

Original Article

Tolterodine reduces veratridine-augmented late I_{Na} , reverse- I_{NCX} and early afterdepolarizations in isolated rabbit ventricular myocytes

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Aim: The augmentation of late sodium current ($I_{Na,L}$) not only causes intracellular Na^+ accumulation, which results in intracellular Ca^{2+} overload via the reverse mode of the Na^+/Ca^{2+} exchange current (reverse- I_{NCX}), but also prolongs APD and induces early afterdepolarizations (EAD), which can lead to arrhythmia and cardiac dysfunction. Thus, the inhibition of $I_{Na,L}$ is considered to be a potential way for therapeutic intervention in ischemia and heart failure. In this study we investigated the effects of tolterodine (Tol), a competitive muscarinic receptor antagonist, on normal and veratridine (Ver)-augmented $I_{Na,L}$, reverse- I_{NCX} and APD in isolated rabbit ventricular myocytes, which might contribute to its cardioprotective activity.

Methods: Rabbit ventricular myocytes were prepared. The $I_{Na,L}$ and reverse- I_{NCX} were recorded in voltage clamp mode, whereas action potentials and Ver-induced early afterdepolarizations (EADs) were recorded in current clamp mode. Drugs were applied via superfusion.

Results: Tol (3–120 nmol/L) concentration-dependently inhibited the normal and Ver-augmented $I_{Na,L}$ with IC_{50} values of 32.08 nmol/L and 42.47 nmol/L, respectively. Atropine (100 μ mol/L) did not affect the inhibitory effects of Tol (30 nmol/L) on Ver-augmented $I_{Na,L}$. In contrast, much high concentrations of Tol was needed to inhibit the transient sodium current ($I_{Na,T}$) with an IC_{50} value of 183.03 μ mol/L. In addition, Tol (30 nmol/L) significantly shifted the inactivation curve of $I_{Na,T}$ toward a more depolarizing membrane potential without affecting its activation characteristics. Moreover, Tol (30 nmol/L) significantly decreased Ver-augmented reverse- I_{NCX} . Tol (30 nmol/L) increased the action potential duration (APD) by 16% under the basal conditions. Ver (20 μ mol/L) considerably extended the APD and evoked EADs in 18/24 cells (75%). In the presence of Ver, Tol (30 nmol/L) markedly decreased the APD and eliminated EADs (0/24 cells).

Conclusion: Tol inhibits normal and Ver-augmented $I_{Na,L}$ and decreases Ver-augmented reverse- I_{NCX} . In addition, Tol reverses the prolongation of the APD and eliminates the EADs induced by Ver, thus prevents Ver-induced arrhythmia.

Keywords: tolterodine; veratridine; ventricular myocytes; late sodium current; reverse- Na^+/Ca^{2+} exchange current; early afterdepolarization; arrhythmia; cardioprotection

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Introduction

Previous studies indicated that late sodium current ($I_{Na,L}$), which is tetrodotoxin (TTX)-sensitive^[1], was increased under various pathological conditions, such as long QT syndrome III, myocardial hypertrophy, heart failure, ischemia, anoxia, and oxidative stress^[2–10]. Although it has a much smaller

amplitude than the transient sodium current ($I_{Na,T}$) under normal conditions, $I_{Na,L}$ plays an important role in determining the plateau of action potential (AP) and action potential duration (APD) under the above pathological conditions^[11, 12]. The augmentation of $I_{Na,L}$ not only causes intracellular Na^+ accumulation, which results in intracellular Ca^{2+} overload via the reverse mode of the Na^+/Ca^{2+} exchange current (I_{NCX}), but also prolongs APD and induces early afterdepolarizations (EAD)^[11–17], which can lead to arrhythmia and cardiac dysfunction. Thus, the inhibition of $I_{Na,L}$ is considered to be a potential target for therapeutic intervention in ischemia and heart

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failure^[1, 11–13, 18–23]. Although several compounds, including RSD1235^[24] and AZD7009^[25], have been reported to block $I_{Na,L}$, their effective doses were above micromolar^[26, 27]. Moreover, these drugs inhibit $I_{Na,L}$ and $I_{Na,T}$ simultaneously, which can induce cardiac conduction block. Currently, only ranolazine has been approved by the FDA as an effective $I_{Na,L}$ blocker for clinical use^[3, 13, 18, 28, 29]. GS-458967, another efficient $I_{Na,L}$ specific inhibitor, has not yet been approved for clinical use and was found to have some potential adverse effects to the central nervous system and peripheral nervous system^[29]. Thus, more research on high affinity $I_{Na,L}$ blockers is urgently needed.

Tolterodine (Tol), a muscarinic antagonist, is used in the symptomatic treatment of patients with frequent micturition, urgent micturition and urinary incontinence induced by bladder overactivity, with a peak serum concentration of 7–58 nmol/L^[30]. Recently, Tol has been reported to affect cardiac ion channels^[30, 31]. Kang *et al* reported that Tol could inhibit HERG channel currents (I_{Kr} on human) expressed on CHO cells and L-type calcium currents ($I_{Ca,L}$) in guinea pig ventricular myocytes, with an IC_{50} of 17, 143 (1 Hz), and 1084 nmol/L (0.1 Hz), respectively. The APD at 90% repolarization (APD₉₀) was increased by 4%, 8%, and 16%, respectively, in the presence of 3, 10, and 30 nmol/L Tol in guinea pig ventricular myocytes. Based on these results, Kang *et al* concluded that Tol did not prolong APD significantly because it blocked both HERG and L-type calcium channels, thus offsetting the effects on APD^[31]. However, this conclusion needs to be further verified. For example, 30 nmol/L Tol significantly inhibited HERG current but slightly decreased $I_{Ca,L}$; thus, in this condition, the prolongation of APD₉₀ should far exceed 16%, which was different from the reported result of a 16% increase in APD₉₀. Furthermore, QT interval prolongation was rarely observed in patients with the application of Tol^[32, 33]. We speculate that Tol can inhibit other inward currents associated with APD. Veratridine (Ver)^[34], an activator of $I_{Na,L}$ is suitable for evoking EAD because it can increase APD to the required extent^[35, 36]. Accordingly, this study aims to explore the possible antiarrhythmic mechanisms of Tol by investigating the effects of Tol on normal and Ver-induced $I_{Na,L}$ reverse I_{NCX} and APD in rabbit ventricular myocytes.

Materials and methods

Cardiomyocyte isolation

Rabbits of either sex, weighing 1500–2000 g, were purchased from the Animal Experimental Center of Wuhan University of Science and Technology, Wuhan, China, and were anesthetized with ketamine (Fujian Gutian Pharmaceutical Co, Ltd, Gutian, Fujian, China, 30 mg/kg iv) and xylazine (Shanghai Shifeng Biological Technology Co, Ltd, Shanghai, China, 7.5 mg/kg im) 10 min after an intravenous injection of 2000 U of heparin. All efforts were made to minimize animal discomfort and suffering during the experimental process. The heart was excised and retrogradely perfused on a modified Langendorff apparatus with Ca^{2+} -free Tyrode's solutions bubbled with 100% O_2 and maintained at 37°C. The heart was then perfused with Ca^{2+} -free Tyrode's solution containing collagenase type

I (0.1 mg/mL) and BSA (0.5 mg/mL) for 40–50 min, and then with KB solution for another 5 min. The left ventricle was cut into small chunks and gently agitated in KB solution. The cells were filtered through nylon mesh and stored in KB solution at 4°C. The use of animals 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” and was approved by the Institutional Animal Care and Use Committee of the Medical College of Wuhan University of Science and Technology (Wuhan, China).

Whole-cell patch-clamp technique

The conventional whole-cell patch-clamp technique using an EPC-10 amplifier (HEKA Electronic, Lambrecht, Germany) was applied to record transmembrane potentials and ion currents in either current clamp or voltage clamp mode. Patch electrodes were pulled with a two-stage puller (PP-83, Narishige Group, Tokyo, Japan), and their resistances were in the range of 1.0–1.5 M Ω after filling with pipette solution. An 80% compensation of series resistance was achieved without ringing. Currents were filtered at 2 kHz, digitized at 10 kHz, and stored on a computer hard drive for further analysis. All experiments were performed at room temperature (22–24°C).

The current-voltage (I - V) relationship of $I_{Na,L}$ was determined by 300 ms depolarizing pulses to potentials ranging from -90 mV to +40 mV in 10 mV increments from a holding potential of -120 mV. $I_{Na,L}$ was measured at the 200 ms of the depolarizing pulse to avoid the influence of $I_{Na,T}$ on $I_{Na,L}$ ^[37]. The voltage dependence of steady-state inactivation for $I_{Na,T}$ was determined using 100 ms conditioning pulses from a holding potential of -120 mV, ranging from -100 mV to -45 mV in 5 mV increments, followed by a test pulse to -10 mV for 100 ms. The voltage dependence of steady-state activation for $I_{Na,T}$ was determined using a holding potential of -120 mV, ranging from -90 mV to +60 mV in 5 mV increments. The maximum value of $I_{Na,T}$ was measured. The I_{NCX} was induced by a ramp-pulse protocol ranging from +60 mV to -120 mV continuing for 2000 ms from a holding potential of -40 mV. I_{NCX} was measured as the current sensitive to 5 mmol/L Ni^{2+} at +50 and -100 mV. AP was recorded in current clamp mode by using whole-cell patch-clamp techniques and was evoked by depolarizing current pulses (5 ms duration, 1.5 times the threshold intensity, 0.25 Hz) in ventricular myocytes.

Solutions

Regular Tyrode's solution contained the following (in mmol/L): 135 NaCl, 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, 0.33 NaH₂PO₄, 10 HEPES and 10 glucose (pH 7.4, adjusted with NaOH).

The KB solution contained the following (in mmol/L): 70 KOH, 40 KCl, 3 MgCl₂, 20 KH₂PO₄, 0.5 EGTA, 50 L-glutamic acid, 20 taurine, 10 HEPES, and 10 glucose (pH 7.4, adjusted with KOH).

To record $I_{Na,L}$, the pipette solution contained the following (in mmol/L): 120 CsCl, 1 CaCl₂, 5 MgCl₂, 5 Na₂ATP, 10 TEACl, 11 EGTA and 10 HEPES (pH 7.3, adjusted with CsOH). The bath solutions contained the following (in mmol/L): 135

NaCl, 5.4 CsCl, 1.8 CaCl₂, 1 MgCl₂, 0.3 BaCl₂, 0.33 NaH₂PO₄, 10 HEPES, 10 glucose and 0.001 nicardipine (pH 7.4, adjusted with NaOH).

To record $I_{Na,T}$, the pipette solution contained the following (in mmol/L): 120 CsCl, 1 CaCl₂, 5 MgCl₂, 5 Na₂ATP, 10 TEACl, 11 EGTA, and 10 HEPES (pH 7.3, adjusted with CsOH). The bath solutions contained the following (in mmol/L): 30 NaCl, 105 CsCl, 1 CaCl₂, 1 MgCl₂, 0.001 nicardipine, 10 HEPES and 10 glucose (pH 7.4, adjusted with CsOH).

To record I_{NCX} , the pipette solution contained the following (in mmol/L): 120 CsOH, 20 NaCl, 10 CaCl₂, 50 aspartic acid, 3 MgCl₂, 20 EGTA, 5 MgATP and 10 HEPES (pH 7.3, adjusted with CsOH). The bath solution contained the following (in mmol/L): 140 NaCl, 2 CaCl₂, 2 MgCl₂, 5 HEPES and 10 glucose (pH 7.4, adjusted with NaOH). In addition, 20 μmol/L ouabain, 1.0 mmol/L BaCl₂, 2.0 mmol/L CsCl and 1.0 μmol/L nicardipine were added to block Na⁺/K⁺ exchange current, potassium current and $I_{Ca,L}$, respectively.

To record AP, the pipette solution contained the following (in mmol/L): 120 potassium aspartate, 1 CaCl₂, 1 MgSO₄, 4 Na₂ATP, 0.25 Na₃GTP and 10 HEPES (pH 7.3, adjusted with KOH). The bath solution was Tyrode's solution.

Drugs and reagents

TTX and collagenase type I were provided by Tocris (Ellisville, MO, USA) and Gibco (Invitrogen, Paisley, UK), respectively. BSA, taurine, and HEPES were purchased from Roche (Basel, Switzerland). All other chemicals were obtained from Sigma Chemical (Saint Louis, MO, USA). Tol and Ver were stored in the dark at room temperature and -20°C, respectively. On the day of experimentation, these two drugs were dissolved in dimethyl sulfoxide to create stock solutions from which test solutions were made. The stock solution was diluted with cell external solution to achieve the desired final concentrations immediately before the experiments. The final concentration of dimethyl sulfoxide was less than 0.1%. When the two drugs were used sequentially in an experiment, we applied the second drug after the effect of the first drug achieved a stable level.

Data analysis

All data are presented as the mean±SD and were analyzed using FitMaster (v2x32, HEKA) and SPSS 13.0 software. Current density (pA/pF), *ie*, the amplitude of the current divided by membrane capacitance, was used for analysis. Figures were plotted with Origin (V7.5, OriginLab Co, MA, USA). Student's *t*-test was used to determine the difference between two groups of data. Statistical analysis was performed using one-way analyses of variance (ANOVA), followed by the Scheffé test for multiple comparisons. A *P*-value<0.05 was considered statistically significant.

Current tracings were fitted using Hill or Boltzmann functions with Origin 7.5. The fractional blockade was calculated using the following equation: Fractional blockade=($I_{control}-I_{drug}$)/ $I_{control}$, where $I_{control}$ and I_{drug} are the current amplitudes in the absence and presence of Tol, respectively. Concentration-

effect curves were fitted using the Hill equation as follows: ($I_{control}-I_{drug}$)/ $I_{control}=B_{max}/[1+(IC_{50}/D)^n]$, where B_{max} was the maximum blockade of currents, IC_{50} was the concentration of Tol for half-maximum blockade, D was the concentration of Tol, and n was the Hill coefficient. For all IC_{50} values, all current levels were measured at -20 mV.

Steady-state activation data and inactivation relationships of $I_{Na,T}$ were fitted to the Boltzmann equation as follows: $Y=1/[1+\exp(V_m-V_{1/2})/k]$, 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}), respectively.

Results

Effects of Tol on $I_{Na,L}$ under normal conditions

$I_{Na,L}$ was recorded with 300 ms voltage steps from a holding potential of -120 mV to -20 mV at 0.5 Hz. After the application of 4 μmol/L TTX, $I_{Na,T}$ did not change significantly, whereas $I_{Na,L}$ was almost completely blocked with densities changing from 0.335±0.017 to 0.076±0.012 ($n=8$, $P<0.01$ vs control; Figure 1). In addition, the increased currents induced by 20 μmol/L Ver (from 0.253±0.015 to 1.613±0.032) were almost completely blocked by 4 μmol/L TTX (0.081±0.009; $n=6$, $P<0.01$ vs 20 μmol/L Ver; Figure 1A). These results showed that the increased recorded currents were exactly $I_{Na,L}$ (Figure 1A).

The current-voltage relationship of $I_{Na,L}$ was recorded. Tol (6, 10, 30, or 120 nmol/L) inhibited $I_{Na,L}$ in a concentration-dependent manner (Figure 1B, 1C). The inhibition rates of $I_{Na,L}$ (at -20 mV) were 11.7%±2.1% ($n=10$, $P<0.05$ vs control), 29.7%±9.0% ($n=10$, $P<0.01$ vs 6 nmol/L), 54.2%±9.3% ($n=10$, $P<0.01$ vs 10 nmol/L), 79.4%±9.6% ($n=10$, $P<0.05$ vs 30 nmol/L) at 6, 10, 30, 120 nmol/L Tol, respectively, and the value of IC_{50} was 32.08 nmol/L (Figure 1).

Effects of Tol on increased $I_{Na,L}$ induced by Ver

At 20 μmol/L, Ver increased currents densities of $I_{Na,L}$ from 0.448±0.015 to 1.601±0.062 ($n=10$, $P<0.01$). Tol (3, 6, 10, 30, or 120 nmol/L) suppressed increased $I_{Na,L}$ induced by Ver in a concentration-dependent manner (Figures 2A–2C). The inhibition rates of $I_{Na,L}$ (at -20 mV) were 8.3%±1.1% ($n=10$, $P<0.05$ vs 20 μmol/L Ver), 12.9%±3.2% ($n=10$, $P<0.01$ vs 3 nmol/L Tol), 32.4%±5.2% ($n=10$, $P<0.01$ vs 6 nmol/L Tol), 49.9%±8.9% ($n=10$, $P<0.05$ vs 10 nmol/L Tol), and 68.6%±9.0% ($n=10$, $P<0.05$ vs 30 nmol/L Tol) at 3, 6, 10, 30, 120 nmol/L Tol, respectively. The value of IC_{50} was 42.47 nmol/L (Figure 2D, 2E).

Effects of atropine on increased $I_{Na,L}$ induced by Ver

We studied whether the blocking effect of Tol on $I_{Na,L}$ was achieved by its binding to the muscarinic (M) receptor. Atropine, a high-performance M receptor antagonist, was used. After the application of 20 μmol/L Ver, the current density of $I_{Na,L}$ was increased from 0.310±0.008 pA/pF to 1.668±0.048 pA/pF ($n=8$, $P<0.01$) and was not significantly changed after giving 100 μmol/L atropine (1.656±0.039 pA/pF, $n=8$, $P>0.05$ vs Ver). However, after the application of 30 nmol/L Tol, the

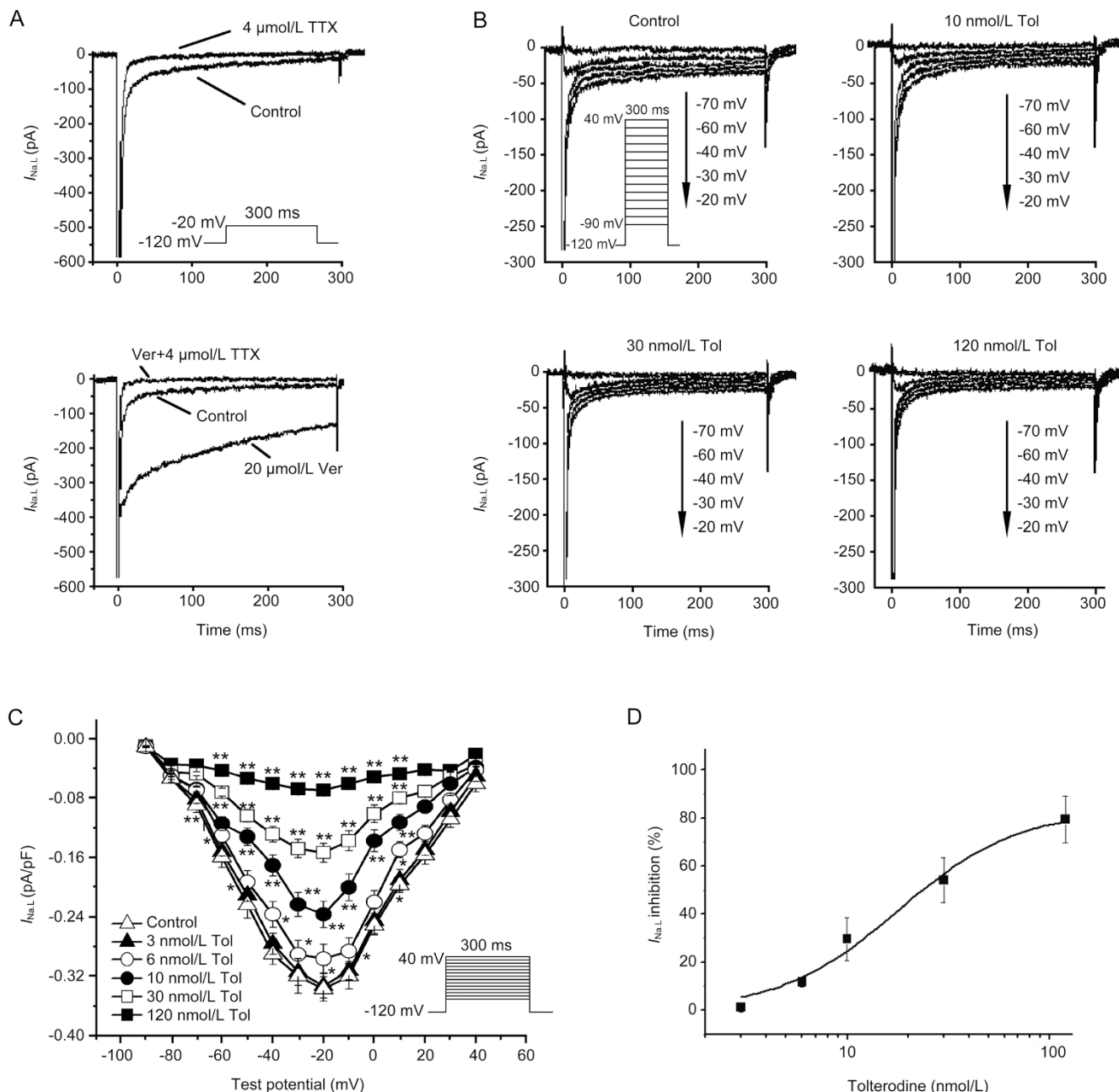


Figure 1. Effects of tolterodine (Tol) on normal $I_{Na,L}$ in rabbit ventricular myocytes. (A) The blockade of 4 $\mu\text{mol/L}$ TTX on normal and Ver-increased $I_{Na,L}$. (B) Typical whole-cell $I_{Na,L}$ traces recorded in the absence (control) and presence of 10, 30, and 120 nmol/L Tol. (C) The effects of Tol on the current-voltage relationship of $I_{Na,L}$. * $P < 0.05$, ** $P < 0.01$ vs control. (D) The concentration-effect curves were plotted based on data from panel C and fitted using the Hill equation (used all currents values at -20 mV).

current density of $I_{Na,L}$ was decreased to 0.689 ± 0.019 pA/pF ($n=8$, $P < 0.01$ vs atropine, Figures 2F). These results indicated that Tol's blockade of $I_{Na,L}$ was independent of its effect on the M receptor signaling pathway.

Effects of Tol on $I_{Na,T}$

At concentration of 1 $\mu\text{mol/L}$ Tol did not change the density of $I_{Na,T}$ ($n=10$, $P > 0.05$ vs control), whereas 10, 30, 100, and 300 $\mu\text{mol/L}$ Tol decreased the magnitudes of $I_{Na,T}$ in a concentra-

tion-dependent manner (Figure 3A). The suppression rates of $I_{Na,T}$ (at -20 mV) were $14.2 \pm 2.3\%$ ($n=10$, $P < 0.05$ vs 1 $\mu\text{mol/L}$ Tol), $25.8 \pm 5.3\%$ ($n=10$, $P < 0.01$ vs 10 $\mu\text{mol/L}$ Tol), $43.7 \pm 5.4\%$ ($n=10$, $P < 0.01$ vs 30 $\mu\text{mol/L}$ Tol), and $55.3 \pm 6.8\%$ ($n=10$, $P < 0.05$ vs 100 $\mu\text{mol/L}$ Tol) at 10, 30, 100, and 300 $\mu\text{mol/L}$ Tol, respectively, and the value of IC_{50} was 183.03 $\mu\text{mol/L}$ (Figures 3A, 3B).

The steady-state activation and inactivation curves for $I_{Na,T}$ were also examined. For the steady-state activation of $I_{Na,T}$, the

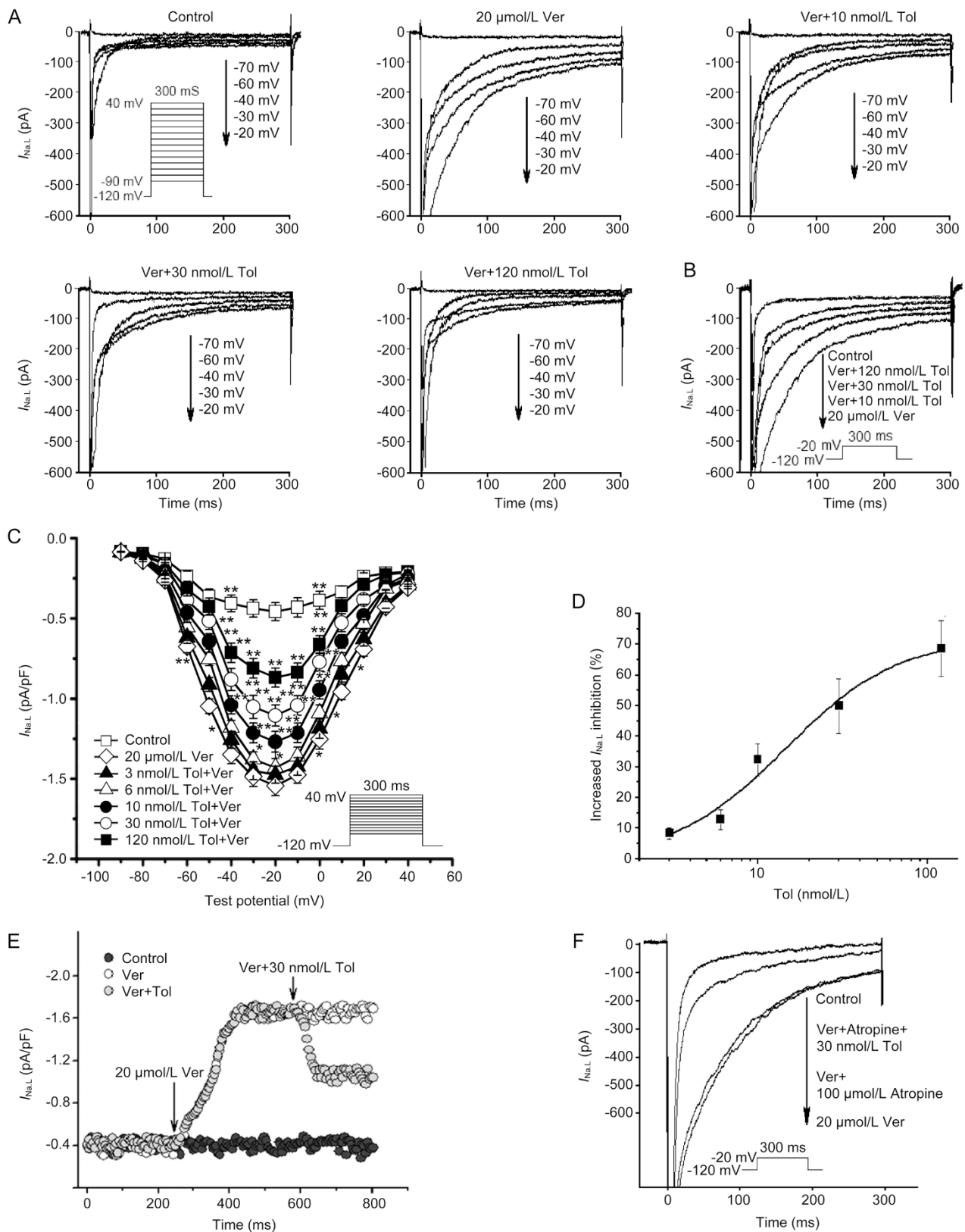


Figure 2. Effects of tolterodine (Tol) on increased $I_{\text{Na,L}}$ induced by Ver in rabbit ventricular myocytes. (A) Tol 10, 30, and 120 nmol/L suppressed increased $I_{\text{Na,L}}$ by 20 $\mu\text{mol/L}$ Ver in a concentration-dependent manner. (B) The current recordings at -20 mV from Panel A. (C) Effects of Tol at 3–120 nmol/L on the current-voltage relationship of $I_{\text{Na,L}}$ induced by 20 $\mu\text{mol/L}$ Ver. * $P < 0.05$, ** $P < 0.01$ vs 20 $\mu\text{mol/L}$ Ver. (D) The concentration-effect curves were plotted based on data from panel C (at -20 mV) and fitted using the Hill equation. (E) The time courses of effects of Ver and Tol on $I_{\text{Na,L}}$ (at -20 mV). (F) In the presence of 100 $\mu\text{mol/L}$ atropine, 30 nmol/L Tol decreased the increased $I_{\text{Na,L}}$ by 20 $\mu\text{mol/L}$ Ver.

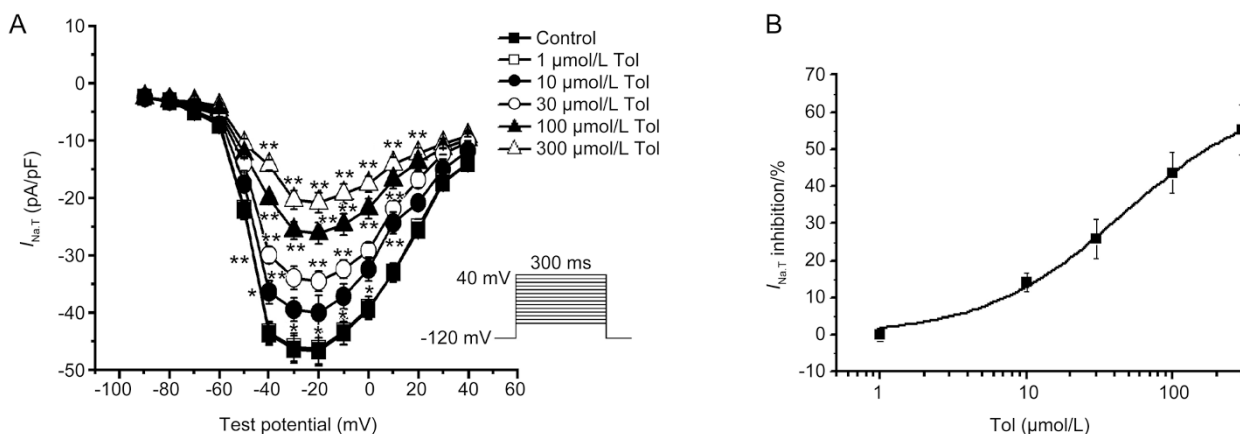


Figure 3. Effects of Tol on $I_{Na,T}$ on rabbit ventricular myocytes. (A) The effects of Tol (1, 10, 30, 100, or 300 $\mu\text{mol/L}$) on the current-voltage relationships of $I_{Na,T}$. * $P < 0.05$, ** $P < 0.01$ vs control. (B) The concentration-effect curves are plotted based on data from panel A and fitted using the Hill equation.

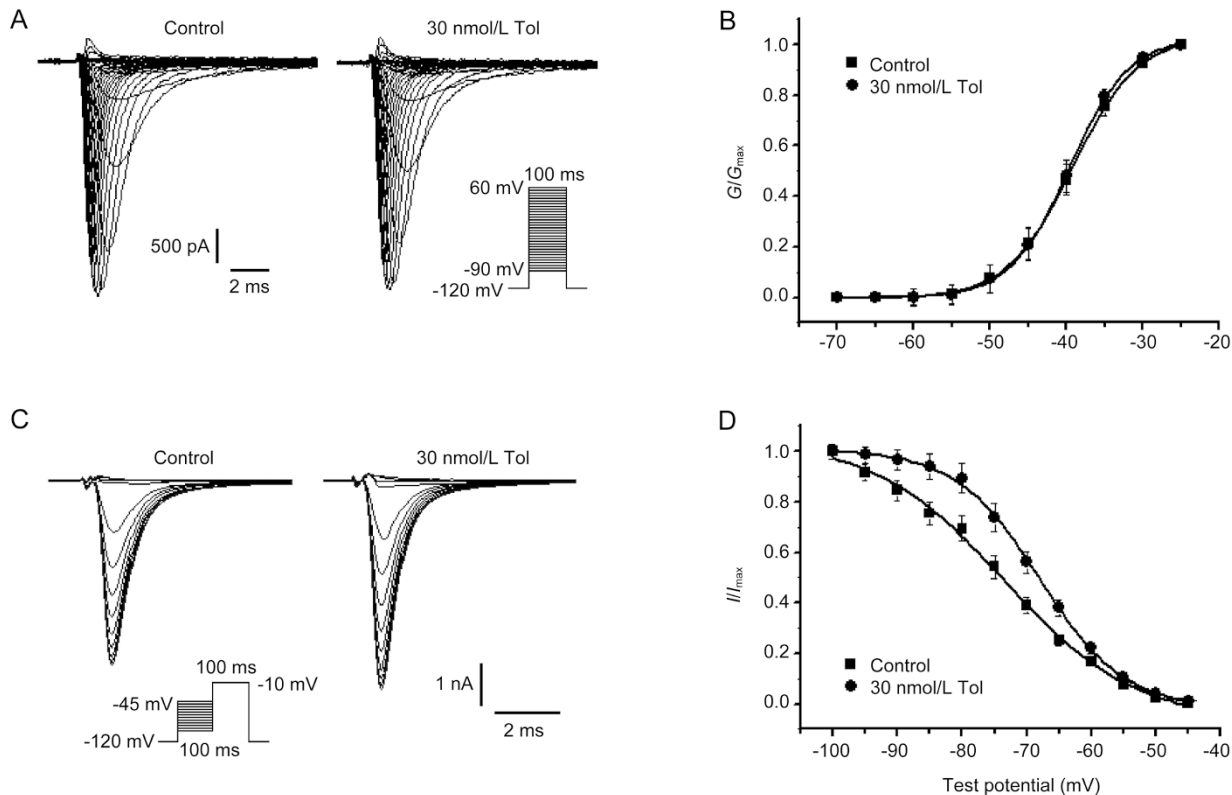


Figure 4. Effects of Tol on steady-state activation and inactivation of $I_{Na,T}$ in rabbit ventricular myocytes. (A and C) The steady-state activation and inactivation current recordings of $I_{Na,T}$ in myocytes before and after superfusion with 30 nmol/L Tol. (B and D) The steady-state activation and inactivation curves of $I_{Na,T}$ before and after application of 30 nmol/L Tol. Conductivities were normalized against the maximum conductivity on each condition (B). Currents were normalized against the maximum currents at -100 mV for each cell (D). The data were fitted using the Boltzmann equation.

values of $V_{1/2}$ were -39.23 ± 0.08 mV and -39.64 ± 0.11 mV ($n=7$, $P > 0.05$), and the values of k were 4.29 ± 0.07 and 3.89 ± 0.10 ($n=7$, $P > 0.05$) before and after application of 30 nmol/L Tol, respectively (Figure 4A, 4B). The steady-state inactivation of $I_{Na,T}$, $V_{1/2}$ changed from -74.69 ± 1.68 mV to -68.08 ± 0.35 mV ($n=7$,

$P < 0.05$) after the application of 30 nmol/L Tol, and k changed from 11.31 ± 2.07 to 6.41 ± 0.32 ($n=7$, $P < 0.05$) (Figure 4C, 4D). The inactivation curves of $I_{Na,T}$ shifted towards more positive potential with 30 nmol/L Tol.

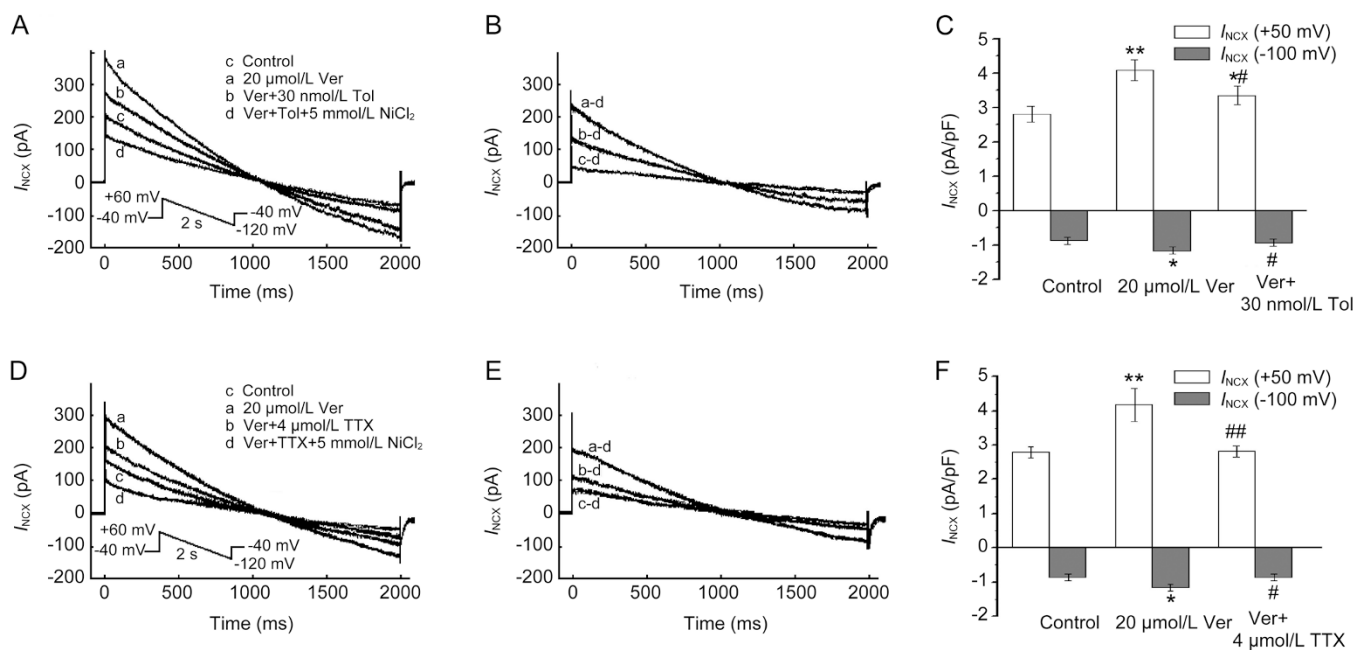


Figure 5. Effects of Tol and TTX on increased I_{NCX} induced by Ver. (A and D) The current-time relationship curves were constructed under conditions of control (no drug, trace c), 20 μ Mol/L Ver (trace a), 20 μ Mol Ver plus 30 nmol/L Tol (trace b, A) or 20 μ Mol/L Ver plus 4 μ Mol/L TTX (trace b, D), and 5 mmol/L Ni^{2+} (trace d) in the presence of 20 μ Mol/L Ver. (B and E) Ni^{2+} -sensitive I_{NCX} obtained by subtracting the data in trace d from the data in traces a, b and c in panel A or panel D. (C and F) Mean current densities of I_{NCX} under different conditions. * P <0.05, ** P <0.01 vs control. # P <0.05, ## P <0.01 vs Ver.

Effects of Tol on reverse I_{NCX} induced by Ver

At concentration of 20 μ Mol/L Ver increased the forward I_{NCX} from -0.872 ± 0.102 pA/pF to -1.165 ± 0.109 pA/pF ($n=10$, P <0.05) and the reverse I_{NCX} from 2.806 ± 0.235 pA/pF to 4.101 ± 0.304 pA/pF ($n=10$, P <0.01). In the presence of 20 μ Mol/L Ver, 30 nmol/L Tol decreased these values to -0.933 ± 0.109 pA/pF and 3.357 ± 0.259 pA/pF ($n=10$, P <0.05 vs Ver for both), respectively (Figures 5A–5C). In another group experiment, 4 μ Mol/L TTX also inhibited the increased forward and reverse I_{NCX} induced by Ver (Figures 5D–5F).

Effects of Tol on AP

The experiments were divided into four groups. In group 1, 30 nmol/L Tol prolonged APD_{90} to some extent ($n=12$, P <0.05 vs

control) (Figure 6A, Table 1). In group 2, 200 nmol/L E-4031 (an I_{kr} specific antagonist) significantly prolonged APD_{90} ($n=12$, P <0.05 vs control) and 30 nmol/L Tol plus E-4031 further extended APD_{90} slightly ($n=12$, P >0.05 vs E-4031) (Figure 6B, Table 1). In group 3, 20 μ Mol/L Ver significantly extended APD_{90} and evoked EAD in 18 of 24 cells (75%). In the presence of Ver, 30 nmol/L Tol reversed APD_{90} and eliminated EADs induced by Ver from 18/24 to 0/24 cells (Figure 6C, Table 2). In group 4, the myocytes were sequentially treated with no drug (control), 20 μ Mol/L Ver, and Ver plus 4 μ Mol/L TTX. TTX also shortened the prolongation of APD_{90} ($n=12$, P <0.05 vs Ver) and eliminated EADs induced by Ver from 8/12 to 0/12 (Figure 6D, Table 2). In all groups, Tol had no effect on the resting potential, AP amplitude and V_{max} (Tables 1, 2).

Table 1. The effects of Tol and E-4031 on AP waveforms in rabbit ventricular myocytes. Values are expressed as mean \pm SD. $n=12$. * P <0.05 vs control. ^{NS} P >0.05 vs E-4031.

Treatment	Resting potential (mV)	Action potential amplitude (mV)	V_{max} (V/s)	APD_{90} (ms)
Control	-90.9 ± 1.3	129.6 ± 2.4	296.6 ± 22.3	265.44 ± 12.68
Tol (30 nmol/L)	-91.1 ± 1.4	129.1 ± 2.1	290.5 ± 23.7	$306.77 \pm 13.43^*$
Control	-90.4 ± 1.5	126.7 ± 2.2	278.7 ± 20.9	268.07 ± 15.31
E-4031 (100 nmol/L)	-90.7 ± 1.1	126.8 ± 2.6	279.2 ± 19.8	$634.89 \pm 35.76^*$
E-4031 (100 nmol/L)+Tol (30 nmol/L)	-90.9 ± 1.2	126.9 ± 2.6	279.9 ± 17.8	647.35 ± 38.72^{NS}

V_{max} , maximum rate of depolarization; APD_{90} , action potential duration at 90% repolarization.

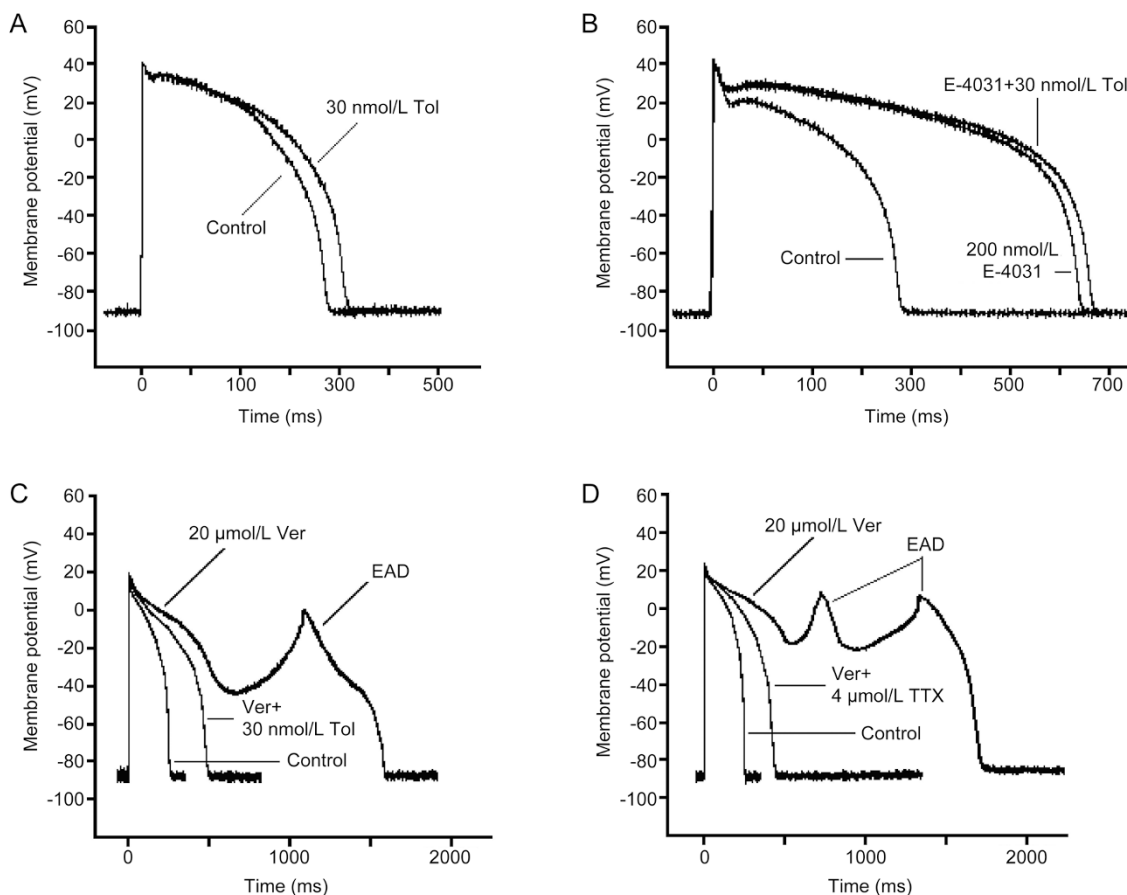


Figure 6. Effects on Tol on APs in rabbit ventricular myocytes. (A) The effects of 30 nmol/L Tol on normal AP waveforms. (B) The effects of 30 nmol/L Tol on AP waveforms induced by 200 nmol/L E-4031. The myocytes were sequentially treated with no drug (control), 200 nmol/L E-4031, and 200 nmol/L E-4031 plus 30 nmol/L Tol. (C and D) The effects of 30 nmol/L Tol (C) or 4 μmol/L TTX (D) on AP waveforms induced by 20 μmol/L Ver. The myocytes were sequentially treated with no drug (control), 20 μmol/L Ver, and 20 μmol/L Ver plus 30 nmol/L Tol (C) or 20 μmol/L Ver plus 4 μmol/L TTX (D).

Table 2. The effects of Tol and TTX on AP waveforms induced by veratridine in rabbit ventricular myocytes. Values are expressed as mean±SD. *n*=15 for Tol treatment group. *n*=12 for TTX treatment group. **P*<0.05 vs control. #*P*<0.05 vs veratridine (Ver 20 μmol/L).

Treatment	Resting potential (mV)	Action potential amplitude (mV)	<i>V</i> _{max} (V/s)	APD ₉₀ (ms)
Control	-90.2±1.3	121.6±7.4	281.3±16.5	276.78±14.54
Ver (20 μmol/L)	-89.9±1.8	120.2±5.0	284.1±13.5	1654.73±78.83*
Tol (30 nmol/L)	-89.8±1.8	120.6±5.8	285.4±12.5	480.09±22.42*#
Control	-91.9±1.5	198.6±6.7	274.9±21.8	265.11±15.82
Ver (20 μmol/L)	-92.5±1.6	199.2±6.1	277.2±22.2	1763.45±81.32*
Ver (20 μmol/L) + 4 μmol/L TTX	-91.8±1.3	199.5±6.4	275.9±20.7	493.36±24.74*#

*V*_{max}, maximum rate of depolarization; APD₉₀, action potential duration at 90% repolarization.

Discussion

Tol, a competitive, nonselective antimuscarinic compound, is developed for the treatment of an overactive bladder^[32, 38, 39]. Millions of patients have benefited from Tol treatment^[40]. Furthermore, little has been reported regarding the fatal proar-

rhythmic effects of Tol in either early clinical tests or follow-up studies^[32, 33, 38, 41]. In recent years, studies have shown that Tol can reduce heart rate variability, while its effect on arrhythmia remains unknown^[42, 43].

In this study, Tol inhibited normal and Ver-augmented

$I_{Na,L}$ in a concentration-dependent manner, with IC_{50} values of 32.08 nmol/L and 42.47 nmol/L, respectively (Figures 1, 2). In the presence of atropine, 30 nmol/L Tol also decreased the enlarged $I_{Na,L}$ induced by Ver, which demonstrated that its blocking effects on $I_{Na,L}$ were not a result of M receptor signaling pathway blockage (Figure 2F). Tol did not modify the steady-activation curve but shifted the steady-inactivation curve of $I_{Na,T}$ toward a more positive voltage, which indicated that Tol accelerated the rate of inactivation of sodium channels (Figure 4D).

Tol also inhibited $I_{Na,T}$ in a concentration-dependent manner (Figure 4). In contrast with an IC_{50} of 32.08 nmol/L for $I_{Na,L}$, the IC_{50} for $I_{Na,T}$ was 183.03 μ mol/L, which indicated that Tol specifically inhibited $I_{Na,L}$. The ratio of suppressions of $I_{Na,L}$ and $I_{Na,T}$ ($IC_{50}I_{Na,T}/IC_{50}I_{Na,L}$) was 5705, compared with values of 38 and 2.7 for ranolazine and lidocaine, respectively^[18], which shows that Tol has a high affinity blockade effect on $I_{Na,L}$. Thus, selectively inhibiting $I_{Na,L}$ with only negligible effect on $I_{Na,T}$ at nmol/L concentration, Tol could have little effect on membrane depolarization, cell excitability and conductivity, thereby preventing arrhythmogenic and other adverse effects.

Under pathological conditions, the increase in $I_{Na,L}$ results in a rise of intracellular Na^+ and subsequent intracellular Ca^{2+} overload by increasing reverse I_{NCX} . The accumulation of Ca^{2+} can cause cell injury, electrical activity disorder and ventricular systolic dysfunction, which aggravates myocardial ischemia and hypoxia. The entire process can develop into a vicious circle^[3, 4, 13, 14]. Our previous study indicates that 2 μ mol/L TTX (no report regarding its effects on ion channels except $I_{Na,L}$) suppresses the reverse I_{NCX} under normal and hypoxic conditions by inhibiting $I_{Na,L}$ ^[11]. In this study, Tol inhibited the reverse I_{NCX} increased by Ver (Figures 5A–5C). Therefore, we speculate that Tol can protect myocytes from injury by preventing abnormal Ca^{2+} influx caused by the increase in reverse I_{NCX} .

Kang *et al*^[31] reported that Tol blocked both the HERG channels and L-type Ca^{2+} channels simultaneously but did not significantly prolong APD, (a maximal 16% extension of APD_{90}). In their study, 30 nmol/L Tol significantly inhibited HERG current but slightly decreased Ca^{2+} current. According to these results, APD_{90} should have been prolonged significantly, which would be markedly different from their report of just a 16% extension of APD_{90} . In our present study 30 nmol/L Tol blocked normal and Ver-increased $I_{Na,L}$. In our AP study, 30 nmol/L Tol extended APD_{90} by 16% under normal conditions, which was consistent with the result reported by Kang *et al*, and had little effects on the prolongation of APD induced by E-4031 (Figure 6B and Table 1). Based on the above-mentioned results, we speculate that Tol's simultaneous inhibition of I_{Kr} and $I_{Na,L}$ can explain the fact that 30 nmol/L Tol weakly prolonged the APD in normal ventricular myocytes. Ver significantly extended APD_{90} and evoked EAD in 18 of 24 cells (75%). In the presence of Ver, 30 nmol/L Tol reversed APD_{90} and eliminated EADs induced by Ver (Figure 6C and Table 2), which indicates that Tol inhibits arrhythmia induced by Ver. As a multi-ion channel blocker, Tol has complex electrophysi-

ological properties. It is possible that Tol inhibits arrhythmias by inhibiting $I_{Na,L}$ reversing prolongation of APD_{90} and eliminating EADs induced by Ver.

In conclusion, Tol inhibited normal and Ver-increased $I_{Na,L}$ in a concentration-dependent manner and decreased Ver-augmented reverse I_{NCX} . In addition, Tol reversed the prolongation of APD and eliminated EADs induced by Ver. These findings point to the potential of Tol as a high affinity $I_{Na,L}$ blocker to inhibit arrhythmias induced by increased $I_{Na,L}$ with few undesirable adverse effects.

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Author contribution

Chao WANG, An-tao LUO, and Ji-hua MA designed the research; Chao WANG, Lei-lei WANG, Chi ZHANG, Zhen-zhen CAO, and Pei-hua ZHANG performed the research; Chao WANG, Lei-lei WANG, Chi ZHANG, Zhen-zhen CAO, Pei-hua ZHANG, Xin-rong FAN, and Ji-hua MA analyzed the data; Chao WANG, Lei-lei WANG, Chi ZHANG, and An-tao LUO wrote the paper.

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