shifted steady state inactivation curves toward more negative potentials, but also delayed the recovery time from inactivation. The former suggests that the rate of sodium channel inactivation was accelerated, whereas the latter indicates that the duration of sodium channel recovery from the inactivation state to the resting state was prolonged. This demonstrates that MFA can interact with the inactivation state of sodium channels. When using trains of stimuli, the amplitudes of I_{Na} evoked by the first impulse failed to change before and after the superfusion of MFA. This may relate to the lower affinity of MFA on the resting state of sodium channels.

Here, we found that MFA inhibited I_{Na} in a positive rate-dependent manner. The faster the frequency of stimulation, the larger number of times that channels became activated and inactivated in unit time and the larger number of times of channel opened and closed. Thus, high frequency stimulation increased the probability of MFA binding to the receptor site of the sodium channel, thereby reducing the number of sodium channels shifting from the resting state to the activation state. At the same time, due to the shortened resting phase, the number of sodium channels shifting from the inactivation state to the resting state became reduced. A large number of sodium channels accumulate in the inactivation state, thus leaving only a small proportion of sodium channels able to be activated. In other words, the amount of channel opening time was reduced in unit time[20–22]. The more frequently channels open (ie, the faster the heart rate is), the stronger the blocking effect of MFA is, which resembles the effects of class I antiarrhythmic agents[12, 23] that are preferred in the treatment of tachyarrhythmia[24].

To clarify either the activation or inactivation state of sodium channels bounded by MFA, we investigated the onset of sodium channel blockage. We found that the blocked fraction of sodium channels was 13.37% with a conditioning pulse

of 2 ms, which was long enough to allow sodium channels to be fully activated. In contrast, MFA significantly blocked I_{Na} when prepulses were longer than 10 ms, suggesting that MFA has a higher affinity for the inactivation state of sodium channels; however, it does have an effect on the activation of sodium channels to some degree.

I_{Na} is involved in the AP upstroke phase and propagation and is also a key target of class I antiarrhythmic agents[25]. Facilitating sodium channel activation and causing abnormally large increases in the "window current" may amplify Na^+ accumulation during many pathological conditions, such as myocardial ischemia or hypoxia, leading to elevated cytosol Ca^{2+} through the reverse Na^+-Ca^{2+} exchanger, followed by contractile dysfunction and ultimately cell death[26, 27]. Inhibition of intracellular Na^+ entry and prevention of Na^+ overload may therefore be cardioprotective[28, 29]. MFA shifted the steady-state inactivation curve of I_{Na} toward negative potentials, reduced the Na^+ "window current", stabilized the cell membrane and made ectopic activity more difficult to trigger, which is an important mechanism to inhibit arrhythmia by sodium channel blockers[30, 31]. It has been reported that MFA can alleviate cardiac injury after myocardial ischemia[32] and post-reperfusion arrhythmias[13, 14]. Thus, MFA can block sodium channels, prevent intracellular Na^+ overload and post-ischemic arrhythmias and improve myocardial function recovery.

In addition, it is widely reported that other flavonoid drugs can also block sodium channels. For instance, total flavonoids of astragalus (TFA) can significantly inhibit the I_{Na} of rat isolated ventricular myocytes after acute myocardial infarction[33]; flavone in resina draconis (FRD) can significantly inhibit the tetrodotoxin-sensitive and tetrodotoxin-resistant sodium current in rat trigeminal ganglion neurons[34, 35]; genistein can block sodium channels in rat fetal brain neurons[36]; and puerarin can inhibit I_{Na} in rat dorsal root ganglion neurons[37] and ventricular myocytes[38]. In clinical practice, some drugs have been applied to treat heart diseases. Thus, some flavonoid compounds are able to inhibit arrhythmia by blocking sodium channels, elevating threshold excitability, retarding conduction, depressing automaticity in ectopic pacemakers and eliminating reentrant movement. In addition, in comparison to other class I antiarrhythmic agents, MFA has more myocardial protective effects, including increasing coronary blood flow[4, 5], protecting the aorta from atherosclerosis[6], possessing weak β-receptor blocking action[8], suppressing the spontaneous and evoked automaticity in the atrium[39] and blocking calcium channels[10, 11]. In conclusion, we believe that MFA has significant potential to be developed as a novel anti-arrhythmic agent.

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Author contribution

Ji-hua MA and Xin-rong FAN designed the research; Xin-rong FAN, Jun-lian XING and Pei-hua ZHANG performed the research; Xin-rong FAN analyzed the data; and Xin-rong FAN and Ji-hua MA wrote the paper.

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