Original Article

Effects of diltiazem and propafenone on the inactivation and recovery kinetics of fKv1.4 channel currents expressed in *Xenopus* oocytes

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Aim: To investigate the effects of diltiazem, an L-type calcium channel blocker, and propafenone, a sodium channel blocker, on the inactivation and recovery kinetics of fKv1.4, a potassium channel that generates the cardiac transient outward potassium current. **Methods:** The cRNA for fKv1.4 Δ N, an N-terminal deleted mutant of the ferret Kv1.4 potassium channel, was injected into *Xenopus* oocytes to express the fKv1.4 Δ N channel in these cells. Currents were recorded using a two electrode voltage clamp technique. **Results:** Diltiazem (10 to 1000 µmol/L) inhibited the fKv1.4 Δ N channel in a frequency-dependent, voltage-dependent, and concentration-dependent manner, suggesting an open channel block. The IC₅₀ was 241.04±23.06 µmol/L for the fKv1.4 Δ N channel (at +50 mV), and propafenone (10 to 500 µmol/L) showed a similar effect (IC₅₀=103.68±10.13 µmol/L). After application of diltiazem and propafenone, fKv1.4 Δ N inactivation was bi-exponential, with a faster drug-induced inactivation and a slower C-type inactivation. Diltiazem increased the C-type inactivation rate and slowed recovery in fKv1.4 Δ N channels. However, propafenone had no effect on either the slow inactivation time constant or the recovery.

Conclusion: Diltiazem and propatenone accelerate the inactivation of the Kv1.4 Δ N channel by binding to the open state of the channel. Unlike propatenone, diltiazem slows the recovery of the Kv1.4 Δ N channel.

Keywords: inactivation; recovery; Kv1.4; potassium channel; diltiazem; propafenone; two electrode voltage clamp technique

Acta Pharmacologica Sinica (2011) 32: 465-477; doi: 10.1038/aps.2010.234

Introduction

Transient outward potassium currents (I_{to}) contribute to the early repolarization phase of the cardiac action potential^[1, 2]. Two types of I_{to} are known: I_{to} (fast), which shows fast recovery kinetics, and I_{to} (slow), which shows slow recovery kinetics that are related to accumulated inactivations^[3]. As the major component of I_{to} (slow), the Kv1.4 channel plays an important role in the repolarization of cardiac myocytes. Kv1.4 channels were inactivated by two well-established processes: Nand C-type inactivation. N-type inactivation results from the occlusion of the intracellular side of the pore by a "ball and chain" mechanism formed by the NH₂ terminus of the channel molecule^[4-9], while C-type inactivation involves conformational changes on the extracellular side of the pore^[10]. These two mechanism are coupled^[5]; C-type inactivation is more rapid in the presence of N-type inactivation^[11] and can be affected by open channel blockers. Recovery from inactivation

Received 2010-06-28 Accepted 2010-12-18

is controlled by the slower C-type mechanism^[11], which makes it physiologically important.

The L-type calcium channel blocker diltiazem and the sodium channel blocker propafenone are widely used in clinics for the treatment of cardiovascular diseases of hypertension, cardiac angina (for diltiazem) and arrhythmias^[12-14]. The therapeutic effects are generally believed to be related to the L-type calcium channel (for diltiazem) and the sodium channel (for propafenone). Recent studies demonstrated that diltiazem inhibited the hKv1.5 channel, which conducts ultra rapid delayed rectifier currents (I_{kur}), and I_{to} , encoded by Kv4.3 by binding to the open and the inactivated states of the channels^[15-17]. There is evidence that diltiazem decreases Kv1.4 channel currents expressed in the oocytes of *Xenopus laevis*^[16], and propafenone was shown to be an open channel antagonist of Kv1.4 channel currents^[18], but their detailed characteristics have not been studied.

The present study, in which we used an N-terminal deletion construct of Kv1.4 (Kv1.4 Δ N) that lacks rapid N-type inactivation but exhibits robust C-type inactivation^[19], was therefore designed for the following: (1) to study the properties of

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diltiazem blockade of the fKv1.4 Δ N channel; (2) to study the effect of diltiazem on Kv1.4 channel C-type inactivation and recovery, and (3) to compare the electrophysiological effects of diltiazem on fKv1.4 Δ N with those of propafenone.

Materials and methods

Molecular biology

The constructs and sequences of the cDNA fKv1.4 Δ N used in this study have been previously described^[19-21] and were a gift from professor Randall L RASMUSSON (University at Buffalo, SUNY). The construction of fKv1.4 Δ N was performed by removal of 2–146 amino acid residues from the N-terminal domain of Kv1.4, which results in the loss of the fast component of inactivation but leaves the C-type inactivation pathway intact^[19-21]. Transcribed fKv1.4 Δ N cRNA was prepared *in vitro* using an mMessage mMachine kit (T3 kit, Ambion, USA).

Isolation of oocytes and incubation

Oocytes were collected from mature female *Xenopus laevis* frogs (Chinese Academy of Science, Beijing, China). Frogs were anesthetized (immersion in 1.5 g/L tricaine) for 30 min, followed by surgical removal of the ovarian lobes through a lateral incision in the lower abdomen. The incision was then sutured, and the frog was allowed to recover in a container with a small amount of water. When the frogs did not produce a high quality of oocytes, they were humanely killed *via* a high dose of tricaine. All procedures were approved by the Institutional Animal Care and Use Committee of the Wuhan University of China.

The follicular layer was removed enzymatically by placing the ovarian lobes in a collagenase-containing, Ca^{2+} -free OR_2 solution (mmol/L: 82.5 NaCl, 2 KCl, 1 MgCl₂ and 5 Hepes, pH 7.4, with 1–1.5 mg/mL collagenase (Type I, Sigma, USA). The oocytes were gently shaken for about 1 h and washed several times with Ca^{2+} -free OR_2 solution as previously described^[13]. Finally, defolliculated (stage IV) oocytes were selected and placed in ND96 solution (mmol/L): 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂ and 5 Hepes, pH 7.4. Each oocyte was injected with about 25–50 nL of fKv1.4 Δ N cRNA using a microinjector (WPI, Sarasota) and incubated in an 18 °C environment in ND96 solution with 100 IU/mL penicillin for over 16 h.

Electrophysiology

The experiment was carried out using a two electrode voltage clamp technique. Oocytes were clamped using a preamplifier CA-1B (DAGAN, USA), and the current signals were filtered at 2.5 kHz. Microelectrodes were fabricated from 1.5 mm o.d. borosilicate glass tubing using a two-stage puller (NARISHIGE, Japan) to produce electrodes with resistances of 0.5–1.0 M Ω when filled with 3 mmol/L KCl. Currents were recorded at room temperature (20–24 °C). Recordings were made in 2 mol/L [K⁺]_o. Diltiazem and propafenone were separately dissolved in distilled water with a stock solution of 100 mmol/L. During recording, oocytes were continuously perfused with a control (ND96) or drug-containing ND96 solution. Whenever drugs were used, 10 min of perfusion

time was used to allow equilibration of the drug with the oocytes. After this wash-on period, a series of 500 ms depolarizing pulses (from -90 mV to +50 mV at a frequency of 1 Hz for 1 min) was employed to ensure a steady-state block before beginning the experimental protocols^[20].

Data analysis

Data were recorded with a personal computer installed with pCLAMP 9.0 (Axon, USA) and analyzed using Clampfit 9.0 (Axon, USA) and Microsoft Excel software (Microsoft, USA). Unless otherwise stated, raw data traces were not leakage or capacitance subtracted. Data are shown as means±SEM. Significant differences were determined using Student's paired *t*-tests.

Results

Effects of diltiazem on fKv1.4 Δ N currents Voltage-, concentration-, and frequency-dependent blockade of diltiazem on fKv1.4 Δ N currents

Figure 1A shows representative fKv1.4 Δ N current traces recorded by applying 5 s pulses from -100 mV to +50 mV in 10 mV increments in the control, in the presence of 250 µmol/L diltiazem, and after the drug washout. The fKv1.4 Δ N currents were substantially inhibited by the application of 250 µmol/L diltiazem and the effect recovered after washout of the drug for 10 min.

Peak-voltage relationships from the control, 250 μ mol/L diltiazem-treated and drug washout oocyte groups were plotted against clamp potential in Figure 1B. In this figure, the $I_{\rm DIL}/I_{\rm CON}$ ratio was plotted as a function of the membrane potential. Diltiazem decreased the peak currents at transmembrane potentials positive to the activation threshold (-30 mV). The blockade increased steeply in the voltage range coinciding with that of channel activation (between -40 mV and -20 mV) and remained constant at voltages above this range. The peak current was blocked by 52.21%±4.63% when the cell membrane was depolarized to +50 mV in 250 μ mol/L diltiazem, and the effect was reversed by 95% after the drug washout. There was a voltage dependence to the action of diltiazem, a phenomenon typical of open channel block.

Figure 1C shows the concentration dependence of fKv1.4 Δ N current inhibition by diltiazem. Inhibition of the currents in a concentration-dependent manner was measured at the end of a 300 ms pulse of +50 mV. A nonlinear least-squares fit of the Hill equation to the individual data points yielded an apparent dissociation constant, K_D , for an open channel blockade of 241.04±23.06 µmol/L (*n*=5).

To evaluate the longer-term effects of exposing the fKv1.4 Δ N channel to diltiazem, we applied a series of 500 ms depolarizing pulses from -90 mV to +50 mV with a frequency of 1 Hz for a period of 1 min. Figure 2 shows the peak currents elicited by this protocol, before and after exposure to 250 µmol/L diltiazem, normalized to the first peak current under control conditions. With increasing pulse numbers, currents in both the control and the diltiazem-treated groups decreased. The first pulse of the pulse train in the presence of 250 µmol/L



Figure 1. Voltage- and concentration-dependent blockade of fKv1.4 Δ N currents by diltiazem. (A) Channels were expressed in *Xenopus* oocytes and recorded with the two electrode voltage clamp technique. Currents were obtained by applying 5 s pulses to potentials (P₁) ranging from -100 mV to +50 mV and were followed by the tail currents obtained upon repolarization to +50 mV (P₂) under control conditions (a), then in the presence of 250 µmol/L diltiazem (b), and finally after 10 min of washout (c). (B) Current-voltage relationships of fKv1.4 Δ N channels under control conditions, in the presence of 250 µmol/L diltiazem, and after the drug washout for 10 min. Currents were normalized to the peak current at +50 mV under control conditions. The *I*_{DIL}/*I*_{CON} ratio was plotted as a function of the membrane potential. Data are shown as means±SEM. (*n*=5). (C) Dose-response relationships for diltiazem inhibition of fKv1.4 Δ N channels at 2 mmol/L [K⁺]₀. Data were obtained upon repolarization to -90 mV after 1 s pulses to +50 mV, holding potential -90 mV. All values shown were normalized to the peak current in the absence of drug in 2 mmol/L [K⁺]₀. Continuous line was derived by fitting the data to the Hill equation: *f*=*K*_D/(*K*_D+*D*), where *f* is fractional current, *K*_D is the apparent dissociation constant, and *D* is the diltiazem concentration. Symbols and error bar are means±SEM. fKv1.4 Δ N current was reduced to 50% by 241.04±23.06 µmol/L.



Figure 2. Frequency-dependent block of fKv1.4 Δ N channels by diltiazem. Currents were elicited by applying a series of depolarising pulses from -90 mV to +50 mV with a frequency of 1 Hz in the absence (A) and in the presence of 250 µmol/L diltiazem (B). The peak currents shown in Panel A and B were normalized to the maximum control value without drug and plotted in Panel C. As pulse number increased, currents in both control and diltiazem-treated groups decreased. In control cells, there was a use-dependent reduction in the magnitude of the peak current. When cells were exposed to 250 µmol/L diltiazem for 10 min before stimulation, there was a reduction in the magnitude of the first peak current compared to the control value and then a use-dependent reduction in current with diltiazem was much greater than that seen in control.

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diltiazem showed a decrease relative to the pre-drug control, and the magnitude of this reduction in current was similar to that seen under steady-state conditions when a sufficiently long recovery time was allowed between test pulses. In both the control and the 250 μ mol/L diltiazem protocols, there was a use-dependent decrease in the peak current when stimulated at 1 Hz, but this use-dependent decrease was considerably greater in 250 μ mol/L diltiazem than under the control conditions. In control oocytes, the peak current decayed mono-exponentially from 100% to 78.86%. In contrast, in 250 μ mol/L diltiazem the current decayed from 44.14% to 23.07%.

Effect of diltiazem on the steady inactivation of peak Kv1.4 ΔN currents

Figure 3A shows the time-dependent progression of the channel from the rapid open block conformation into a diltiazeminduced block during a single depolarizing step from -90 mV to +50 mV. For comparison, all current traces were normalized to the peak values under control conditions. In control conditions, the rate of inactivation of fKv1.4 Δ N was monoexponential with a time constant of 2.32±0.41 s (*n*=6). In the presence of 10 to 1000 µmol/L diltiazem, inactivation became bi-exponential, with a dominant fast exponential.

Figure 3B(a) shows steady-state inactivation as a function of holding potential for the fKv1.4 Δ N channel both before and after exposure to diltiazem. The relationships were determined from the two pulse protocol by calculating the ratio of the magnitude of the peak current in P₂ to the maximum of the P₂ obtained when P₁ was -100 mV. In this panel, diltiazem can be seen to shift the voltage dependence of inactivation to the left. In order to correct the visual error caused by different minimum values, the steady-state inactivation relationships were renormalized [Figure 3B(b)], thus preferably displaying the drift of the steady inactivation curve before and after diltiazem treatment. Figure 3B(b) also shows this shift to the left. The half-inactivation (V_{1/2}) and slope factor (k) values from



Figure 3. Effect of diltiazem on the steady inactivation of peak Kv1.4 Δ N currents. (A) The time-dependent progression of channel currents. Currents were elicited by applying 1 s pulses from -90 mV to +50 mV in the absence and presence of increasing concentrations of diltiazem. For comparison, all current traces were normalized to the peak values under control conditions. The smooth continuous line superimposed on each trace is the best fit of an exponential function, used to determine the inactivation time constant (s). The control trace was best fitted by a mono-exponential function (Chebyshev method) (a), whereas in the presence of 10 µmol/L-1000 µmol/L diltiazem, inactivation was best fitted by a bi-exponential function (Levenberg-Marquardt) (b-f). (B) Steady-state inactivation relationships (a). The steady-state inactivation for each P₁ voltage was calculated as the magnitude of the peak current in P₂ compared with that from the maximum of the P₂ obtained when P₁ was -100 mV. Average data are shown as mean±SEM. Steady-state inactivation relationships are shown: fKv1.4 Δ N (\blacksquare) and fKv1.4 Δ N+250 µmol/L diltiazem (\bullet). Continuous lines represent the fit of the data to a Boltzmann equation: $f=1/\{1+\exp^*[(V-V_{1/2})/k)]\}$. Steady-state inactivation relationships were re-normalized (b).

fKv1.4ΔN without diltiazem and fKv1.4ΔN with 250 µmol/L diltiazem presented in Figure 4A (a and b) are similar. No statistical difference was observed between the control and diltiazem conditions. The *K* was 4.58±0.75 (*n*=6) in the control and 5.06±0.78 (*n*=6) in the diltiazem treated group, and the *V*_{1/2} was -38.38±0.81 mV in the control and -39.23±0.85 mV (*n*=6) in the diltiazem treated group.

Inactivation of fKv1.4 Δ N is best fitted by a single exponential function (Figure 4B), with an inactivation time constant ($\tau_{inactivation}$) that averaged 2.32±0.41 s (*n*=6) at +50 mV. In the presence of diltiazem, the inactivation of fKv1.4 Δ N is best fitted with a bi-exponential function, with τ_{fast} =0.41±0.04 s and τ_{slow} =1.78±0.29 s at +50 mV (*n*=6) [Figure 4A(c)], where τ_{fast} represents the time constant of inactivation induced by the drug and τ_{slow} represents the time constant of C-type inactivation. We found that C-type inactivation was obviously accelerated

by 250 μmol/L diltiazem at +50 mV. Over the range 0 mV to +50 mV, there is no voltage sensitivity to $\tau_{inactivation}$ (*P*>0.05, *n*=6). In the presence of diltiazem, τ_{fast} is voltage independent, whereas τ_{slow} is voltage dependent. The time constant of C-type inactivation changes was independent of voltage, indicating that C-type inactivation of the fKv1.4ΔN channel is at least partly independent on activation. Thus, above 0 mV, C-type inactivation has nothing to do with the degree of depolarization of membrane voltage; however, C-type inactivation becomes correlated to activation in the presence of diltiazem.

Figure 4C shows the plot of the $1/\tau_{block}$ as a function of the diltiazem concentration for data obtained at concentrations between 10 µmol/L and 1000 µmol/L. The straight line is the least-squares fit to the equation $(1/\tau_{block}=k_{+1}[d]+k_{-1})$. Slope and intercept with the ordinate axis for the fitted relation yielded a k_{+1} and k_{-1} of $(0.01\pm0.002)\times10^6 \text{ (mol/L)}^{-1}\text{ s}^{-1}$ and



Figure 4. Comparison of the voltage for half-inactivation ($V_{1/2}$) and slope factor (k) from fKv1.4 Δ N without diltiazem and fKv1.4 Δ N with 250 µmol/L diltiazem (A(a) and A(b)). (A (a)) $V_{1/2, control}=-38.38\pm0.81$ mV (n=6), $V_{1/2, diltiazem}=-39.23\pm0.85$ mV (n=6), (A(b)) $K_{control}=4.58\pm0.75$ (n=6), $K_{diltiazem}=5.06\pm0.78$ (n=6). Average data are shown as means±SEM (^{a}P >0.05 vs control), (A(c)) The effect of diltiazem on the rate of inactivation of fKv1.4 Δ N channels. The time constant of inactivation was acquired by fitting the current trace elicited at +50 mV (P_1) ranging from the beginning of the peak of P_1 to the end of 5 s. $\tau_{inactivation, control}=2.32\pm0.41$ s (n=6). In the presence of diltiazem, $\tau_{fast}=0.41\pm0.04$ s and $\tau_{slow}=1.78\pm0.29$ s (n=6). Average data are shown as means±SEM ($^{b}P<0.05$ vs control). (B) Diltiazem alters the rate of inactivation for fKv1.4 Δ N channels. Inactivation of fKv1.4 Δ N channels is well fitted by a single exponential function (Chebyshev method), and is voltage independent (\blacksquare) over the range 0 mV to +50 mV. In the presence of diltiazem, the inactivation of fKv1.4 Δ N is best fitted with a bi-exponential function (Levenberg-Marquardt). Over the range 0 mV to +50 mV, τ_{fast} is voltage independent (\bullet), whereas τ_{slow} is voltage dependent (\blacktriangle). (C) The reciprocal of the diltiazem-induced fast time constant ($1/\tau_{plock}$) at +50 mV as a function of the diltiazem concentration for data obtained at concentrations in the range between 10 µmol/L and 1000 µmol/L. The straight line is the least-squares fit to equation: $1/\tau_{plock}=k_{+1}[d]+k_{-1}$, where τ_{block} is the time constant of development of block, k_{+1} and k_{-1} are the apparent association rate constant and the apparent dissociation rate constant, respectively. The dotted lines is the 95% confidence interval of the fit, each point represents the means±SEM of 6 experiments.

2.67±0.25 s⁻¹, respectively.

Effects of diltiazem on the recovery kinetics of fKv1.4 Δ N currents The rate of recovery from inactivation of the Kv1.4 channel is governed by recovery from C-type inactivation. We measured the effect of diltiazem on the rate of recovery from inactivation in the fKv1.4 Δ N channel using a standard gapped pulse protocol with a variable interstimulus interval. The ratio of the magnitude of the first and second pulse peak currents was used as an indication of the degree of the recovery from inactivation.

Figure 5A shows the fraction of fKv1.4 channels recovered plotted against the interstimulus interval. In the presence of

250 μmol/L diltiazem, there is a dramatic decrease in the rate of recovery of fKv1.4ΔN channels when compared to the control. The envelope of peak ratios was best fitted with a mono-exponential function. The presence of 250 μmol/L diltiazem increased the time constant for the rate of recovery from inactivation in fKv1.4ΔN. The mean time constants for recovery were 1.73±0.10 s (*n*=5) in the control and 2.66±0.14 s (*n*=5) in the diltiazem treated group (*P*<0.05; Figure 5B). The half time constant of recovery (*t*_{1/2}) for fKv1.4ΔN was 1.01±0.03 s in the control and 1.67±0.05 s in the presence of 250 μmol/L diltiazem (*n*=5, *P*<0.05; Figure 6). Diltiazem shifted the recovery from inactivation curve to the right and slowed the recovery time constant and *t*_{1/2}.



Figure 5. Effect of diltiazem on the rate of recovery from inactivation in fKv1.4 Δ N. Recovery from inactivation was measured using a standard variable interval gapped pulse protocol. An initial 5 s pulse (P₁) from -90 mV to +50 mV was followed by a second pulse (P₂) to +50 mV after an interval of between 0.1 s and 20 s. (A) The ratio of the peak current elicited by the P₁ and P₂ pulses (P₂/P₁) is plotted against pulse interval to show the recovery from inactivation. The recovery of inactivation was best fitted using the function: *f*=1-A*exp(-T/*t*), where *t* is duration (in s), τ is the time constant, A is the amplitude of the current. Recovery curves for fKv1.4 Δ N and fKv1.4 Δ N+diltiazem, holding potential=-90 mV. (B) Comparison of recovery rate data from fKv1.4 Δ N without and with 250 µmol/L diltiazem. The mean time constants for recovery were 1.73±0.10 s (*n*=5) in control and 2.66±0.14 s (*n*=5) in the diltiazem treated group (^bP<0.05 vs control).



Figure 6. (A) Average recovery time course for fKv1.4 Δ N without diltiazem and with 250 µmol/L diltiazem. Data were normalized between 0 and 1 presented with intervals on a log scale. (B) $t_{1/2}$ for fKv1.4 Δ N was 1.01±0.03 s (*n*=5) and $t_{1/2}$ was 1.67±0.05 s (*n*=5) in the presence of 250 µmol/L diltiazem (^b*P*<0.05 vs control).

Effects of propatenone on fKv1.4 Δ N currents

Voltage-, concentration-, and frequency-dependent blockade of propafenone on fKv1.4 ΔN currents

Figure 7A shows typical fKv1.4 Δ N current traces recorded by applying 5 s pulses from -100 mV to +50 mV followed by the tail currents obtained upon repolarization to +50 mV under control conditions, in the presence of 100 µmol/L propafenone, and after the drug washout. As shown, 100 µmol/L propafenone decreased fKv1.4 Δ N currents, with the effect recovered upon a 10 min washout.

Peak-voltage relationships under control conditions, 100 µmol/L propafenone, and after washout are shown in Figure 7B. In this figure, the I_{PRO}/I_{CON} ratio is plotted as a function of the membrane potential. Propafenone substantially decreased the current amplitude at potentials positive to -30 mV. The I_{PRO}/I_{CON} ratio was plotted as a function of the membrane

potential; the blockade increased steeply in the voltage range coinciding with that of channel activation (between -40 mV and -20 mV), and it remained constant thereafter. The peak current was blocked by $51.82\% \pm 2.35\%$ when depolarized to +50 mV in 100 µmol/L propafenone, and the effect was reversed by 96% after the drug washout. There was a voltage dependence related to the action of propafenone, a phenomenon typical of open channel block.

The concentration dependence of open channel propafenone block of peak fKv1.4 Δ N currents at 2 mmol/L [K⁺]_o is shown in Figure 7C. Data were obtained upon repolarization to -90 mV after 1 s pulses to +50 mV (holding potential: -90 mV). The concentration dependence of the blockade of fKv1.4 Δ N currents was best fitted to the Hill equation. The K_D value for open channel block of fkv1.4 Δ N was 103.68±11.25 µmol/L (*n*=5).



Figure 7. Voltage- and concentration-dependent blockade by propafenone on fKv1.4 Δ N currents. (A) Current recordings from two-electrode voltage clamp of oocytes expressing fKv1.4 Δ N. Currents were obtained by applying 5 s pulses to potentials (P₁) ranging from -100 mV to +50 mV followed by the tail currents obtained upon repolarization to +50 mV (P₂) under control conditions (a), then in the presence of 100 µmol/L propafenone (b), and finally after 10 min of washout (c). (B) Current-voltage relationships of fKv1.4 Δ N channels under control conditions, in the presence of 100 µmol/L propafenone, and after the drug washout for 10 min. Currents were normalized to the peak current at +50 mV under control conditions. The *I*_{PRO}/*I*_{CON} ratio was plotted as a function of the membrane potential. Data are shown as means±SEM (*n*=5). (C) Dose-response relationships for propafenone inhibition of fKv1.4 Δ N channels at 2 mmol/L [K⁺]_o. Data were obtained upon repolarization to -90 mV after 1 s pulses to +50 mV, holding potential -90 mV. All values shown were normalized to the peak current in the absence of drug in 2 mmol/L [K⁺]_o. Continuous line was derived by fitting the data to the Hill equation: *f*=*K*_D/(*K*_D+*D*), where *f* is fractional current, *K*_D is the apparent dissociation constant, and *D* is the propafenone concentration. Symbols and error bar are means±SEM. fKv1.4 Δ N current was reduced to 50% by 103.68±11.25 µmol/L.

To determine the longer-term effects of exposing the fKv1.4∆N channel to propafenone, we applied a series of 500 ms depolarizing pulses from -90 mV to +50 mV with a frequency of 1 Hz for a period of 1 min. Figure 8 shows the peak currents recorded by this protocol, before and after exposure to 100 µmol/L propafenone, normalized to the first peak current under control conditions. With increasing pulse numbers, the currents in both the control and the propafenone-treated groups decreased. The first pulse of the pulse train in the presence of 100 µmol/L propafenone showed a decrease relative to the pre-drug control, and the magnitude of this reduction was similar to that seen under steady-state conditions when an adequately long recovery time was allowed between test pulses. In both the control and the 100 µmol/L propafenone protocols, there was a use-dependent decrease in the peak current when stimulated at 1 Hz, but this use-dependent decrease was much greater in 100 µmol/L propafenone than in the control. In control oocytes, the peak current decayed mono-exponentially from 100% to 74.52%. By contrast, in 100 µmol/L propafenone, the current decreased from 49.72% (due to initial rapid open channel block) to 35.20% in the steady state.

Effect of propatenone on the steady inactivation of peak Kv1.4 ΔN currents

The time-dependent progression of the channel from the rapid open block conformation into a propafenone-induced block was studied during a single depolarizing step from -90 mV to +50 mV (Figure 9A). For comparison, all current traces were normalized to the peak values under control conditions. In control conditions, the rate of inactivation of fKv1.4 Δ N was mono-exponential with a time constant of 2.32±0.41 s (*n*=6). In the presence of 10 to 500 µmol/L propafenone, inactivation became bi-exponential, with a dominant fast exponential.

Figure 9B(a) shows the inactivation curves in the absence and presence of 100 μ mol/L propafenone. The steady-state inactivation relationships were determined from the two pulse protocol by calculating the ratio of the magnitude of the peak current in P₂ to the maximum of the P₂ obtained when P₁ was -100 mV. In this panel, propafenone can be seen to shift the voltage dependence of inactivation to the left. However, after steady-state inactivation relationships were renormalized [Figure 9B(b)], we found that propafenone did not shift the voltage dependence of inactivation. Under control conditions, the $V_{1/2}$ and K values averaged -41.29±5.21 mV and 1.13±0.09 (n=6), and 100 µmol/L propafenone did not modify either the $V_{1/2}$ (-50.62±6.77 mV) or the K (1.62±0.27) (n=6) [Figure 10A(a and b)].

Inactivation of fKv1.4 Δ N is best fitted by a single exponential function (Figure 10B), with $\tau_{\text{inactivation}}$ =2.32±0.41 s (*n*=6) at +50 mV. In the presence of propafenone, the inactivation of fKv1.4 Δ N is best fitted with a bi-exponential function, with τ_{fast} =0.44±0.03 s and τ_{slow} =2.23±0.23 s (*n*=6) at +50 mV [Figure 10A(c)]. We found that C-type inactivation was not shifted by 100 µmol/L propafenone at +50 mV. Over the range of 0 mV to +50 mV, there was no voltage sensitivity to $\tau_{\text{inactivation}}$ (*P*>0.05, *n*=6). In the presence of propafenone, both τ_{fast} and τ_{slow} were voltage independent.

Figure 10C shows the plot of the $1/\tau_{block}$ as a function of the propafenone concentration for data obtained at concentrations between 10 µmol/L and 500 µmol/L. The straight line is the least-squares fit to the equation $(1/\tau_{block}=k_{+1}[d]+k_{-1})$, and the apparent association and dissociation rate constants were $(0.02\pm0.002)\times10^6 \text{ (mol/L)}^{-1}\cdot\text{s}^{-1}$ and $1.87\pm0.15 \text{ s}^{-1}$, respectively.

Effects of propatenone on the recovery kinetics of fKv1.4 ΔN currents

The effects of propafenone on the recovery kinetics of the fKv1.4 Δ N channel expressed in *Xenopus* oocytes are presented in Figure 11A. The fraction of fKv1.4 channels recovered was plotted against the interstimulus interval. The mean time constants of recovery from the steady-state inactivation were 1.78±0.09 s (*n*=5) in the control and 1.86±0.14 s (*n*=5) in the propafenone treated group (*P*>0.05; Figure 11B). In the presence of 100 µmol/L propafenone, there is no significant change in the rate of recovery of fKv1.4 Δ N compared to the control. The half time constant of recovery for fKv1.4 Δ N was 1.14±0.04 s in the control and 1.49±0.05 s in the presence of 100 µmol/L propafenone (*n*=5, *P*>0.05; Figure 12). These results



Figure 8. Frequency-dependent block of fKv1.4 Δ N channel by propafenone. Currents were elicited by using a series of depolarising pulses from -90 mV to +50 mV with a frequency of 1 Hz in the absence (A) and in the presence of 100 µmol/L propafenone (B). The peak currents shown in Panel A and B were normalized to the maximum control value without drug and plotted in Panel C. As pulse number increased, currents in both control and propafenone-treated groups decreased. In control cells, there was a use-dependent reduction in the magnitude of the peak current. When cells were exposed to 100 µmol/L propafenone for 10 min before stimulation, there was a reduction in the magnitude of the first peak current compared to the control value and then a use-dependent component. The use-dependent reduction in current with propafenone was obviously greater than that seen in control.



Figure 9. Effect of propafenone on the steady inactivation of peak Kv1.4 Δ N currents. (A) The time-dependent progression of channel currents. Currents were recorded by using 1 s pulses from -90 mV to +50 mV in the absence and presence of increasing concentrations of propafenone. For comparison, all current traces were normalized to the peak values under control conditions. The smooth continuous line superimposed on each trace is the best fit of an exponential function, used to determine the inactivation time constant (s). The control trace was best fitted by a mono-exponential function (Chebyshev method) (a), whereas in the presence of 10–500 µmol/L propafenone, inactivation was best fitted by a bi-exponential function (Levenberg-Marquardt) (b–f). (B) Steady-state inactivation relationships (a). The steady-state inactivation for each P₁ voltage was calculated as the magnitude of the peak current in P₂ compared with that from the maximum of the P₂ obtained when P₁ was -100 mV. Average data are shown as mean±SEM. Steady-state inactivation relationships are shown: fKv1.4 Δ N (**■**) and fKv1.4 Δ N+100 µmol/L propafenone (**●**). Continuous lines represent the fit of the data to a Boltzmann equation: *f*=1/{1+exp*[(V-V_{1/2})/k)]}. Steady-state inactivation relationships were re-normalized (b).

indicate that propatenone does not affect the recovery of fKv1.4 Δ N channels from inactivation.

Discussion

The L-type calcium channel blocker diltiazem and the sodium channel blocker propafenone have been reported to block several cloned potassium channels, including Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv2.1, Kv4.2, and hERG channel currents^[16, 21-24]. For instance, diltiazem, at concentrations of 0.01 nmol/L to 500 µmol/L, suppressed the hKv1.5 potassium channel expressed in mouse fibroblasts with an estimated IC₅₀ of 42.3 µmol/L^[17]. But in human atrial myocytes, I_{Kur} was blocked by diltiazem at relatively low concentrations (IC₅₀=11.2 µmol/L)^[25]. In Chinese hamster ovary cells, diltiazem (109.9 µmol/L) was reported to suppress the Kv4.3 channel by 50%^[17]. Diltiazem blocked I_{to} (fast) in human atrial myocytes with an IC₅₀ of 29.2 µmol/L^[26]. Propafenone was shown to depress hERG chan-

nel currents in human embryonic kidney cells with an IC₅₀ of 440 µmol/L^[22], and it was shown to block hKv1.5 channels in a concentration-, voltage-, time- and use-dependent manner with an IC₅₀ value of 4.4 mmol/L^[23]. Propafenone was also shown to inhibit $I_{\rm to}$ in rabbit atrial myocytes and rat ventricular myocytes^[26], as well as to inhibit the hyperpolarization-activated inward current in isolated human atrial myocytes^[27] and $I_{\rm Kr}$ in sinoatrial node cells, rabbit atrial myocytes and guinea pig ventricular myocytes^[26, 28-29].

Limited data are available for the Kv1.4 potassium channel. In *Xenopus* oocytes, diltiazem and propafenone have been reported to reduce fKv1.4 potassium channel currents, and 100 µmol/L diltiazem and 100 µmol/L propafenone were reported to suppress Kv1.4 potassium channel tail currents by 10% and 11%, respectively^[16]. Propafenone was shown to be an open channel antagonist of Kv1.4 channel currents^[18], but the mechanism of the drug block was not examined. Our find-

www.nature.com/aps Zhang D et al С Aa b -60 2500 -50 1.5 Time constant (ms) 2000 ZZZ Tslow -40 Tfast V_{1/2}/mV 1500 1.0 -30 \leq 1000 -20 0.5 500 -10 0.0 0 0 100 µmol/L Control Control 100 µmol/L Control 100 µmol/L Propafenone Propafenone Propafenone Ba b 2500 12 2000 9 Time constant (ms) $1/\tau$ block (s⁻¹) 1500 Control τ_{fast} 100 µmol/L propafenone 100 µmol/L propafenone 6 1000 500 3 0 ά 10 20 30 40 50 100 200 300 400 0

Figure 10. Comparison of the voltage for $V_{1/2}$ and K from fKv1.4 Δ N without propafenone and fKv1.4 Δ N with 100 μ mol/L propafenone [A(a) and A(b)]. [A(a)] V_{1/2, control}=-41.29±5.21 mV (n=6), V_{1/2, propagenone}=-50.62±6.77 mV (n=6); [A(b)] K_{control}=1.13±0.09 (n=6), K_{propagenone}=1.62±0.27 (n=6); [A(c)] The effect of propafenone on the rate of inactivation of fKv1.4ΔN channels. The time constant of inactivation was acquired by fitting the current trace elicited at +50 mV (P1) ranging from the beginning of the peak of P1 to the end of 5 s. $\tau_{inactivation, control} = 2.32 \pm 0.41$ s (n=6). In the presence of propafenone, τ_{rast}=0.44±0.03 s and τ_{slow}=2.32±0.23 s (n=6). Average data are shown as means±SEM (^aP>0.05, ^bP<0.05 vs control). (B) Effect of propafenone on the rate of inactivation for fKv1.4ΔN channels. Inactivation of fKv1.4ΔN channels is well fitted by a single exponential function (Chebyshev method), and is voltage independent (a) over the range of 0 mV to +50 mV. In the presence of propafenone, the inactivation of fKv1.4ΔN is best fitted with a biexponential function (Levenberg-Marquardt). Over the range of 0 mV to +50 mV, both τ_{rast} (•) and τ_{slow} (\blacktriangle) are voltage independent. (C) The reciprocal of the propafenone-induced fast time constant $(1/\tau_{block})$ at +50 mV as a function of the propafenone concentration for data obtained at concentrations in the range between 10 and 500 μ mol/L. The straight line is the least-squares fit to equation: $1/\tau_{block} = k_{\star 1}[d] + k_{\star 1}$, where τ_{block} is the time constant of development of block, k+1 and k1 are the apparent association rate constant and the apparent dissociation rate constant, respectively. The dotted lines is the 95% confidence interval of the fit, each point represents the means±SEM of 6 experiments.

ings agree with these reports; however, we found that blockade required relatively high drug concentrations.

Voltage/mV

In the current study, it has been shown that both diltiazem and propafenone are blockers of the fKv1.4 channel. Starting with concentrations of 10 µmol/L, up to 50% of the fKv1.4 channel currents were blocked with 241 µmol/L diltiazem and 103 µmol/L propafenone. Although the concentrations required were higher, in interpreting the results, it has to be further considered that in the oocyte expression system, a fivefold to ten fold higher concentration of antiarrhythmic drugs is needed to obtain an effect comparable to that seen in mammalian cells lines^[16]. Thus, it can be assumed that in cardiomyocytes, both diltiazem and propafenone have an even stronger effect on the Kv1.4 channel than that reported in this study in Xenopus oocytes.

Diltiazem and propafenone are different types of antiarrhythmic drugs. The electrophysiological effects of the two

drugs on fKv1.4ΔN channel inactivation have been determined. Both drugs decrease fKv1.4 Δ N channel currents in voltage-, concentration-, and frequency-dependent manners. However, the difference between the two drugs is three fold. First, our results have demonstrated that diltiazem exhibits a similarly high affinity for fKv1.4 channels and that the concentration of blockade is slightly higher than that needed to block L-type calcium channels^[16], while propafenone exhibits a higher binding affinity for fKv1.4 channels compared with diltiazem. Second, in the presence of diltiazem, the magnitude of the peak current is obviously reduced, and the rate of inactivation is increased compared with the control. The $\tau_{inactivation}$ values we found were 2.32±0.21 s and 1.78±0.19 s in the absence and presence of 250 µmol/L diltiazem, respectively; however, 100 µmol/L propafenone did not increase the C-type inactivation time constant. Third, diltiazem slows recovery from inactivation, but propafenone has no effect on this process.

Propafenone/µmol·L-1

Τ

500



Figure 11. Effect of propatenone on the rate of recovery from inactivation in fKv1.4 Δ N. Recovery from inactivation was measured using a standard variable interval gapped pulse protocol. An initial 5 s pulse (P₁) from -90 mV to +50 mV was followed by a second pulse (P₂) to +50 mV after an interval between 0.1 and 20 s. (A) The ratio of the peak current elicited by the P₁ and P₂ pulses (P₂/P₁) is plotted against pulse interval to show the recovery from inactivation. The recovery of inactivation was best fitted using the function: $f=1-A*\exp(-\tau/t)$, where *t* is duration (in s), τ is the time constant, *A* is the amplitude of the current. Recovery curves for fKv1.4 Δ N and fKv1.4 Δ N+propatenone, holding potential=-90 mV. (B) Comparison of recovery rate data from fKv1.4 Δ N without and with 100 µmol/L propatenone. The mean time constants for recovery were 1.78±0.09 s (*n*=5) in control and 1.86±0.14 s (*n*=5) in the propatenone treated group (^aP>0.05 vs control).



Figure 12. (A) Average recovery time course for fKv1.4 Δ N without propafenone and with 100 µmol/L propafenone. Data were normalized between 0 and 1 presented with intervals on a log scale. (B) $t_{1/2}$ for fKv1.4 Δ N was 1.14±0.04 s (*n*=5) and $t_{1/2}$ was 1.49±0.05 s (*n*=5) in the presence of 100 µmol/L propafenone (^a*P*>0.05 vs control).

Diltiazem induced a voltage-dependent block of fKv1.4 channels that increased over the voltage range of channel activation. When channel activation reached saturation, the block induced by diltiazem remained increased, an effect that resembles the action of propafenone on fKv1.4^[18], and there was a voltage dependence to the action of both drugs.

The rate of fKv1.4 current decay in the control could be fitted to a single exponential function, and in the presence of diltiazem and propafenone, the inactivation became biexponential, characterized by the extremely fast drug-induced inactivation and the relatively slower C-type inactivation. The diltiazem-induced C-type inactivation was much faster than that seen under control conditions, which may be explained by the mechanism in which binding of drug to the intracellular site of the channel triggers a conformational change at the external mouth of the pore that facilitates C-type inactivation. This phenomenon was also observed in the same channel induced by quinidine and verapamil^[18, 20]. However, propafenone did not increase the C-type inactivation time constant, demonstrating that binding of propafenone to the channel did not induce a conformational change at the external mouth of the pore.

The two drug-induced extra component of inactivation [rapid inactivation(τ_{fast})] had a time constant that was much faster than that of slow inactivation; therefore, this fast time constant can be considered to represent the interaction of the drug with the open state. Using the time constants of development for fKv1.4 blockade obtained in the range of 10–1000 µmol/L (for diltiazem) and 10–500 µmol/L (for propafenone), the k_{+1} and k_{-1} constants for diltiazem and propafenone were obtained. Assuming a first order reaction drug/channel inter-

action, the ratio $k_{.1}/k_{+1}$ would give the apparent IC₅₀ of 267 µmol/L (for diltiazem) and 113 µmol/L (for propafenone)^[30, 31]. This estimate was independent of but similar to the IC₅₀ calculated from the respective concentration-response curve. The similarity of the IC₅₀ values obtained by the two independent methods supports the open-channel block model used to calculate the rate constants for the Kv1.4 channel. Diltiazem and propafenone-induced blockade of Kv1.4 channels developed during depolarization, and no blockade happened when the channel closed, which strongly suggests that both drugs are open state blockers of the Kv1.4 channel.

From this study, we found that diltiazem blockade of the fKv1.4 Δ N channel was a complex process that may involve more than one conformational state. First, diltiazem blocks the open channel with rapid kinetics as fast as activation kinetics because diltiazem does not change the steady state activation. Second, diltiazem binds to the channel and blocks it in a voltage- and time-dependent manner. Finally, the binding of diltiazem to the channel enhances C-type inactivation. Based on previous results showing that retardation of C-type inactivation dramatically reduced E-4031 binding affinity in hERG channels^[32], we concluded that an interaction between drug binding and C-type inactivation exists.

Clinically, diltiazem is widely used as an anti-arrhythmic and anti-anginal drug. It has been reported that the cardiac action potentials in mice were lengthened by 10 nmol/L diltiazem, a finding that may be explained by diltiazem-induced blockade of the I_{to} (slow) currents generated by the Kv1.4 channel^[33]. The Kv1.4 channel, as the major component of I_{to} (slow), which in turn is a major contributor to phase 1 and the early part of phase 2 of the action potential, plays an important role in the repolarization of the endocardial region of the left ventricle^[34]. Therefore, the reduction of Kv1.4 induced by diltiazem prolongs action potential durations. Propafenone has a similar effect.

In this study, we found that in response to the faster frequency stimulations, fKv1.4ΔN channel currents were significantly reduced. Opening the channel with a faster frequency may facilitate entry of the drug into the channel. These conformation-specific drug-binding properties lead to some clinically important issues, such as use dependence. The effects of micromolar diltiazem on fKv1.4 are moderate but are likely to be enhanced during tachyarrhythmias due to the frequency dependence of the drug's action, and propafenone shares these characteristics. Decreasing recovery rates could attenuate the shortening of the action potential duration caused by diltiazem-induced inhibition of L-type calcium channels. Because diltiazem and propafenone have different antiarrhythmic effects, their blockade actions on the Kv1.4ΔN channel must be considered when diltiazem is applied in combination with propafenone or other potassium channel blockers such as amiodarone.

Acknowledgements

We thank Dr RASMUSSON (University at Buffalo, SUNY) for the fKv1.4 Δ N cDNA.

Author contribution

Dong ZHANG, Hui CHEN, and Shi-min WANG designed the research; Dong ZHANG, Hui CHEN, Sheng-ping CHAO, and Xue-jun JIANG performed the research; Dong ZHANG and Shi-min WANG analyzed the data; Dong ZHANG and Hui CHEN wrote the paper.

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