Full Paper

Ranolazine Attenuates the Enhanced Reverse Na^+ - Ca^{2+} Exchange Current via Inhibiting Hypoxia-Increased Late Sodium Current in Ventricular Myocytes

Xiao-Jing Wang¹, Lei-Lei Wang¹, Chen Fu¹, Pei-Hua Zhang¹, Ying Wu¹, and Ji-Hua Ma^{1,*}¹Cardio-Electrophysiological Research Laboratory, Medical College, Wuhan University of Science and Technology, Wuhan, Hubei 430065, China

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Abstract. Ranolazine (RAN), a novel antianginal agent, inhibits the increased late sodium current ($I_{\text{Na,L}}$) under many pathological conditions. In this study, the whole-cell patch-clamp technique was used to explore the effects of RAN on $I_{\text{Na,L}}$ and reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange current (I_{NCX}) in rabbit ventricular myocytes during hypoxia. Tetrodotoxin (TTX) at 2 μM or RAN at 9 μM decreased significantly $I_{\text{Na,L}}$ and reverse I_{NCX} under normoxia and RAN had no further effects on both currents in the presence of TTX. RAN (3, 6, and 9 μM) attenuated hypoxia-increased $I_{\text{Na,L}}$ and reverse I_{NCX} in a concentration-dependent manner. Hypoxia-increased $I_{\text{Na,L}}$ and reverse I_{NCX} were inhibited by 2 μM TTX, whereas 9 μM RAN applied sequentially did not further decrease both currents. In another group, after both currents were decreased by 9 μM RAN, 2 μM TTX had no further effects in the presence of Ran. In monophasic action potential (MAP) recording, early after-depolarizations (EADs) were suppressed by RAN (9 μM) during hypoxia. In conclusion, RAN decreased reverse I_{NCX} by inhibiting $I_{\text{Na,L}}$ in normoxia, concentration-dependently attenuated the increase of $I_{\text{Na,L}}$, which thereby decreased the reverse I_{NCX} , and obviously relieved EADs during hypoxia.

Keywords: hypoxia, ranolazine, late sodium current, sodium-calcium exchanger, patch-clamp technique

Introduction

Late sodium current ($I_{\text{Na,L}}$), also known as persistent sodium current, is a tetrodotoxin (TTX)-sensitive inward current with slow inactivation kinetics and has relatively smaller current amplitude compared with peak sodium current under physiological conditions. However, due to its property of staying active protractedly, $I_{\text{Na,L}}$ plays a significant role in regulating intracellular Na^+ concentration ($[\text{Na}^+]_i$), maintaining a plateau of action potential (AP), and determining action potential duration (APD) in pathological conditions (1–4). It has been shown that $I_{\text{Na,L}}$ is increased under many pathological conditions such as myocardial ischemia, hypoxia, chronic heart failure, and congenital or acquired long Q-T syndrome

(2–10). The increase of $I_{\text{Na,L}}$ enhances the risk of arrhythmia possibly in two ways. First, the increased $I_{\text{Na,L}}$ prolongs APD, causing arrhythmia by EADs (4, 5, 10–12). Second, the increased $I_{\text{Na,L}}$ causes intracellular Na^+ overload which subsequently leads to intracellular Ca^{2+} overload via the enhanced reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange current (I_{NCX}) (2–4, 6, 13–16), eventually resulting in arrhythmia, dysfunction of cell contraction, and cell injury (5, 17–19). Therefore inhibition of $I_{\text{Na,L}}$ is considered to be a new potential therapeutic target for treat patients afflicted with heart failure or ischemic heart disease (18, 20, 21). Approved by the US Food and Drug Administration (FDA) as an antianginal drug in 2006 (11, 22), ranolazine (RAN) was first applied as an inhibitor of $I_{\text{Na,L}}$ in clinical treatment (12, 23). Experimentally, RAN can relieve intracellular Na^+ and Ca^{2+} overload caused by myocardial ischemia via blocking $I_{\text{Na,L}}$ (21, 24–27). So it has been administered as an important adjuvant drug in the treatment of myo-

*Corresponding author. mjhua@wust.edu.cn

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cardial ischemia and acute myocardial infarction by improving myocardial diastolic function (25, 28).

Antzelevitch et al. have reported that RAN, at therapeutic concentrations of 1 – 10 μ M, inhibits many cardiac ion channels, including $I_{Na,T}$, $I_{Na,L}$, I_{Kr} , $I_{Ca,L}$, and I_{NCX} , and inhibits $I_{Na,L}$ more effectively than any other cardiac ion channels (29 – 31). However, the effect of RAN (< 10 μ M) on hypoxia-increased $I_{Na,L}$ and reverse I_{NCX} in isolated ventricular myocytes has not been reported. If RAN can attenuate reverse I_{NCX} , it is unclear whether it reduces reverse I_{NCX} directly or does so via inhibiting $I_{Na,L}$. In the study, $I_{Na,L}$ or I_{NCX} was recorded alternatively to fill the above-mentioned literature gap by testing the effect of RAN and/or TTX during hypoxia.

Materials and Methods

Isolation of rabbit ventricular myocytes

This use of animals in the investigation was approved by the Institutional Animal Care and Use Committee of Wuhan University of Science and Technology and conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication no. 85 – 23, revised 1996) and the Guide for the Care and Use of Laboratory Animals of Hubei Province, China. The animals were maintained at ambient temperature (20°C – 25°C) with unlimited access to food and water. Adult New Zealand white rabbits (1.6 – 2 kg, Wuhan, China) were heparinized (2000 U) and anesthetized with sodium pentobarbital (55 mg/kg, iv). Hearts were excised rapidly, and perfused retrogradely on a Langendorff apparatus for 5 min with a Ca^{2+} -free oxygenated Tyrode’s solution [135 mM NaCl, 5.4 mM KCl, 1.0 mM $MgCl_2$, 0.33 mM NaH_2PO_4 , 10 mM HEPES, and 10 mM glucose (pH 7.4, adjusted with NaOH)] and then perfused with enzyme-containing solution (0.1 g/L collagenase type I) and 0.5 g/L bovine serum albumin (BSA) in the same solution for 40 – 50 min. The perfusate was finally changed to KB solution containing 70 mM KOH, 40 mM KCl, 50 mM glutamic acid, 20 mM taurine, 20 mM KH_2PO_4 , 3 mM $MgCl_2$, 0.5 mM EGTA, 10 mM glucose, and 10 mM HEPES (pH 7.4, adjusted with KOH) for 5 min. All solutions were bubbled with 100% O_2 and maintained at 37°C. 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 with BSA (0.5 g/L) at 25°C until use.

Induction of hypoxia

In order to simulate ideal hypoxic conditions, cardiomyocytes were transferred into a customized perfusing chamber. Hypoxia was achieved by bubbling 100% N_2

into the perfusate solution (for I_{NCX} recording without glucose) and saturated for at least 50 min. The recording chamber was perfused with a constant flow of extracellular solution for 10 – 15 min. Meanwhile, constant flow of 100% N_2 passed through a catheter into the radially arrayed slots, and the recording chamber was precisely controlled to create a stable laminar layer of N_2 on the bath level to prevent O_2 in the air from diffusing into the solution (6).

Drugs and reagents

To record I_{NCX} , the intracellular pipette solution contained 20 mM NaCl, 10 mM $CaCl_2$, 50 mM aspartic acid, 3 mM $MgCl_2$, 20 mM EGTA, 10 mM HEPES, 5 mM MgATP, and 120 mM CsOH (pH 7.3, adjusted with CsOH). The bath solution contained 140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, and 10 mM glucose (without glucose during hypoxia), adjusted to pH 7.4 with NaOH. In addition, 1 mM $BaCl_2$, 2 mM CsCl, 20 μ M ouabain, and 1 μ M nicardipine were used to block the K^+ channels, Na^+ - K^+ pump, and L- Ca^{2+} channels, respectively.

HEPES was obtained from Amresco (Solon, OH, USA). Tetrodotoxin (TTX) was purchased from Tocris Bioscience (Ellisville, MO, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA). Stock solutions of drugs were prepared in water. Each of the stocks was diluted to the required concentrations in the external recording solution immediately before use.

Current recordings

All experiments were performed at room temperature (22°C – 24°C). The isolated ventricular myocytes were placed into a special recording chamber (6) and were perfused with bath solution. Whole-cell patch-clamp techniques were used to record $I_{Na,L}$ and I_{NCX} . Patch electrodes were pulled with a two-stage patch pipette puller (PP-830; Narishige Group, Tokyo). Their resistances were in the range of 1 – 2 M Ω when filled with pipette solution. Capacitance and series resistances were adjusted to obtain minimal contribution of the capacitive transients. A 60% – 80% compensation of the series resistance was usually achieved without ringing. Currents were obtained with a Multiclamp 700B amplifier (Axon Instruments Inc., New York, NY, USA), filtered at 2 kHz, digitized at 10 kHz, and stored on a computer hard disk for further analysis.

Protocol of experiments

In this study, $I_{Na,L}$ and I_{NCX} were recorded alternately at an interval of 5 s and the synchronous trends were tracked to demonstrate the simultaneous changes in $I_{Na,L}$ and reverse I_{NCX} in rabbit ventricular myocytes under sole

hypoxic conditions. The intracellular and extracellular solutions for I_{NCX} recording were employed to confirm that $I_{\text{Na,L}}$ and I_{NCX} could be recorded successively and alternately. Tang et al. (6) reported that there was no significant difference in the increase of the amplitude of $I_{\text{Na,L}}$ during hypoxia when cells were exposed to the solutions for $I_{\text{Na,L}}$ or for I_{NCX} . Isolated cardiomyocytes were perfused in extracellular perfusate saturated with oxygen after cells adhered to the bottom of its chamber (6). Cells were perfused with bath solution for I_{NCX} recording at a constant flow rate (2 ml/min) for 10–15 min. $I_{\text{Na,L}}$ and I_{NCX} were recorded in the absence and then in the presence of drug (TTX or RAN) during hypoxia.

$I_{\text{Na,L}}$ was recorded using depolarizing pulses with a duration of 300 ms applied at 0.25 Hz from a holding potential of -120 mV and measured at 200 ms to eliminate the influence of transient sodium current ($I_{\text{Na,T}}$). Five seconds later, I_{NCX} was elicited by using ramp voltage-clamp pulses from a holding potential of -40 mV to $+60$ mV for 100 ms and then ramped to -120 mV over a period of 2 s (i.e., at 90 mV/s) before returning to -40 mV. I_{NCX} was measured at voltage levels of $+50$ and -100 mV, respectively, as the Ni^{2+} -sensitive current by subtracting the current recorded in the presence from that in the absence of 5 mM NiCl_2 . The current-time relationship was constructed from the declining slope of the ramp pulse.

Monophasic action potential (MAP) recording

The heart was excised using the method mentioned above and retrogradely perfused through the aorta with normal Tyrode's solution [135 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl_2 , 0.33 mM NaH_2PO_4 , 10 mM HEPES, 10 mM glucose, and 1.25 mM CaCl_2 , at pH 7.4] gassed with 95% O_2 and 5% CO_2 . The temperature of the perfusate was maintained at $37.0^\circ\text{C} \pm 1^\circ\text{C}$ with a water bath. Control rate to 0.5–1 Hz with a multi-channel physiological signal acquisition and processing system (Chengdu Instrument Factory, Chengdu, China) after the sinus node was ablated by cauterization. Epicardial electrodes made of Ag-AgCl wire were placed on the left ventricular free wall below the level of the atrio-ventricular node. Electrical stimuli were delivered with the electrodes, and the signals were amplified by the system and were visible on a computer screen. Anoxic conditions could be well-simulated by occluding the perfusion tube during MAP recording.

Data analysis

Whole-cell recordings were analyzed using clampfit 9.0 (Axon Instruments, Inc., Foster City, CA, USA). Figures were plotted using Origin software (v7.0; OriginLab Co., Northampton, MA, USA). Statistical

significance between two groups or multiple groups was respectively evaluated by Student's *t*-test or one-way analysis of variance (ANOVA). All values were expressed as mean $\pm$ S.D., and $P < 0.05$ was considered to be statistically significant.

Results

Effects of TTX and RAN on $I_{\text{Na,L}}$ and I_{NCX} in normoxia

$I_{\text{Na,L}}$ and I_{NCX} were recorded alternately at an interval of 5 s first in the absence and then in the presence of 2 μM TTX or 9 μM RAN (Fig. 1). After the application of 2 μM TTX or 9 μM RAN, the mean current density of inward I_{NCX} did not change obviously, whereas $I_{\text{Na,L}}$ and reverse I_{NCX} were attenuated significantly (Fig. 1).

Under normoxic conditions, 2 μM TTX and 9 μM RAN were applied sequentially. The amplitude of $I_{\text{Na,L}}$ and reverse I_{NCX} was decreased by 2 μM TTX ($n = 6$, $P < 0.01$; Fig. 2), but did not further decrease after adding 9 μM RAN in the presence of TTX. Meanwhile, inward I_{NCX} remained unchanged throughout the experiment ($n = 6$, $P > 0.05$; Fig. 2).

The concentration-dependent inhibition of RAN on hypoxia-increased $I_{\text{Na,L}}$ and reverse I_{NCX}

$I_{\text{Na,L}}$ and I_{NCX} were recorded alternately at an interval of 5 s after 10 min of hypoxia. Electrogenic I_{NCX} was measured to investigate whether the reverse I_{NCX} was activated by the increase of $I_{\text{Na,L}}$ enhanced by hypoxia or attenuated with the decrease of $I_{\text{Na,L}}$ by RAN. The results showed that the mean current density of both $I_{\text{Na,L}}$ and reverse I_{NCX} was increased during hypoxia and depressed obviously after application of RAN (3, 6, and 9 μM) in a concentration dependent manner, while no noticeable change was observed with inward I_{NCX} in the course of the experiment (Fig. 3).

The inhibitory effects of TTX and RAN in different application sequences on hypoxia-increased $I_{\text{Na,L}}$ and reverse I_{NCX}

To determine whether RAN reduced the hypoxia-increased I_{NCX} directly or did so via inhibiting $I_{\text{Na,L}}$, the extracellular solutions were sequentially added with TTX and RAN in cardiac muscle cells during hypoxia. Hypoxia-increased $I_{\text{Na,L}}$ and reverse I_{NCX} were inhibited significantly by 2 μM TTX after both currents approached stability in hypoxia, while they did not further change after 9 μM RAN was dissolved into the bath solution (Fig. 4). In another group of experiments, 9 μM RAN and 2 μM TTX were applied sequentially. (Fig. 5: A–D). The hypoxia-increased $I_{\text{Na,L}}$ and reverse I_{NCX} both decreased obviously after 9 μM RAN was added into the bath, while no change was observed when 2 μM

Fig. 1. Inhibitory effects of TTX and RAN on I_{NaL} and reverse I_{NCX} in normoxia. A and B: I_{NaL} was decreased obviously by 9 μ M RAN (A) or 2 μ M TTX (B) under the normoxic condition. C: Histograms show the mean current densities of I_{NaL} (Values are expressed as mean $\pm$ S.D., $n = 7$. $**P < 0.01$ vs. Control.) D: The current–time relationship (I – T) curves were shown under no drug (control, trace a), 9 μ M RAN (trace b), and 5 mM Ni^{2+} (trace c) in the presence of 9 μ M RAN. E: Ni^{2+} -sensitive I_{NCX} were obtained by subtracting the data in trace c from the data in traces a and b in panel D. F: Histograms show the mean current densities of I_{NCX} obtained from panel E. G: Similar to panel D, the I – T curves were constructed from the negative slope region of a ramp voltage clamp under conditions of control (no drug, trace d), 2 μ M TTX (trace e), and 5 mM Ni^{2+} (trace f). H: Ni^{2+} -sensitive I_{NCX} were obtained by subtracting the data in trace f from the data in traces d and e in panel G. I: Mean current densities of I_{NCX} under control or the presence of 2 μ M TTX. Histograms F and I show that I_{NCX} were measured at voltage levels of +50 mV and –100 mV, respectively. The positive values indicate the mean current densities of I_{NCX} measured at the voltage level of +50 mV, and the negative values represent the other one. Values are expressed as the mean $\pm$ S.D., $n = 7$. $**P < 0.01$ vs. control.

TTX was added (Fig. 5: A–D). Meanwhile, it should be noted that inward I_{NCX} was independent of the drug sequences and remained unchanged all the way (Fig. 4 and 5: A–D).

The increase of I_{NaL} enhances the risk of arrhythmia possibly in two ways as mentioned above of which one is causing arrhythmia by EADs (4, 5, 10–12). To investigate the preventive effect of RAN on EADs, we conducted two groups of experiments by Langendorff rabbit hearts during hypoxia (Fig. 5: E, F). The number of hearts showing EAD (9/10 hearts) in the control group was more than that in the RAN group (6/12 hearts). The Chi-square test, the classical statistical analysis, showed that RAN significantly reduced the incidence of EADs ($P < 0.05$). Further, RAN delayed the average appearing

time of EADs from 3.72 ± 0.97 min ($n = 9$) to 5.5 ± 0.45 min ($n = 6$, $P < 0.05$) during hypoxia (Fig. 5: E, F).

Discussion

The hypoxia- or acute ischemia-increased I_{NaL} would lead to intracellular Na^+ overload in cardiomyocytes and ultimately resulting in intracellular Ca^{2+} overload via enhancing reverse I_{NCX} , which causes cell injury and arrhythmia, such as EADs, myocardial contracture, and even cell apoptosis and death (6, 14–16, 19, 25, 32–37). Furthermore, exposure of the heart to long hypoxia would damage cells seriously and increase the incidence of cardiac arrhythmias. In brief, the increased I_{NaL} plays a vital role in Ca^{2+} overload via the reverse

Fig. 2. Inhibition of 2 μ M TTX and 9 μ M RAN on I_{NaL} and I_{NCX} under the normoxic condition. A: A significant change appeared after adding TTX but not after RAN. B: Histograms show the mean current densities of I_{NaL} . C: I_{NCX} values were obtained by subtracting $NiCl_2$. D: The mean current densities of I_{NCX} . Values are expressed as the mean $\pm$ S.D., $n = 6$. ** $P < 0.01$ vs. control. No mark means there was no significant differences among groups.

Fig. 3. The concentration-dependent inhibition of RAN on hypoxia-increased I_{NaL} and reverse I_{NCX} . A: RAN inhibited hypoxia-increased I_{NaL} in a concentration dependent manner. B: Histograms show the mean current densities of I_{NaL} . C: RAN inhibited hypoxia-increased reverse I_{NCX} . D: Mean current densities of I_{NCX} were obtained from panel C. Values are expressed as the mean $\pm$ S.D., $n = 8$. ** $P < 0.01$ vs. control, ## $P < 0.01$ vs. hypoxia, ++ $P < 0.01$ vs. 3 μ M RAN, && $P < 0.01$ vs. 6 μ M RAN.

I_{NCX} , and thus lowering the density of $[Na^+]_i$ or applying a sodium-channel blocker can inhibit the increase of intracellular Ca^{2+} concentration (13). RAN, approved by the FDA in 2006 as an antianginal drug (11, 22), can inhibit many cardiac ion channels, and it is particularly effective against I_{NaL} at relatively low concentrations

(< 10 μ M) (29, 30, 38–40). In recent years, many studies show that RAN inhibits I_{NaL} under pathological conditions and thus alleviates Ca^{2+} overload and cell damage (37, 41–45). In addition, RAN remarkably attenuates ischemia-induced myocardial diastolic Ca^{2+} overload, improves left ventricular function during isch-

Fig. 4. TTX (2 μ M) and RAN (9 μ M) inhibited the increase of I_{NaL} and reverse I_{NCX} during hypoxia. **A:** 2 μ M TTX inhibited I_{NaL} during hypoxia but the amplitude of I_{NaL} changed little after adding 9 μ M RAN. **B:** Histograms show the mean current densities of I_{NaL} obtained from panel A. **C:** I_{NCX} was obtained by subtracting $NiCl_2$. **D:** Histograms show the mean current densities of I_{NCX} . Values are expressed as the mean $\pm$ S.D., $n = 8$. ** $P < 0.01$ vs. control, ## $P < 0.01$ vs. hypoxia.

emia/reperfusion, and protects cardiac cells from necrosis (12, 17, 18, 22, 27, 34, 40, 46). However, to our knowledge, the effect of RAN on hypoxia-increased I_{NaL} has not been studied to date. In addition, little information is available about the inhibition of RAN ($< 10 \mu$ M) on hypoxia-increased reverse I_{NCX} before this investigation. Although RAN has been reported to inhibit reverse I_{NCX} at high concentration (100 μ M), whether it directly attenuates the hypoxia-enhanced reverse I_{NCX} or not at therapeutically relevant concentrations ($< 10 \mu$ M) in ventricular myocytes remains to be determined conclusively (29, 30). In this study, I_{NaL} and I_{NCX} were recorded simultaneously and alternately at an interval of 5 s in the same cell during hypoxia, and then the myocytes were treated with RAN ($< 10 \mu$ M) or TTX (2 μ M) to observe their effects on the two aforementioned currents.

The result demonstrated that I_{NaL} and reverse I_{NCX} could be inhibited significantly by 2 μ M TTX or 9 μ M RAN under normoxia (Fig. 1), while inward I_{NCX} remained unchanged. Since existing literature could not reveal whether RAN directly reduces I_{NCX} or does so via inhibiting I_{NaL} in normoxia, we intentionally addressed the question in this study by adding 2 μ M TTX and 9 μ M RAN in order (Fig. 2). The data suggest that I_{NaL} and reverse I_{NCX} were inhibited by 2 μ M TTX during normoxia, but both currents did not decrease any more with the intervention of 9 μ M RAN. As is well known, TTX, a specific inhibitor of the sodium channel, only

blocks I_{NaL} at the concentration of 2 μ M (2–4, 6–8, 14). If RAN worked directly on I_{NCX} , reverse I_{NCX} would have continued decreasing after it was added rather than remain the same. That is to say, RAN attenuated reverse I_{NCX} through inhibiting I_{NaL} under normoxia.

Recently, it has been reported that reverse I_{NCX} increases along with I_{NaL} augmentation during hypoxia and decreases along with the I_{NaL} diminishment by 2 μ M TTX in hypoxic cells (6), but it has remained unclear whether the hypoxia-increased I_{NaL} and reverse I_{NCX} could be inhibited by RAN. So the following experiments were designed to clarify this issue by studying the effects of RAN or TTX on enhanced I_{NaL} and reverse I_{NCX} during hypoxia. The hypoxia-increased I_{NaL} was reduced by 3 μ M RAN to a certain extent (Fig. 3: A, B) while reverse I_{NCX} was decreased accordingly (Fig. 3: C, D). The increasing concentration of RAN resulted in the reinforcement of the inhibition effects on I_{NaL} and reverse I_{NCX} , while inward I_{NCX} remained the same in the course of the experiment (Fig. 3). The data shows that the hypoxia-increased I_{NaL} , which may contribute to increasing reverse I_{NCX} , could be inhibited by RAN in a concentration-dependent manner. In order to determine whether RAN reduced the hypoxia-increased I_{NCX} directly or via inhibiting I_{NaL} , the extracellular solutions were sequentially added with 2 μ M TTX and 9 μ M RAN during hypoxia. The hypoxia-increased I_{NaL} was significantly decreased with the intervention of 2 μ M TTX, and meanwhile the reverse I_{NCX} was also attenuated

Fig. 5. Effects of RAN (9 μ M) on increased I_{NaL} and EADs during hypoxia. **A:** 9 μ M RAN inhibited I_{NaL} during hypoxia but the amplitude of I_{NaL} changed little after adding 2 μ M TTX. **B:** Mean current densities of I_{NaL} . **C:** Hypoxia-increased I_{NCX} was decreased by RAN, but not changed by adding TTX afterwards. **D:** Mean current densities of I_{NCX} . Values are expressed as the mean $\pm$ S.D., $n = 6$. ** $P < 0.01$ vs. control, ## $P < 0.01$ vs. hypoxia. **E:** EADs occurred at 3.72 ± 0.97 min ($n = 9$) during hypoxia. **F:** EADs were delayed to 5.5 ± 0.45 min ($n = 6$, $P < 0.05$) by adding RAN.

obviously along with it (Fig. 4). However, no further changes in I_{NaL} and reverse I_{NCX} were observed after adding 9 μ M RAN (Fig. 4). On the other hand, the increased I_{NaL} and reverse I_{NCX} were both alleviated by 9 μ M RAN in the presence of hypoxia, but did not change when treated with 2 μ M TTX after that (Fig. 5: A–D). In other words, the data from the latter two experiments complementarily proved that hypoxia-increased reverse I_{NCX} was decreased by RAN via blocking I_{NaL} . The result clearly confirmed that RAN ($< 10 \mu$ M) and TTX (2 μ M), as inhibitors of I_{NaL} , significantly reduced hypoxia-increased reverse I_{NCX} by inhibiting I_{NaL} .

RAN, an effective I_{NaL} inhibitor in therapeutic concentrations ($< 10 \mu$ M), can obviously lower the density of $[Na^+]_i$ through inhibiting I_{NaL} and subsequently relieve Ca^{2+} overload via the attenuation of reverse I_{NCX} during hypoxia or ischemia in cardiac muscle cells. The result, in accordance with what Soliman et al. recently reported,

validates that RAN can reduce ischemia-induced Ca^{2+} overload (31). Soliman et al. also reported that RAN inhibits RM NCX1.1 currents (IC_{50} 1.7 μ M) in cultured tsA201 cells (31). However, in this study, RAN decreased the hypoxia-increased reverse I_{NCX} by inhibiting I_{NaL} . The contrast between our result and that of Soliman et al. may derive from the difference in species of the experimental animals.

Hypoxia could do damage to cell function and induce EADs in myocardial cells (47). Previous studies showed that RAN suppressed EADs induced by many pathological conditions, such as ATX α , veratridine, and H_2O_2 (37, 48, 49). However, to date, there is no report showing that RAN inhibits hypoxia-induced EADs. In this study, the results of RAN reducing the incidence and delaying the occurrence time of EADs indicated that RAN has a preventive effect on hypoxia-induced EADs.

In conclusion, the data indicate that RAN ($< 10 \mu$ M)

decreases reverse I_{NCX} through inhibiting $I_{Na,L}$ under both normoxia and hypoxia conditions and that the inhibitory effects of RAN on hypoxia-increased $I_{Na,L}$ and reverse I_{NCX} are concentration-dependent and that suppressing effects of RAN ($< 10 \mu\text{M}$) on EADs are statistically significant.

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Conflicts of Interest

The authors declare no conflicts of interest for this manuscript.

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