

## Original Article

# 18 $\beta$ -Glycyrrhetic acid preferentially blocks late Na current generated by $\Delta$ KPQ Nav1.5 channels

Yi-mei DU<sup>1, #</sup>, Cheng-kun XIA<sup>2, #</sup>, Ning ZHAO<sup>1</sup>, Qian DONG<sup>1</sup>, Ming LEI<sup>1, 3</sup>, Jia-hong XIA<sup>2, \*</sup>

<sup>1</sup>Ion Channelopathy Research Center, Institute of Cardiology and <sup>2</sup>Department of Cardiovascular Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China, <sup>3</sup>Cardiovascular Research Group, School of Clinical and Laboratory Sciences, University of Manchester, Manchester, M13 9NT, UK

**Aim:** To compare the effects of two stereoisomeric forms of glycyrrhetic acid on different components of Na<sup>+</sup> current, HERG and Kv1.5 channel currents.

**Methods:** Wild-type (WT) and long QT syndrome type 3 (LQT-3) mutant  $\Delta$ KPQ Nav1.5 channels, as well as HERG and Kv1.5 channels were expressed in *Xenopus* oocytes. In addition, isolated human atrial myocytes were used. Two-microelectrode voltage-clamp technique was used to record the voltage-activated currents.

**Results:** Superfusion of 18 $\beta$ -glycyrrhetic acid (18 $\beta$ -GA, 1–100  $\mu$ mol/L) blocked both the peak current ( $I_{Na,P}$ ) and late current ( $I_{Na,L}$ ) generated by WT and  $\Delta$ KPQ Nav1.5 channels in a concentration-dependent manner, while 18 $\alpha$ -glycyrrhetic acid (18 $\alpha$ -GA) at the same concentrations had no effects. 18 $\beta$ -GA preferentially blocked  $I_{Na,L}$  ( $IC_{50}=37.2\pm 14.4$   $\mu$ mol/L) to  $I_{Na,P}$  ( $IC_{50}=100.4\pm 11.2$   $\mu$ mol/L) generated by  $\Delta$ KPQ Nav1.5 channels. In human atrial myocytes, 18 $\beta$ -GA (30  $\mu$ mol/L) inhibited 47% of  $I_{Na,P}$  and 87% of  $I_{Na,L}$  induced by *Anemonia sulcata* toxin (ATX-II, 30 nmol/L). Superfusion of 18 $\beta$ -GA (100  $\mu$ mol/L) had no effects on HERG and Kv1.5 channel currents.

**Conclusion:** 18 $\beta$ -GA preferentially blocked the late Na current without affecting HERG and Kv1.5 channels.

**Keywords:** anti-arrhythmia agent; 18 $\beta$ -glycyrrhetic acid; Nav1.5 channel; HERG channel; Kv1.5 channel; human atrial myocyte; *Anemonia sulcata* toxin; long QT syndrome

Acta Pharmacologica Sinica (2012) 33: 752–760; doi: 10.1038/aps.2012.22; published online 21 May 2012

## Introduction

Activation of the cardiac voltage-gated sodium channel (Nav1.5) generates two types of inward currents, a large peak transient current (<3 ms) ( $I_{Na,P}$ ) and another current of weak intensity that spans the action potential (>300 ms). The transient current initiates the rapid upstroke of the action potential, whereas the late persistent sodium current ( $I_{Na,L}$ ) does not have a well-defined function, although it has been shown to affect the duration of the action potential. Recently, the role of  $I_{Na,L}$  in controlling cardiac action potential repolarization and its importance in arrhythmogenesis has received increased attention<sup>[1]</sup>.

An abnormal increase in  $I_{Na,L}$  current, produced by the delayed opening of Na<sup>+</sup> channels, prolongs action potential repolarization and can lead to failed repolarization (early after-depolarizations) and Na<sup>+</sup>-induced Ca<sup>2+</sup> overloading that

triggers delayed after-depolarizations, calcium oscillations, and rapid tachyarrhythmia such as ventricular tachycardia (VT) or fibrillation<sup>[1]</sup>. The reduction of  $I_{Na,L}$  would therefore be expected to have therapeutic potential<sup>[2–4]</sup>. For example, ranolazine, a relatively selective inhibitor of  $I_{Na,L}$ , has been shown to be effective in reducing angina and the incidence of non-sustained VT in patients with ischemic heart disease<sup>[5–7]</sup>.

Pronounced  $I_{Na,L}$  was also observed in the congenital long QT syndrome (LQTS) caused by the mutation of Nav1.5. The most severe defect observed to date has been associated with the  $\Delta$ KPQ mutation. The loss of three amino acids in the intercellular linker between domains 3 and 4 has been associated with LQTS. In the clinic, LQTS can most commonly be produced as an adverse effect to the drug due to blockade of the rapid component of the delayed rectifier potassium current,  $I_{Kr}$ <sup>[8]</sup>. HERG expresses a rapid delayed rectifier current ( $I_{Kr}$ ), and the testing of potential drugs for their ability to block HERG is required for drug approval<sup>[8]</sup>. Kv1.5 conducts ultra-rapid delayed rectifier current ( $I_{Kur}$ ) in the human atria<sup>[9, 10]</sup>, and the loss-of-function mutation might also result in LQTS and cardiac arrest<sup>[11]</sup>.

# These authors contributed equally to the paper.

\* To whom correspondence should be addressed.

E-mail xiajihong@hotmail.com

Received 2011-12-24 Accepted 2012-02-28

Recently, we reported that glycyrrhetic acid (GA), an active ingredient of licorice<sup>[12]</sup>, blocks both  $I_{NaP}$  and  $I_{NaL}$ <sup>[13]</sup>. However, GA exists in two different stereoisomeric forms, the *trans* form and the *cis* form. The two forms have different physical and chemical properties and pharmacological effects<sup>[14]</sup>. Therefore, the purpose of the present study was to determine the action of 18 $\beta$ -GA and 18 $\alpha$ -GA on  $I_{NaP}$  and  $I_{NaL}$  using wild-type (WT) and mutant  $\Delta$ KPQ Nav1.5 channels expressed in *Xenopus* oocytes. Our results indicate that 18 $\beta$ -GA blocked WT and  $\Delta$ KPQ Nav1.5 channels; however, 18 $\alpha$ -GA had no significant effect on either channel. We further characterized the inhibition of WT and  $\Delta$ KPQ Nav1.5 channels by 18 $\beta$ -GA and have demonstrated that 18 $\beta$ -GA preferentially blocks the  $I_{NaL}$  produced by  $\Delta$ KPQ Nav1.5 channels in a concentration-dependent tonic manner. Moreover, we investigated the effects of 18 $\beta$ -GA on HERG and Kv1.5 channels and found that 18 $\beta$ -GA had no obvious effects on either channel. Finally, we evaluated the blockage effects of 18 $\beta$ -GA on  $I_{NaP}$  and  $I_{NaL}$  induced by ATX-II in human atrial myocytes, to further determine the prospects of this drug for treatment in human cardiovascular disease.

## Materials and methods

### Drugs

18 $\alpha$ -GA and 18 $\beta$ -GA were purchased from Sigma (USA) and prepared initially as a 100 mmol/L stock solution by dissolving in 100% DMSO. Before use in an experiment, the stock solution was diluted with ND96 solution to reach the desired final concentration. The percentage of DMSO in the final solution was  $\leq 0.1\%$ , which alone showed no detectable effect on the sodium current. ATX-II was purchased from Sigma (USA) and dissolved in distilled water.

### In vitro transcription of cRNA and functional expression in *Xenopus* oocytes

In vitro transcription of cRNAs and the isolation of oocytes were performed as previously described<sup>[13, 15, 16]</sup>. The plasmid pTracer-SV40 containing WT or  $\Delta$ KPQ human Nav1.5 genes was a kind gift from Dr Thomas ZIMMER of Friedrich Schiller University Jena. HERG was subcloned into the pSP64 plasmid, which was a kind gift from Prof Michael C SANGUINETTI (University of Utah, USA). The human Kv1.5 gene (a gift from Dr Maria L GARCIA, Merck & Co, Inc, USA) was subcloned into a pCI-neo vector. Stage IV and V oocytes were injected with cRNA and then incubated in ND96 solution supplemented with 100 U/mL penicillin, 100 U/mL streptomycin and 2.5 mmol/L sodium pyruvate at 18°C for 3 to 7 d before use in voltage-clamp experiments. ND96 solution contains (in mmol/L) 96 NaCl, 2 KCl, 1.8 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, and 5 HEPES; the pH was adjusted to 7.5 with NaOH. The amount of cRNA injected was varied according to the purpose of the experiment. For  $I_{NaP}$ , the amount of cRNA was adjusted to yield peak currents in the range of 4–8  $\mu$ A to minimize space clamp heterogeneities and series resistance errors;  $I_{NaL}$  measurements required peak current amplitudes of greater than 10  $\mu$ A to

maximize the signal.

### Two-microelectrode voltage clamp

Standard two-microelectrode voltage clamp techniques and a TEV-200A amplifier (Dagan Corporation) were used to record currents at room temperature (22–24°C). Glass microelectrodes were filled with 3 mol/L KCl, and their tips were broken to obtain resistances of 0.5 to 1.5 M $\Omega$ . pCLAMP software (version 9.0; Molecular Devices, Union City, CA, USA) and a 1322A analog/digital interface (Molecular Devices) were used to generate voltage commands.

The voltage protocols used to obtain currents are described in the results. The data for activation and steady-state inactivation were fitted with a simple Boltzmann function:  $I/I_{max} = \{1 + \exp[(V_m - V_{1/2})/k]\}^{-1}$ , where  $I/I_{max}$  is the relative current,  $V_{1/2}$  is the half-maximum voltage of activation or inactivation, and  $k$  is the slope factor. The recovery time course was fitted with the biexponential function:  $I/I_{max} = A_o + A_f(1 - \exp[-t/\tau_f]) + A_s(1 - \exp[-t/\tau_s])$ , where  $\tau$  and  $A$  are the time constants and the corresponding relative amplitude, respectively.

The amplitudes of  $I_{NaP}$  were measured as maximal amplitudes during the first 5 ms of the depolarizing pulse and the  $I_{NaL}$  as the mean current amplitude of the last 10 ms of the pulse. The concentration required for a 50% block of current (IC<sub>50</sub>) was determined by fitting the data to a Hill equation using five concentrations of drug (6–27 oocytes/point).

### Human atrial myocyte isolation and whole-cell patch clamp

Human atrial myocytes were enzymatically dissociated as described previously<sup>[17]</sup>. Right atrial appendage tissues were obtained during atriectomy in patients undergoing coronary artery bypass grafting. All patients were free of supraventricular tachyarrhythmia and symptomatic congestive heart failure, and all atrial tissues were grossly normal at the time of cardiac surgery. Using a patch-clamp amplifier (Axon-200 B, Molecular Devices), the whole-cell patch clamp technique was used to record the  $I_{Na}$ . The series resistance averaged 1.6 $\pm$ 0.4 M $\Omega$  after compensating for approximately 80% of the initial value. The pipette solution contained (in mmol/L) 120 CsCl, 1 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 5 Na<sub>2</sub>ATP, 10 TEACl, 11 EGTA and 10 HEPES (pH 7.3 with CsOH). The bath solution for  $I_{NaP}$  recording contained (in mmol/L) 25 NaCl, 105 CsCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 0.05 CdCl<sub>2</sub>, 10 HEPES and 10 glucose (pH 7.4 with CsOH). The bath solution used to measure the  $I_{NaL}$  induced by ATX-II recording contained (in mmol/L) 135 NaCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 0.05 CdCl<sub>2</sub>, 10 HEPES and 10 glucose (pH 7.4 with NaOH).

### Data analysis

pCLAMP 9.0 and Origin 7.5 (Microcal Software, Northampton, MA, USA) software were used for data acquisition and analysis. Values are expressed as the mean $\pm$ SD. Analyses of variance (ANOVA) for repeated measures and the Holm-Sidak multiple comparison post-test or Student's *t*-test (InStat 2.04; GraphPad Software) were employed to determine statis-

tical significance ( $P < 0.05$ ).

## Results

### Effects of 18 $\alpha$ -GA and 18 $\beta$ -GA on WT and $\Delta$ KPQ Nav1.5 channels

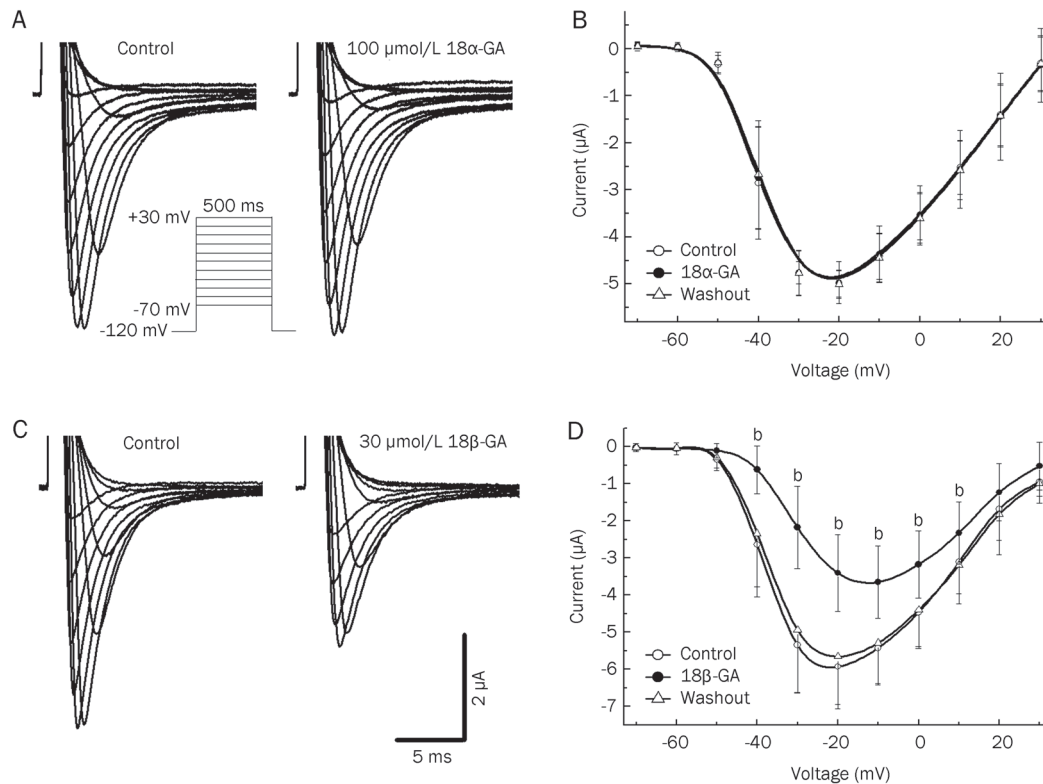
We first compared the effects of 18 $\alpha$ -GA and 18 $\beta$ -GA on  $I_{Na}$  produced by WT (Figure 1) and  $\Delta$ KPQ (Figure 2) Nav1.5 channels expressed in *Xenopus* oocytes. Currents were elicited by a series of 500-ms depolarizing steps from a hold potential of -120 mV with an interpulse interval of 10 s. Original  $I_{Na,P}$  traces of WT Nav1.5 channel-mediated current before and after superfusion with 100  $\mu$ mol/L 18 $\alpha$ -GA or 30  $\mu$ mol/L 18 $\beta$ -GA are shown in Figures 1A and 1C, respectively. Figures 2A and 2C show one typical current trace of  $\Delta$ KPQ Nav1.5 channel current elicited by a depolarizing pulse to -20 mV, which demonstrates the incomplete inactivation of  $I_{Na,L}$  and the effects of 100  $\mu$ mol/L 18 $\alpha$ -GA and 100  $\mu$ mol/L 18 $\beta$ -GA, respectively. Application of 18 $\beta$ -GA significantly inhibited the  $I_{Na}$  produced by both WT and  $\Delta$ KPQ Nav1.5 channels (Figures 1D and 2D), while 18 $\alpha$ -GA had no obvious effects on WT and  $\Delta$ KPQ Nav1.5 channels (Figures 1B and 2B).

### Concentration-dependent tonic block of WT and $\Delta$ KPQ Nav1.5 channels by 18 $\beta$ -GA

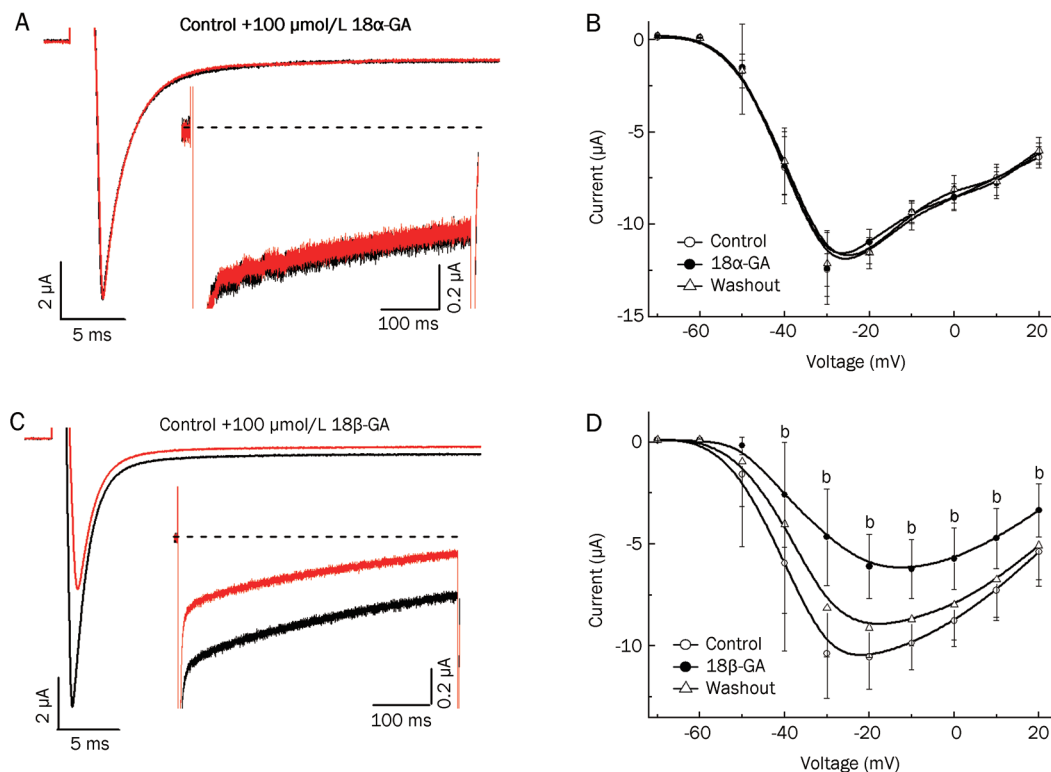
We then examined the dose-dependent and tonic blockage

effects of 18 $\beta$ -GA on WT and  $\Delta$ KPQ Nav1.5 channels. Oocytes were held at -120 mV, and currents were evoked by depolarization to -20 mV every 10 s. This infrequent pulsing protocol should minimize the effects of a frequency-dependent block, therefore providing a reasonable estimate of the extent to which 18 $\beta$ -GA induces a tonic block of  $I_{Na}$ . The upper panel of Figure 3A shows the experimental recordings of  $I_{Na,P}$  from WT Nav1.5 channels before and after the successive application of 1, 30, and 100  $\mu$ mol/L 18 $\beta$ -GA. The upper panels of Figures 3B and 3C show the superimposed  $I_{Na,P}$  and  $I_{Na,L}$  traces of  $\Delta$ KPQ Nav1.5 channels and the blocking effects of 18 $\beta$ -GA at 1, 30 and 100  $\mu$ mol/L, respectively.

Because there was no detectable  $I_{Na,L}$  in WT Nav1.5 channels, only the suppression of  $I_{Na,P}$  was analyzed. The lower panels of Figure 3 show the concentration-response curves for 18 $\beta$ -GA after application to the WT and  $\Delta$ KPQ Nav1.5 channels. The smooth lines represent the best fits of the data using the Hill equation, with the parameters of the fits shown in Figure 3. The Hill coefficients of 18 $\beta$ -GA binding to WT and  $\Delta$ KPQ Nav1.5 channels were not significantly different from 1, suggesting that only one drug molecule is necessary to block the channel. The 18 $\beta$ -GA block of  $I_{Na,P}$  and  $I_{Na,L}$  in  $\Delta$ KPQ Nav1.5 channels exhibited  $IC_{50}$  values of  $100.4 \pm 11.2$   $\mu$ mol/L and  $37.2 \pm 14.4$   $\mu$ mol/L, respectively. These results demonstrated



**Figure 1.** Effects of 18 $\alpha$ -GA and 18 $\beta$ -GA on  $I_{Na,P}$  mediated by the WT Nav1.5 channel. (A) and (C) Representative current traces elicited by 500-ms test pulses from -70 mV to 30 mV with 10 mV increments at 0.1 Hz in typical oocytes perfused before and after in 100  $\mu$ mol/L 18 $\alpha$ -GA (A) or 30  $\mu$ mol/L 18 $\beta$ -GA (C). The membrane potential was held at -120 mV. (B) and (D) Averaged current-voltage relationships of  $I_{Na,P}$  before and after perfusion with 100  $\mu$ mol/L 18 $\alpha$ -GA (B) or 30  $\mu$ mol/L 18 $\beta$ -GA (D);  $n=6$  per group; <sup>b</sup> $P < 0.05$ .



**Figure 2.** Effects of 18 $\alpha$ -GA and 18 $\beta$ -GA on  $\Delta$ KPQ Nav1.5 channels. (A) and (C) Original current recordings in the absence and presence of 100  $\mu$ mol/L 18 $\alpha$ -GA (A) or 18 $\beta$ -GA (C). Current was elicited by a depolarizing pulse from a holding potential of -120 mV to -30 mV for 500 ms. Insets show magnified current traces for comparison of the late component. (B) and (D) Averaged current-voltage relationships of  $I_{Na,P}$  before and after perfusion with 100  $\mu$ mol/L 18 $\alpha$ -GA (B) or 18 $\beta$ -GA (D);  $n=6$  per group;  $^bP<0.05$ .

that 18 $\beta$ -GA exhibited greater degrees of tonic inhibition of  $I_{Na,L}$  compared with  $I_{Na,P}$  when applied to  $\Delta$ KPQ Nav1.5 channels.

#### Rate-dependent block of WT and $\Delta$ KPQ Nav1.5 channels by 18 $\beta$ -GA

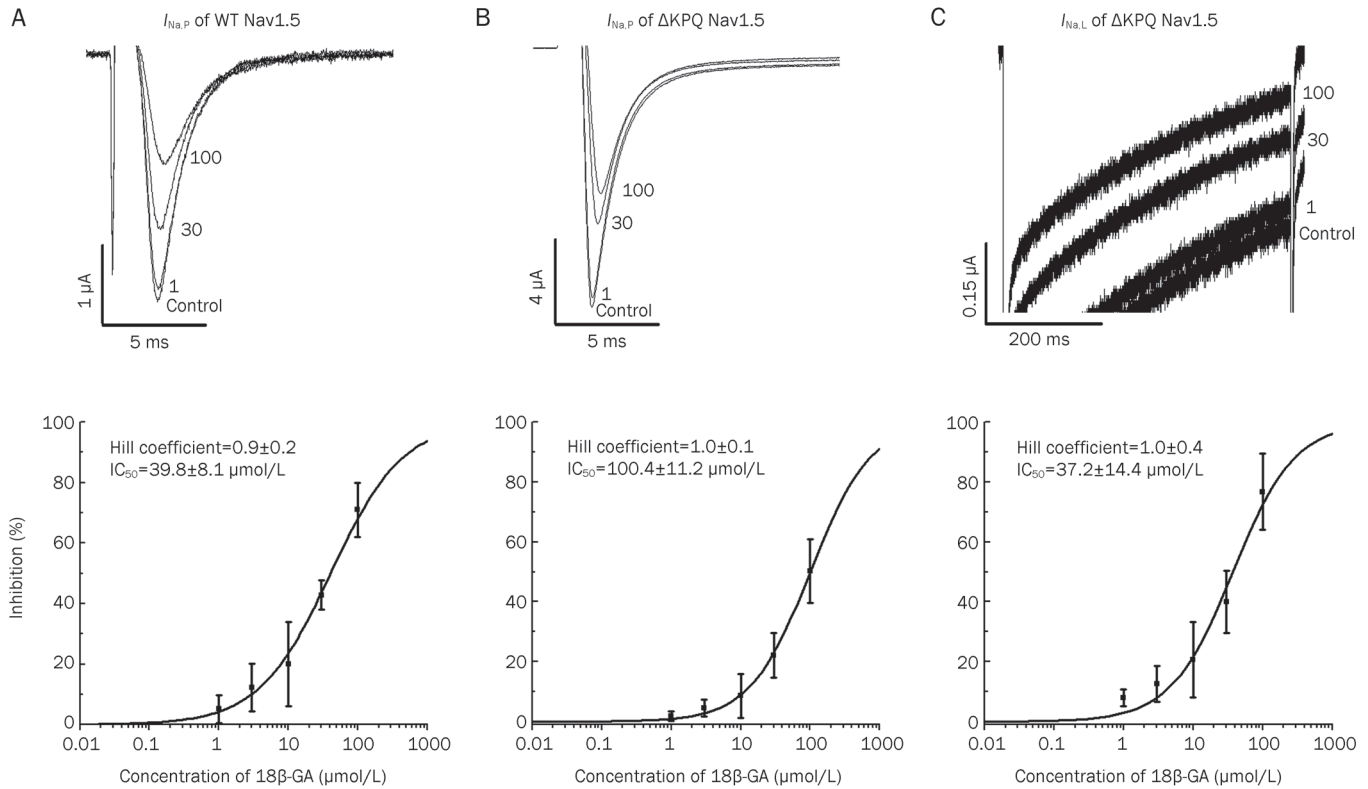
To study the rate-dependent block (phasic block), a series of 30 repetitive impulses to -20 mV from a holding potential of -120 mV were applied at 1, 2, and 4 Hz, according to the method proposed by Rajamani *et al*<sup>[18]</sup>. The amplitude of currents was normalized to the current during the first impulse and plotted as a function of the pulse number. Lidocaine is a clinically used class I anti-arrhythmic agent, and its action on Nav1.5 channels has been studied extensively (reviewed by Sheets *et al*<sup>[19]</sup>). In the presence of 18 $\beta$ -GA (30  $\mu$ mol/L),  $I_{Na,P}$  of WT Nav1.5 channels did not change after 30 repetitive depolarizing events at 4 Hz (approximately 0.93 at the 30th pulse,  $n=6$  in each group, Figure 4A). On the contrary, lidocaine (100  $\mu$ mol/L) caused a significant rate-dependent reduction in the current conducted by the WT Nav1.5 channel ( $P<0.05$  at 1 Hz and 2 Hz,  $P<0.01$  at 4 Hz,  $n=5$ , Figure 4B), which is similar to other reports<sup>[20, 21]</sup>. The  $I_{Na,P}$  mediated by the WT Nav1.5 channel (Figure 4A) shows that the administration of 18 $\beta$ -GA at 100  $\mu$ mol/L caused an additional phasic block of  $I_{Na,P}$  and  $I_{Na,L}$

at  $\Delta$ KPQ Nav1.5 channels ( $P<0.05$ ,  $n=6$ , Figures 4C and 4D).

#### Voltage dependence of the activation and inactivation of WT and $\Delta$ KPQ Nav1.5 channels in the absence and presence of 18 $\beta$ -GA

Voltage-dependent activation and steady-state inactivation of WT and  $\Delta$ KPQ Nav1.5 channels were measured and fitted with Boltzmann equations. The former was evaluated as normalized conductance-voltage relationships, and the latter was determined using a double-pulse as indicated in the protocol diagram in Figure 1S. The parameters are summarized in Table 1. Treatment with 18 $\beta$ -GA significantly shifted the  $V_{1/2}$  of activation curves in the positive direction and  $V_{1/2}$  of the steady-state inactivation curves in the negative direction; these shifts were completely recovered after washout. These results confirmed and extended our previous study on GA<sup>[13]</sup> and suggested that 18 $\beta$ -GA alters the gating function of both WT and  $\Delta$ KPQ Nav1.5 channels.

The recovery time course of WT and  $\Delta$ KPQ Nav1.5 channels from inactivation had fast ( $\tau_f$ ) and slow components ( $\tau_s$ ) (Figure 1S and Table 2). The application of 18 $\beta$ -GA significantly slowed the recovery of WT (30  $\mu$ mol/L) and  $\Delta$ KPQ (100  $\mu$ mol/L) Nav1.5 channels. Table 2 summarizes the above parameters of WT and  $\Delta$ KPQ Nav1.5 channels. These results suggest that 18 $\beta$ -GA could act on both fast and slow inactiva-



**Figure 3.** Concentration-dependent block by 18 $\beta$ -GA of WT  $I_{Na,P}$  (A),  $\Delta$ KPQ  $I_{Na,P}$  (B), and  $I_{Na,L}$  (C).  $I_{Na}$  was elicited by a depolarizing pulse from a holding potential of -120 mV to -30 mV for 500 ms at 0.1 Hz. Representative traces (upper panels) were superimposed before (control) and during perfusion of 18 $\beta$ -GA (1–100  $\mu$ mol/L). (B, C) are traces from the same oocyte on an expanded scale. Summarized dose-response data (lower panels) fitted with the Hill equation.  $IC_{50}$  values and the Hill coefficient are provided in the figure.  $n=6$ –27 oocytes/point.

**Table 1.** Comparative activation and inactivation parameters of WT (at 30  $\mu$ mol/L) and  $\Delta$ KPQ (at 100  $\mu$ mol/L)  $I_{Na,P}$  in the absence (control) and presence of 18 $\beta$ -GA. Mean $\pm$ SD.  $n=6$  per group. <sup>b</sup> $P<0.05$  vs control.

	WT				$\Delta$ KPQ			
	Activation		Inactivation		Activation		Inactivation	
	$V_{1/2}$	$K$	$V_{1/2}$	$K$	$V_{1/2}$	$K$	$V_{1/2}$	$K$
Control	-33.8 $\pm$ 1.8	6.7 $\pm$ 1.6	-81.8 $\pm$ 0.4	7.2 $\pm$ 0.3	-36.2 $\pm$ 1.8	7.0 $\pm$ 1.5	-75.1 $\pm$ 0.4	4.8 $\pm$ 0.4
18 $\beta$ -GA	-25.1 $\pm$ 1.9 <sup>b</sup>	7.7 $\pm$ 1.7	-88.1 $\pm$ 0.3 <sup>b</sup>	6.9 $\pm$ 0.2 <sup>b</sup>	-28.6 $\pm$ 1.6 <sup>b</sup>	7.8 $\pm$ 1.3 <sup>b</sup>	-80.5 $\pm$ 0.5 <sup>b</sup>	5.1 $\pm$ 0.4
Washout	-31.6 $\pm$ 1.7	7.6 $\pm$ 1.5	-83.0 $\pm$ 0.4	7.2 $\pm$ 0.4	-33.2 $\pm$ 1.8	7.0 $\pm$ 1.5	-75.4 $\pm$ 0.4	4.7 $\pm$ 0.4

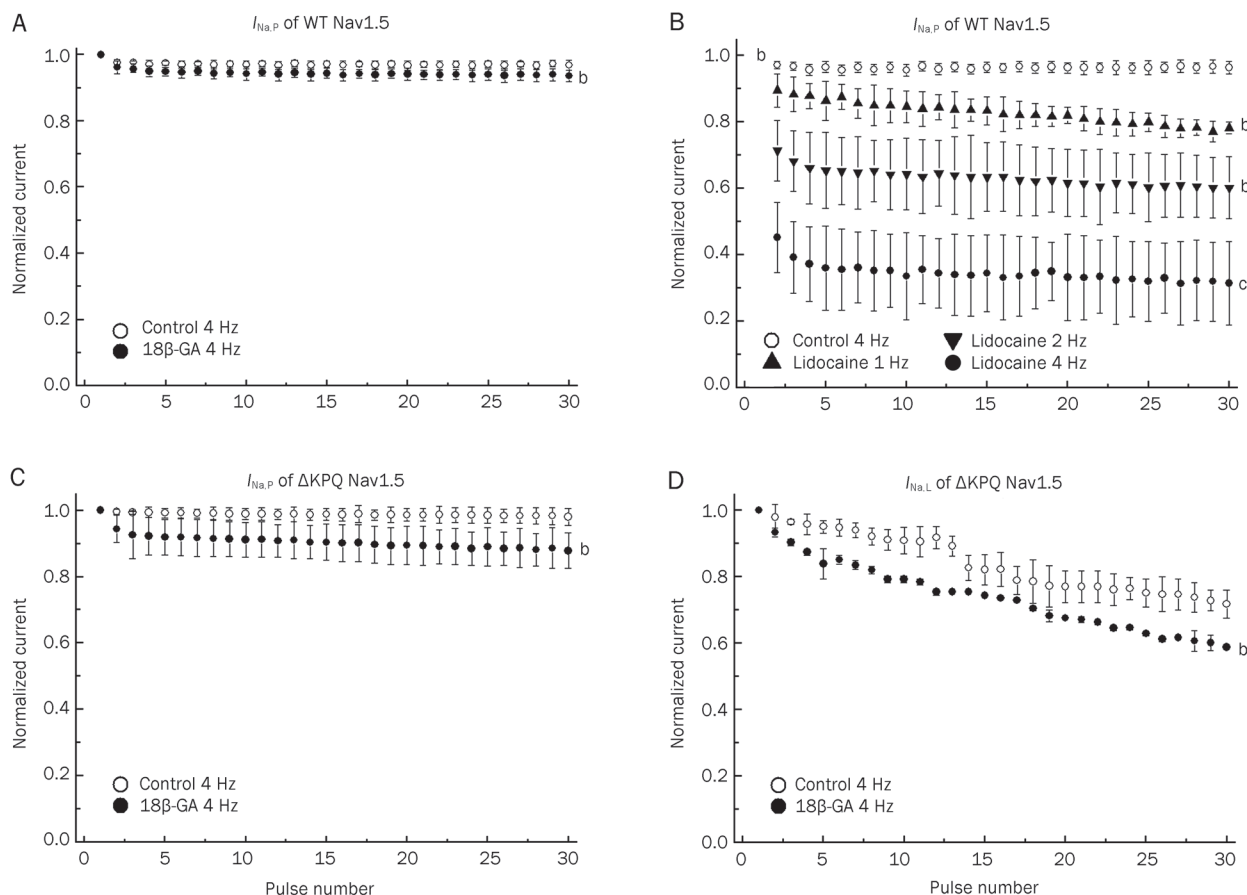
tion components of WT and  $\Delta$ KPQ Nav1.5 channels.

**Table 2.** Recovery inactivation parameters of WT (at 30  $\mu$ mol/L) and  $\Delta$ KPQ (at 100  $\mu$ mol/L)  $I_{Na,P}$  in the absence (control) and presence of 18 $\beta$ -GA. Mean $\pm$ SD.  $n=6$  per group. <sup>b</sup> $P<0.05$  vs control.

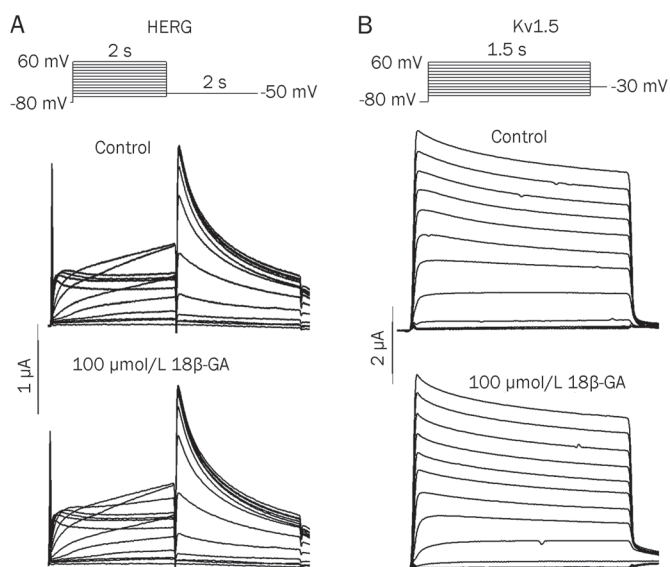
	Control		18 $\beta$ -GA	
	$\tau_f$	$\tau_s$	$\tau_f$	$\tau_s$
WT	11.8 $\pm$ 2.4	261.7 $\pm$ 26.0	22.1 $\pm$ 7.4 <sup>b</sup>	342.7 $\pm$ 36.1 <sup>b</sup>
$\Delta$ KPQ	5.8 $\pm$ 1.1	201.2 $\pm$ 40.5	7.3 $\pm$ 2.0 <sup>b</sup>	272.0 $\pm$ 34.0 <sup>b</sup>

### Effects of 18 $\beta$ -GA on HERG and Kv1.5 channels

Our previous study demonstrated that GA had no significant effect on L-type calcium current ( $I_{Ca,L}$ ) or hyperpolarization-activated inward current ( $I_h$ ) in rabbit sinoatrial node pacemaker cells<sup>[13]</sup>. In this study, we extended our previous study to investigate the effects of 18 $\beta$ -GA on HERG and Kv1.5 channels. Figures 5A and 5B show the experimental current recordings obtained from HERG and Kv1.5 channels before and after the application of 100  $\mu$ mol/L 18 $\beta$ -GA. The voltage protocols are presented in the inset of Figure 5. 18 $\beta$ -GA at 100  $\mu$ mol/L had no inhibitory effects on either HERG ( $n=6$ ) or Kv1.5 channels ( $n=7$ ). No significant effect of 18 $\alpha$ -GA (100



**Figure 4.** Rate-dependent blockade of WT and  $\Delta$ KPQ Nav1.5 channels.  $I_{Na}$  was elicited by a series of 30 depolarizing pulses of -120 mV to -20 mV at different stimulation frequencies. The relative current amplitude elicited by each pulse was normalized to the respective amplitudes the currents elicited by the first pulse and plotted against each pulse number: for WT  $I_{Na,P}$ , 30  $\mu$ mol/L 18 $\beta$ -GA at 4 Hz (A) ( $n=6$ ) and 100  $\mu$ mol/L lidocaine at 1, 2, and 4 Hz (B) ( $n=5$ ); for  $\Delta$ KPQ, 100  $\mu$ mol/L 18 $\beta$ -GA of  $I_{Na,P}$  (C) and  $I_{Na,L}$  (D) at 4 Hz ( $n=6$ ). <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$ .



**Figure 5.** Effects of 18 $\beta$ -GA on HERG (A) and Kv1.5 potassium channels (B) expressed in *Xenopus* oocytes. The voltage protocols are shown in the upper panels. 18 $\beta$ -GA at 100  $\mu$ mol/L had no significant effects on HERG ( $n=6$ ) or Kv1.5 channels ( $n=7$ ).

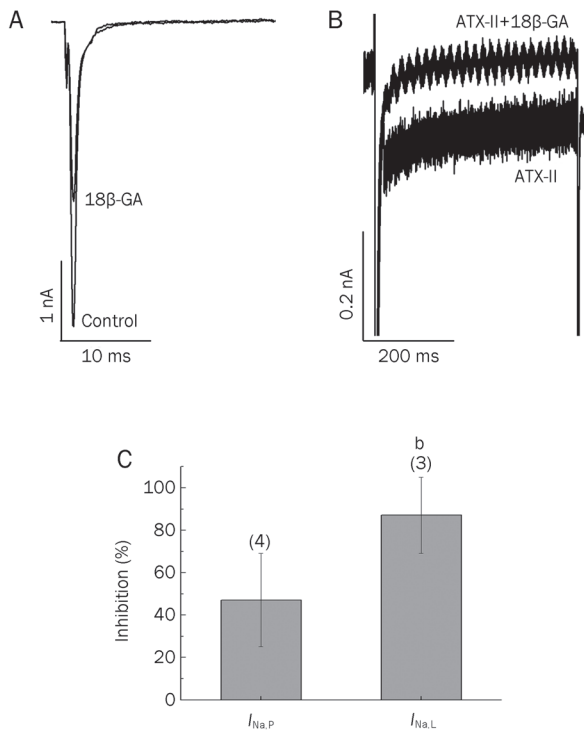
$\mu$ mol/L) on HERG was observed (data not shown).

#### Inhibition of $I_{Na,P}$ and $I_{Na,L}$ induced by ATX-II in human atrial myocytes after exposure to 18 $\beta$ -GA

Figure 6A shows superimposed typical recordings of  $I_{Na,P}$  in the absence and presence of 30  $\mu$ mol/L 18 $\beta$ -GA in freshly isolated human atrial myocytes. Figure 6B shows typical traces of  $I_{Na,L}$  induced by ATX-II (30 nmol/L) and the effect of 18 $\beta$ -GA at 30  $\mu$ mol/L. Currents were obtained with a depolarizing step to -20 mV from a holding potential of -80 mV. 18 $\beta$ -GA at 30  $\mu$ mol/L blocked the  $I_{Na,P}$  and  $I_{Na,L}$  induced by ATX-II by approximately 47% and 87%, respectively ( $P<0.05$ , Figure 6C). These results confirmed the preferential blockage effects of 18 $\beta$ -GA on  $I_{Na,L}$  produced by  $\Delta$ KPQ Nav1.5 channels expressed in *Xenopus* oocytes.

#### Discussion

In the present study, we compared the electrophysiological effects of two stereoisomeric forms of glycyrrhetic acid, 18 $\alpha$ - and 18 $\beta$ -GA, on WT and  $\Delta$ KPQ Nav1.5 channels expressed in *Xenopus* oocytes. The key findings were that 18 $\beta$ -GA inhibited both channels, while 18 $\alpha$ -GA had no significant effects on



**Figure 6.** Effects of 18 $\beta$ -GA on  $I_{Na,P}$  and  $I_{Na,L}$  induced by ATX-II in isolated human atrial myocytes. (A) Original  $I_{Na,P}$  recording in control oocytes and in the presence of 30  $\mu\text{mol/L}$  18 $\beta$ -GA. Current was elicited by a depolarizing pulse from a holding potential of -80 mV to -30 mV for 100 ms. (B) Representative  $I_{Na,L}$  induced by ATX-II (30 nmol/L) in the absence and in the presence of 30  $\mu\text{mol/L}$  18 $\beta$ -GA. Current was elicited by a depolarizing pulse from a holding potential of -80 mV to -30 mV for 500 ms. (C) The bar graph shows the percentage of inhibition of  $I_{Na,P}$  and  $I_{Na,L}$  induced by ATX-II mediated by 30  $\mu\text{mol/L}$  18 $\beta$ -GA. The numbers of cells are indicated in parentheses. <sup>b</sup> $P < 0.05$ .

either WT or  $\Delta\text{KPQ}$  channels. This is the first study to examine the effects of 18 $\beta$ -GA on WT and  $\Delta\text{KPQ}$  Nav1.5 channels as well as HERG and Kv1.5 potassium channels. Our results suggest that 18 $\beta$ -GA preferentially blocks the  $I_{Na,L}$  generated by  $\Delta\text{KPQ}$  Nav1.5 channels in a concentration-dependent tonic manner without significant effects on either HERG or Kv1.5 potassium channels. The greater inhibition of 18 $\beta$ -GA on  $I_{Na,L}$  induced by ATX-II was also observed in human atrial myocytes.

In this study, we compared the pharmacological effects of two stereoisomeric forms of GA (18 $\alpha$ - and 18 $\beta$ -) on WT and  $\Delta\text{KPQ}$  Nav1.5 channels. We observed that 18 $\alpha$ -GA had no significant effects on either WT or  $\Delta\text{KPQ}$  Nav1.5 channels, even at a very high concentration (100  $\mu\text{mol/L}$ ). 18 $\beta$ -GA, however, significantly inhibited both WT and  $\Delta\text{KPQ}$  Nav1.5 channels. Our results demonstrated that 18 $\beta$ -GA but not 18 $\alpha$ -GA blocked WT and  $\Delta\text{KPQ}$  Nav1.5 channels. One possible explanation regarding the difference in blocking effects between the two stereoisomers is the stereochemical structure. Conformational analysis showed that in 18 $\alpha$ -GA, the hydrogen atom of C18 is not in the same plane with the carboxyl chain of C30.

In contrast, in 18 $\beta$ -GA, the hydrogen atom and the carboxyl chain are in the same plane. Consistent with our results, different potencies of 18 $\alpha$ -GA and 18 $\beta$ -GA have been observed in other pharmacological contexts<sup>[22, 23]</sup>. For example, 18 $\alpha$ -GA blocks voltage-gated potassium channels in vascular smooth muscle cells, while 18 $\beta$ -GA does not<sup>[24]</sup>.

Our previous study showed that GA blocked the  $I_{Na,P}$  mediated by the WT Nav1.5 channel (approximately 33% at 90  $\mu\text{mol/L}$ ) and the  $I_{Na,L}$  mediated by the  $\Delta\text{KPQ}$  Nav1.5 channel ( $\text{IC}_{50} = 67 \pm 7.08$   $\mu\text{mol/L}$ )<sup>[13]</sup>. In this study, 18 $\beta$ -GA inhibited the  $I_{Na,P}$  mediated by the WT Nav1.5 channel with an  $\text{IC}_{50}$  of 40  $\mu\text{mol/L}$  and blocked  $I_{Na,P}$  and  $I_{Na,L}$  currents mediated by the  $\Delta\text{KPQ}$  Nav1.5 channel at  $\text{IC}_{50}$  values of 100  $\mu\text{mol/L}$  and 37  $\mu\text{mol/L}$ , respectively. Therefore, 18 $\beta$ -GA was a more potent  $I_{Na,P}$  and  $I_{Na,L}$  blocker than GA; its potency to block  $I_{Na,L}$  was approximately three-fold greater than its potency in blocking the  $I_{Na,P}$  mediated by the  $\Delta\text{KPQ}$  Nav1.5 channel.

Following tonic block, 30 depolarizing pulses were delivered to determine the extent of phasic block, the rate-dependent block, at different frequencies (1, 2, and 4 Hz). For the WT Nav1.5 channel, at 4 Hz, the  $I_{Na,P}$  remaining at the 30th pulse was 94% of the 1st pulse in the presence of 30  $\mu\text{mol/L}$  18 $\beta$ -GA. In contrast to 18 $\beta$ -GA, lidocaine caused a much greater reduction at the 30th pulse *vs* the 1st pulse (~21%). This result implies that 18 $\beta$ -GA caused much less phasic block than lidocaine. Similar results were also found for the  $I_{Na,P}$  and  $I_{Na,L}$  currents of the  $\Delta\text{KPQ}$  Nav1.5 channel. Our results suggest that 18 $\beta$ -GA caused a potent tonic block with little additional phasic block at WT and  $\Delta\text{KPQ}$  Nav1.5 channels, which was different from the effect of lidocaine as well as the reported effects of mexiletine<sup>[25]</sup> and ranolazine<sup>[18]</sup>.

Phasic block is a characteristic of most class I anti-arrhythmic drugs<sup>[19]</sup>, but it also accounts for the unfavorable lethal pro-arrhythmias induced by these drugs<sup>[26]</sup>. 18 $\beta$ -GA, which exhibits less potent phasic block, may prevent excessive blockage of  $I_{Na}$  in the treatment of tachyarrhythmia and may reduce the frequency of bradycardia. We found that  $I_{Na,L}$  exhibited a significant rate-dependent reduction similar to the observation reported by Guo *et al*<sup>[27]</sup>, who suggested that the  $I_{Na,L}$  mediated by the  $\Delta\text{KPQ}$  Nav1.5 channel was more pronounced at low heart rates. This may explain the clinical findings that ventricular tachyarrhythmias and sudden cardiac death in patients with LQT3 tend to occur during sleep or at rest, when the heart rate is slow<sup>[28]</sup>. Ranolazine has recently been used to treat LQT-3 patients expressing the  $\Delta\text{KPQ}$  channel<sup>[29]</sup>. Ranolazine exhibits strong rate-dependent inhibition, as ranolazine produced less inhibition when  $I_{Na,L}$  was more pronounced at a slower heart rate<sup>[18]</sup>. Unlike ranolazine<sup>[18]</sup>, 18 $\beta$ -GA produced a potent tonic block of  $I_{Na,L}$ , which resulted in stronger anti-arrhythmic effects. Recently, a more selective potent  $I_{Na,L}$  blocker, F15845, also characterized by tonic blockade, has been demonstrated to be effective in preventing ischemia-induced arrhythmia<sup>[30]</sup>.

HERG expresses  $I_{Kr}$ , and blockage of HERG is believed to cause LQT, which can induce EAD and a Torsades de-Pointes-type of ventricular arrhythmia, as observed after treatment

with class I or class III anti-arrhythmic drugs including ranolazine<sup>[9, 31, 32]</sup>. Kv1.5 conducts  $I_{Kur}$  in the human atria<sup>[9, 10]</sup>. This channel is also found in the ventricle, but its role remains unknown. 18 $\beta$ -GA at 100  $\mu$ mol/L demonstrated no inhibition of HERG or Kv1.5 channels, which suggested that 18 $\beta$ -GA might not increase QT. However, further study is required to investigate the effects of 18 $\beta$ -GA on QT because other potassium currents, such as transient outward current ( $I_{to}$ ), slow delayed rectifier current ( $I_{Ks}$ ) and inward rectifier current ( $I_{K1}$ ), also determine QT duration.

Up-regulated  $I_{Na,L}$  has been demonstrated to be a major contributor to intracellular  $Na^+$  accumulation during many pathological conditions, such as ischemia or hypoxia, leading to elevated levels of intracellular  $Ca^{2+}$  that have entered the cell through the reverse-mode  $Na^+$ - $Ca^{2+}$  exchanger, which is followed by arrhythmogenesis and ultimately cell death. The inhibition of  $I_{Na,L}$  and prevention of  $Na^+$  overload may therefore be cardioprotective<sup>[3, 33-36]</sup>. 18 $\beta$ -GA preferentially blocks the  $I_{Na,L}$  induced by ATX-II (mimicking  $I_{Na,L}$  activation evoked by ischemia<sup>[36]</sup>) in isolated human atrial myocytes. Experiments on isolated human atrial myocytes confirmed the potency of 18 $\beta$ -GA to reduce  $I_{Na,L}$  in a more physiological environment. Through blockade of  $I_{Na,L}$ , 18 $\beta$ -GA can alleviate  $I_{Na,L}$ -associated arrhythmias<sup>[37]</sup> and cardiac injury after myocardial ischemia<sup>[38]</sup>.

In conclusion, our results show that 18 $\beta$ -GA but not 18 $\alpha$ -GA preferentially blocks  $I_{Na,L}$ . 18 $\beta$ -GA had no significant effects on HERG or Kv1.5 channels. Our results suggest that 18 $\beta$ -GA has significant potential for development as a novel anti-arrhythmic agent, particularly in  $I_{Na,L}$ -associated arrhythmias and myocardial ischemia. Care should be taken in applying the results from heterologous expression studies to the clinical management of patients. Temperature, lipid environment, subunit composition and additional post-translational modifications of  $Na^+$  channels in cardiomyocytes may also affect the degree of blockade by 18 $\beta$ -GA. Nonetheless, further evaluation of the therapeutic potential of 18 $\beta$ -GA is warranted.

### Acknowledgements

This work was supported by the National Natural Science Foundation of China (N<sub>O</sub> 30971243, 81170164 to Dr Yi-mei DU, N<sub>O</sub> 30872542 to Dr Jia-hong XIA) and the Open Foundation of Hubei Key Laboratory of Biological Targeted Therapy N<sub>O</sub> 2010-79 to Dr Cheng-kun XIA.

We thank Drs Thomas ZIMMER (Friedrich Schiller University, Germany), Michael C SANGUINETTI (University of Utah, USA) and Maria L GARCIA (Merck & Co, Inc, USA) for generously providing the cDNA plasmid vector.

### Author contribution

Jia-hong XIA, Ming LEI, and Yi-mei DU designed the research program; Yi-mei DU, Cheng-kun XIA, Ning ZHAO, and Qian DONG performed the research; Yi-mei DU, Cheng-kun XIA, Ming LEI, and Jia-hong XIA analyzed the data and drafted the article.

### Supplementary information

Supplementary figure is available at the Acta Pharmacologica Sinica website.

### References

- 1 Noble D, Noble PJ. Late sodium current in the pathophysiology of cardiovascular disease: consequences of sodium-calcium overload. *Heart* 2006; 92: v1-v5.
- 2 Hale SL, Shryock JC, Belardinelli L, Sweeney M, Kloner RA. Late sodium current inhibition as a new cardioprotective approach. *J Mol Cell Cardiol* 2008; 44: 954-67.
- 3 Morita N, Lee JH, Xie Y, Sovari A, Qu Z, Weiss J N, *et al*. Suppression of re-entrant and multifocal ventricular fibrillation by the late sodium current blocker ranolazine. *J Am Coll Cardiol* 2011; 57: 366-75.
- 4 Shryock JC, Belardinelli L. Inhibition of late sodium current to reduce electrical and mechanical dysfunction of ischaemic myocardium. *Br J Pharmacol* 2008; 153: 1128-32.
- 5 Antzelevitch C, Burashnikov A, Sicouri S, Belardinelli L. Electrophysiological basis for the antiarrhythmic actions of ranolazine. *Heart Rhythm* 2011; 8: 1281-90.
- 6 Jacobshagen C, Belardinelli L, Hasenfuss G, Maier LS. Ranolazine for the treatment of heart failure with preserved ejection fraction: background, aims, and design of the RALI-DHF study. *Clin Cardiol* 2011; 34: 426-32.
- 7 Conti CR. Ion channel therapy of ischemic heart disease: from calcium channel blockers to late sodium current inhibition. *Clin Cardiol* 2011; 34: 66-7.
- 8 Witche HJ. Drug-induced hERG block and long QT syndrome. *Cardiovasc Ther* 2011; 29: 251-9.
- 9 Nerbonne JM. Molecular basis of functional voltage-gated  $K^+$  channel diversity in the mammalian myocardium. *J Physiol* 2000; 525: 285-98.
- 10 Fedida D, Wible B, Wang Z, Fermini B, Faust F, Nattel S, *et al*. Identity of a novel delayed rectifier current from human heart with a cloned  $K^+$  channel current. *Circ Res* 1993; 73: 210-6.
- 11 Nielsen NH, Winkel BG, Kanters JK, Schmitt N, Hofman-Bang J, Jensen HS, *et al*. Mutations in the Kv1.5 channel gene KCNA5 in cardiac arrest patients. *Biochem Biophys Res Commun* 2007; 354: 776-82.
- 12 Fiore C, Eisenhut M, Ragazzi E, Zanchin G, Armanini D. A history of the therapeutic use of liquorice in Europe. *J Ethnopharmacol* 2005; 99: 317-24.
- 13 Du Y, Zhang S, Wu H, Zou A, Lei M, Cheng L, *et al*. Glycyrrhetic acid blocks cardiac sodium channels expressed in *Xenopus* oocytes. *J Ethnopharmacol* 2009; 125: 318-23.
- 14 Wang ZY, Agarwal R, Zhou ZC, Bickers DR, Mukhtar H. Inhibition of mutagenicity in *Salmonella typhimurium* and skin tumor initiating and tumor promoting activities in SENCAR mice by glycyrrhetic acid: comparison of 18 alpha- and 18 beta-stereoisomers. *Carcinogenesis* 1991; 12: 187-92.
- 15 Du YM, Zhang XX, Tu DN, Zhao N, Liu YJ, Xiao H, *et al*. Molecular determinants of Kv1.5 channel block by diphenyl phosphine oxide-1. *J Mol Cell Cardiol* 2010; 48: 1111-20.
- 16 Tu DN, Liao YH, Zou AR, Du YM, Run Q, Wang XP, *et al*. Electropharmacological properties of telmisartan in blocking hKv1.5 and HERG potassium channels expressed on *Xenopus laevis* oocytes. *Acta Pharmacol Sin* 2008; 29: 913-22.
- 17 Xiao H, Wang M, Du Y, Yuan J, Zhao G, Tu D, *et al*. Agonist-like auto-antibodies against calcium channel in patients with dilated cardiomyopathy. *Heart Vessels* 2011. doi: 10.1007/s00380-011-0176-7.
- 18 Rajamani S, El-Bizri N, Shryock JC, Makielski JC, Belardinelli L. Use-



- dependent block of cardiac late Na<sup>+</sup> current by ranolazine. *Heart Rhythm* 2009; 6: 1625–31.
- 19 Sheets MF, Fozzard HA, Lipkind GM, Hanck DA. Sodium channel molecular conformations and antiarrhythmic drug affinity. *Trends Cardiovasc Med* 2010; 20: 16–21.
- 20 Gintant GA, Hoffman BF. Use-dependent block of cardiac sodium channels by quaternary derivatives of lidocaine. *Pflugers Arch* 1984; 400: 121–9.
- 21 Fan XR, Ma JH, Zhang PH, Xing JL. Blocking effect of methylflavonolamine on human Na<sub>v</sub>1.5 channels expressed in *Xenopus laevis* oocytes and on sodium currents in rabbit ventricular myocytes. *Acta Pharmacol Sin* 2010; 31: 297–306.
- 22 Chintharlapalli S, Papineni S, Jutooru I, Mcalees A, Safe S. Structure-dependent activity of glycyrrhetic acid derivatives as peroxisome proliferator-activated receptor (gamma) agonists in colon cancer cells. *Mol Cancer Ther* 2007; 6: 1588–98.
- 23 Zani F, Cuzzoni MT, Daglia M, Benvenuti S, Vampa G, Mazza P. Inhibition of mutagenicity in *Salmonella typhimurium* by *Glycyrrhiza glabra* extract, glycyrrhizinic acid, 18 alpha- and 18 beta-glycyrrhetic acids. *Planta Med* 1993; 59: 502–7.
- 24 Guan BC, Si JQ, Jiang ZG. Blockade of gap junction coupling by glycyrrhetic acids in guinea pig cochlear artery: a whole-cell voltage- and current-clamp study. *Br J Pharmacol* 2007; 151: 1049–60.
- 25 Wang DW, Yazawa K, Makita N Jr, George AL, Bennett PB. Pharmacological targeting of long QT mutant sodium channels. *J Clin Invest* 1997; 99: 1714–20.
- 26 Mestre M, Djellas Y, Carriot T, Cavero I. Frequency-independent blockade of cardiac Na<sup>+</sup> channels by riluzole: comparison with established anticonvulsants and class I anti-arrhythmics. *Fundam Clin Pharmacol* 2000; 14: 107–17.
- 27 Guo D, Lian J, Liu T, Cox R, Margulies KB, Kowey PR, *et al*. Contribution of late sodium current ( $I_{Na-L}$ ) to rate adaptation of ventricular repolarization and reverse use-dependence of QT-prolonging agents. *Heart Rhythm* 2011; 8: 762–9.
- 28 Schwartz PJ, Priori SG, Spazzolini C, Moss AJ, Vincent GM, Napolitano C, *et al*. Genotype-phenotype correlation in the long-QT syndrome: gene-specific triggers for life-threatening arrhythmias. *Circulation* 2001; 103: 89–95.
- 29 Moss AJ, Zareba W, Schwarz KQ, Rosero S, McNitt S, Robinson JL. Ranolazine shortens repolarization in patients with sustained inward sodium current due to type-3 long-QT syndrome. *J Cardiovasc Electro-physiol* 2008; 19: 1289–93.
- 30 Pignier C, Rougier JS, Vie B, Culie C, Verscheure Y, Vacher B, *et al*. Selective inhibition of persistent sodium current by F 15845 prevents ischaemia-induced arrhythmias. *Br J Pharmacol* 2010; 161: 79–91.
- 31 Sanguinetti MC, Jiang C, Curran ME, Keating MT. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the  $I_{Kr}$  potassium channel. *Cell* 1995; 81: 299–307.
- 32 Rajamani S, Shryock JC, Belardinelli L. Rapid kinetic interactions of ranolazine with HERG K<sup>+</sup> current. *J Cardiovasc Pharmacol* 2008; 51: 581–9.
- 33 Lindegger N, Hagen BM, Marks AR, Lederer WJ, Kass RS. Diastolic transient inward current in long QT syndrome type 3 is caused by Ca<sup>2+</sup> overload and inhibited by ranolazine. *J Mol Cell Cardiol* 2009; 47: 326–34.
- 34 Vacher B, Pignier C, Letienne R, Verscheure Y, Le Grand B. F 15845 inhibits persistent sodium current in the heart and prevents angina in animal models. *Br J Pharmacol* 2009; 156: 214–25.
- 35 Wu L, Ma J, Li H, Wang C, Grandi E, Zhang P, *et al*. Late sodium current contributes to the reverse rate-dependent effect of  $I_{Kr}$  inhibition on ventricular repolarization. *Circulation* 2011; 123: 1713–20.
- 36 Belardinelli L, Shryock J C, Fraser H. Inhibition of the late sodium current as a potential cardioprotective principle: effects of the late sodium current inhibitor ranolazine. *Heart* 2006; 92: v6–v14.
- 37 Chen R, Yuan C. Experimental anti-arrhythmic effects of zhigancao (prepared licorice) injection. *Zhongguo Zhong Yao Za Zhi* 1991; 16: 617–9. Chinese.
- 38 Miura T, Ohnuma Y, Kuno A, Tanno M, Ichikawa Y, Nakamura Y, *et al*. Protective role of gap junctions in preconditioning against myocardial infarction. *Am J Physiol Heart Circ Physiol* 2004; 286: H214–21.