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Research Article

Effects of tanshinone IIA on sodium channel currents in rat ventricular myocytes

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BACKGROUND: The function of the heart can be affected by the physiological and pathological changes of various ion channels in myocardial cell membrane, especially voltage-gated ion channels. This study is to explore the effects of tanshinone IIA on I_{Na} and kinetic characteristics of channels in ventricular myocytes of SD rats.

METHODS: Firstly, cut rat heart rapidly after anesthesia. Secondly, obtain single ventricular myocyte by reverse perfusion of Langendorff aorta. Then, record sodium currents by whole-cell patch clamp technique and add tanshinone IIA in the way of extracellular fluid elution and we explored the effects of different concentrations of tanshinone IIA (5,10,20,40,80,160 μM) on I_{Na} Finally, analyse the changes of kinetic characteristics of the sodium channel.

RESULTS: When the concentration of tanshinone IIA was 5 and 10 μM, there was no significant effect on I_{Na}, while the concentration was more than 20 μM, the inhibitory effect on I_{Na} was enhanced with the increase of concentration, among which, 20 and 80 μM tanshinone IIA reduced $I_{Na\text{-}Peak}$ from (-110.61±6.28) pA/pF to (-87.28±5.76) pA/pF and (-54.53±3.15) pA/pF ($P<0.01$, $n=6$). I-V curve of I_{Na} shifted up significantly which affected by tanshinone IIA. However, the trajectory of I-V curve, V_{ac}, V_{Peak} and V_{rev} have not been changed. Tanshinone IIA had no significant effect on the steady-state activation curve of I_{Na}. In addition, the steady-state inactivation curve of I_{Na} shifted to the left significantly, that is, moving to the hyperpolarization. Moreover, the recovery curve of I_{Na} shifted to the right.

CONCLUSIONS: Tanshinone IIA inhibited I_{Na} in a concentration-dependent manner and affected the kinetic characteristics of sodium channel.

Keywords: cardiac muscle cell, sodium channel, tanshinone IIA, patch clamp

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Introduction

In recent years, with the improvement of people's living conditions, the incidence and mortality of cardiovascular diseases such as arrhythmia, myocardial infarction and sudden death have increased rapidly. The prevention and treatment of these diseases has become one of the key points in the current medical work. As far as arrhythmia is concerned, although there are many types of arrhythmias, and the etiology and pathogenesis are complicated [1], but the process of disease must involve the changes of the excitability and electrophysiology of cardiac myocytes [2,3]. Therefore, it is very important to understand the process of change and the cause of change, and to find effective drugs for the treatment of these diseases.

The traditional Chinese medicine Danshen is often used in the prescriptions for the treatment of cardiovascular diseases, and it is the dry root and rhizome of Salvia miltiorrhiza Bge [4]. The function of Danshen are activating blood circulation, regulating meridians, dispelling blood stasis, relieving pain, cooling blood, eliminating carbuncle, eliminating irritation and

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tranquilizing the mind[5]. Qu et al. [6] found that Guanxin Danshen dropping pills could effectively reduce the score of blood stasis syndrome and serum sICAM-1 / sVCAM-1 level in patients with chronic stable angina pectoris and blood stasis syndrome. Wang [7] also expounded the unique advantages of Danshen and its modern preparations in the prevention and treatment of ischemic cardiovascular disease. The main liposoluble component of Danshen is tanshinone, which belongs to the flavonoids of fat-soluble phenanthraquinone, from which more than 10 tanshinone monomers, such as tanshinone I, tanshinone IIA, tanshinone IIB, cryptotanshinone and isocryptotanshinone, were obtained. And it was proved by clinical trial that it was a new drug for treating coronary heart disease because of its remarkable effect and little side effect in the treatment of angina pectoris [8]. Tanshinone IIA is a diterpenoid compound of tanshinone type, which has a wide range of physiological activities, including anti-inflammatory, improving blood circulation, anti-tumor, anti-oxidation, protecting liver and improving liver function, providing protection of spinal cord, renal tubule and renal interstitial injury [9], inhibiting left ventricular hypertrophy and ischemic arrhythmia [10]. The common dosage form for it is sodium tanshinone IIA sulfonate injection.

Arrhythmia is a great threat to human life, including LQTS, SQTS, BRS, CPVT and ARVD / C, etc. In addition, arrhythmia caused by the disorder of ion channels in the myocardial cell membrane are called cardiac ion channel diseases [11]. Many ion channels on the membrane, especially voltage-gated ion channels, such as sodium, potassium and calcium channels, are abnormal in opening and closing, which can lead to changes in ion currents across myocardial membranes, inducing arrhythmias and even sudden death [3]. Therefore , focusing on the function of ion channel of myocardial cells has become a new target to treat arrhythmia by electrophysiological technique. It has been reported that the changes of calmodulin and ion channel protein gene expression in rabbit cardiomyocytes may be the important mechanism of ventricular arrhythmias in acute myocardial infarction. Tanshinone IIA can significantly reduce the incidence of ventricular arrhythmias, and the molecular mechanism of reducing myocardial infarction area may be related to the changes of calmodulin and ion channel protein gene expression [12], prevent and treat AF by improving atrial electrical remodeling induced by down-regulated expression of $K_v1.5$ in rats with AF [13], reduce the density of I_{Kr} and I_{Ks} in hypertrophic cardiomyocytes [14] and inhibit LTCC and decrease the current density of it in a concentration-dependent manner [15]. However, the effect of tanshinone IIA on Na_v in cardiomyocytes is unclear.

In this study, we will study whether tanshinone IIA, an effective component of traditional Chinese medicine Danshen, has an effect on myocardial sodium current and explore the possible mechanism of its anti-arrhythmic effect.

Materials and methods

Animals

SD rats, 200-300 g, which were provided by Yangzhou University Center for Comparative Medicine, and male and female are unlimited.

Medicines

Tanshinone IIA (Chengdu Purse Biotechnology Co., Ltd.) , Collagenase II(Worthington Company), Taurine, CsCl, TEACl, MgATP and HEPES(Sigma Co.), Heparin sodiumand Glucose(Solarbio Co.), EGTA(Fluka Co.), BSA(ICN Co.), TTX(Shanghai Kelamar Reagent Co., Ltd.), L- potassium glutamate (Vetec Co.), all of the NaCl, KCl, $CaCl_2$, KOH, NaOH, $CsOH·H_2O$, $MgCl_2·6H_2O$, $NaH_2PO_4·2H_2O$ and L-glutamic acid were pure product made in china.

Preparation of reagent

Calcium-free Tyrode's solution(mmol/L): NaCl 136.0, KCl 5.5, NaH_2PO_4 0.3, $MgCl_2$ 1.0, HEPES 5.0, Glucose 10.9. The pH was adjusted to 7.35 ~ 7.40 with NaOH.

Tyrode's solution: Adding $CaCl_2$ into calcium-free Tyrode's solution to the final concentration of 1.8 mmol/ L.

Enzyme solution: Adding 0.4 g/L collagenase II, 1 g/L BSA, 0.4 g/L Taurine, 30.0 moL $CaCl_2$ into calcium-free Tyrode's solution.

KB solution: L-glutamic acid 70.0, Taurine 10.0, KCl 25.0, EGTA 0.5, Glucose 11.0 HEPES 5.0, KOH 89.0. The pH was adjusted to 7.35 ~ 7.40 by KOH.

Electrode liquid(mmol/L): CsCl 133.0, NaCl 5.0, TEACl 10.0, EGTA 10.0, HEPES 5.0, MgATP 5.0. The pH was adjusted to 7.30 by CsOH.

Extracellular Fluid(mmol/L): NaCl 135.0, CsCl 5.4,

$MgCl_2$ 1.0, $CaCl_2$ 1.8, HEPES 5.0, Glucose 10.0, $CdCl_2$ 0.1. The pH was adjusted to 7.40 by NaOH.

Tetrodotoxin (TTX): 50 μmol/L(μM) [16].

Tanshinone IIA: Cryopreservation of 100 mmol/L(mM) mother liquor dissolved in ethanol [17], and the corresponding extracellular solution was used to dilute the mother liquor making the final concentration were 5、10、20、40、80、160 μmol/L(μM).

Stimulus package

I_{Na} single stimulation: In the voltage clamp mode, set holding potential at -80 mV, give an square wave stimulation at -20 mV, 30 ms duration.

I_{Na} I-V curves: In the voltage clamp mode, set holding potential at -80 mV, give the square wave string stimulation at -70～90 mV, and the step was 5 mV, duration was 30 ms, the frequency was 0.5 Hz. The I-V curves of I_{Na} were obtained by using the $I_{Na\text{-}Peak}$ as the y axis and the membrane potential as the x axis. The activation current stimulation scheme was as follows: set holding potential at -80 mV, give the square wave string stimulation at -80～10 mV, and the step was 5 mV, duration was 30 ms, the frequency was 0.5 Hz .

I_{Na} activation curve: The original data of the activation curve of I_{Na} was transformed from the partial data of the I-V curve. The figure was completed by taking the relative conductance G/Gmax($G=I/(V-V_{rev})$) as the y axis and the membrane potential as the x axis. The steady-state activation curve was obtained by fitting the Boltzmann equation $G/Gmax= 1/\{1+\exp [(V_{1/2\text{-}ac}-V)/ k] \}$. V_{rev} is the voltage at which the I-V curve intersects the x axis (that is, the voltage from the inward current to the outgoing current, that is, the voltage at y=0), and k is the slope factor.

I_{Na} inactivation curve: In voltage clamp mode, the method of double pulse stimulation was used. Set the holding potential at -80 mV and conditional pulse of depolarization from -140 mV to 20 mV, the step was 10 mV, duration was 50 ms. Then start the repolarization to -30 mV, lasting 25 ms. The figure was completed by setting the relative current I/Imax as y axis and the membrane potential as x axis. The steady-state inactivation curve was obtained by fitting the Boltzmann equation $I/Imax =1/\{ 1 + \exp [(V-V_{1/2\text{-}in})/ k] \}$, k is the slope factor.

I_{Na} recovery curve after inactivation: In voltage clamp mode, the method of double pulse stimulation was used. The stimulation of depolarization to -30 mV lasted for 30 ms. After returning to holding potential for 5 ms, a series of depolarization at -30 mV for 30 ms was given. And the interval between the two pulses was increased by a range of 5 ms, with a total of 15 pulses. Then Fit with One-phase association exponential equation $I/Ipre=1-\exp(-t/\tau)$, I/Ipre is the ratio of the latter pulse to the previous pulse current, t is the interval between two pulses and τ is the recovery time when the channel is reactivated to the maximum current.

Acute isolation of ventricular myocytes from rats

All procedures were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine of Yangzhou University. The isolation of rat ventricular myocytes was improved with reference to relevant report [16]. In short, 2 000 IU kg^{-1} heparin sodiumand was injected intraperitoneally for 10 min, and then 2% pentobarbital sodium (40 $mg \cdot kg^{-1}$) was injected intraperitoneally. After anesthesia, the chest was opened quickly and the heart was cut. Then the free heart was cleaned at 4℃ calcium-free Tyrode's solution, and the aorta was inserted and fixed on the modified Langendorff perfusion frame after simple pruning. The method comprises the following steps of : Firstly , the heart was resuscitated for 5 seconds by the infusion of Tyrode's solution and the internal blood stain was removed. Secondly, the heart was perfused for 5 ~ 10 min with calcium-free Tyrode's solution. Thirdly, perfusing the heart with enzyme solution for 20 ~ 30 min at a speed of about 4 ~ 6 ml/min, until the heart began to swell, transparent, and the droplets became turbid, and the long rod cells appeared at the time of microscopic examination. Finally, the digestion was stopped quickly, and the KB solution was injected into the aortic orifice with 2.5 ml volume syringe to further terminate the digestion and protect the cells. The whole perfusion process maintained constant temperature of 37 ℃ and total oxygen. After the digestion was terminated, the ventricular tissue was cut off quickly and shredded in KB solution. After filtration with 120 mesh screen, filtrate was placed at room temperature for 10 min, then the supernatant was discarded. The precipitate was cleaned with KB solution, and it was repeated for 3 times. Then the cell suspension was filled with total oxygen for 30 min, and then placed 2~3 h standby away from the light.

Electrode and other experimental conditions

The glass microelectrodes used in the experiment were made by a programmed P-97 microelectrode horizontal drawing instrument. An electrode with a tip diameter of 1 ~ 2 μm was made by drawing in five steps, and the electrode liquid was injected at the end of the electrode. The parameters are that the resistance of the electrode is kept at 2 ～ 4 MΩ after entering the extracellular Fluid, and the series resistance compensation was 20%～40%.

Whole-cell patch clamp recording

The whole-cell patch clamp recording was done with reference to relevant report [17].

Statistical methods

PatchMaster Software was used to recorded current. Origin7.0 software was used to transform the experimental data and graphpadprism6.1 was used to analyze and fit the data. SPSS 16.0 software was used for statistical processing of experimental data, and all experimental data were expressed as $\bar{x} \pm S$, and the standard of statistical test was $P<0.05$.

Results

Effects of tanshinone IIA on I_{Na} in rat ventricular myocytes

The sodium current was recorded in accordance with the current stimulation scheme of appendix 2.1. First of all, 50 μM TTX [18], the specific blocker of Na_v, was added to the extracellular fluid, and the current was recorded under this condition. And then, the drug was eluted with extracellular fluid and the current was recorded again. As shown in Fig. 1A and B, the recorded current was an inward current, and the current was significantly suppressed under the influence of 50 μM TTX, and recovered after elution. It can be determined that the recorded current was a sodium current. Then the effects of different concentrations of tanshinone IIA (5,10,20,40,80,160 μM) which was dissoluted by alcohol [19] on I_{Na} in ventricular myocytes were observed after 10 min administration, as shown in Fig. 1C and D, when the concentration of tanshinone IIA was 5 and 10 μM, there was no significant effect on I_{Na}, while the concentration was more than 20 μM, the inhibitory effect on I_{Na} was enhanced with the increase of concentration.

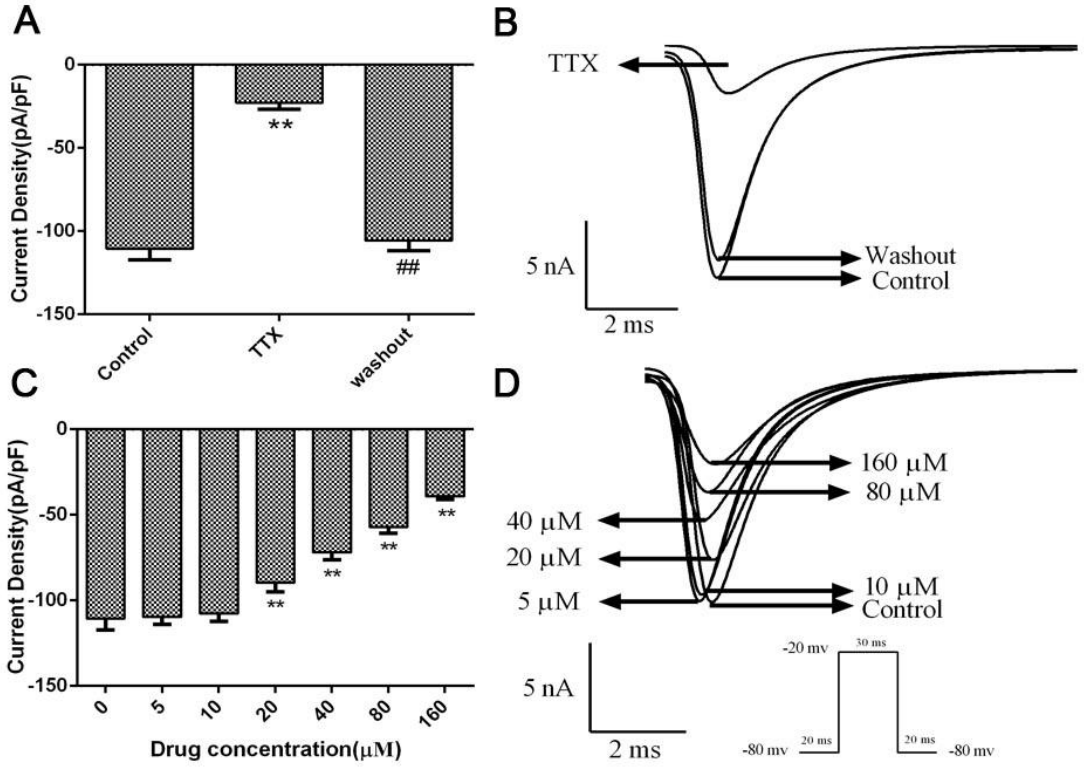

Figure 1. Effects of drugs on I_{Na}. A. The change of $I_{Na\text{-}peak}$ which was affected by 50 μM TTX and eluted by extracellular fluid. B. The single stimulus primary current of I_{Na} which was affected by 50 μM TTX and eluted by extracellular fluid. C. The changes of $I_{Na\text{-}Peak}$ induced by tanshinone IIA at 5, 10, 20, 40, 80 and 160 μM. D. The primary current of I_{Na} under the influence of different concentrations of tanshinone IIA. Compared with control group $^{**}P<0.01$, n=6 and compared with 50 μM TTX, $^{\#\#}P<0.01$, n=6.

Effects of tanshinone IIA on the I-V curve of I_{Na}

The I_{Na} (Fig. 2A) in the presence or absence of tanshinone IIA was recorded by using the stimulation scheme described in appendix 2.2, and the I-V curve of I_{Na} was drawn (Fig. 2B). It can be seen from the figure that tanshinone IIA can significantly move up the I-V curve of I_{Na}, and 20 and 80 μM tanshinone IIA reduced $I_{Na\text{-}Peak}$ from (-110.61±6.28) pA/pF to (-87.28±5.76) pA/pF and (-54.53±3.15) pA/pF respectively(P <0.01， n= 6). However, the trajectory of I-V curve, V_{ac}, V_{Peak} and V_{rev} did not change.

Effects of tanshinone IIA on the activation process of I_{Na}

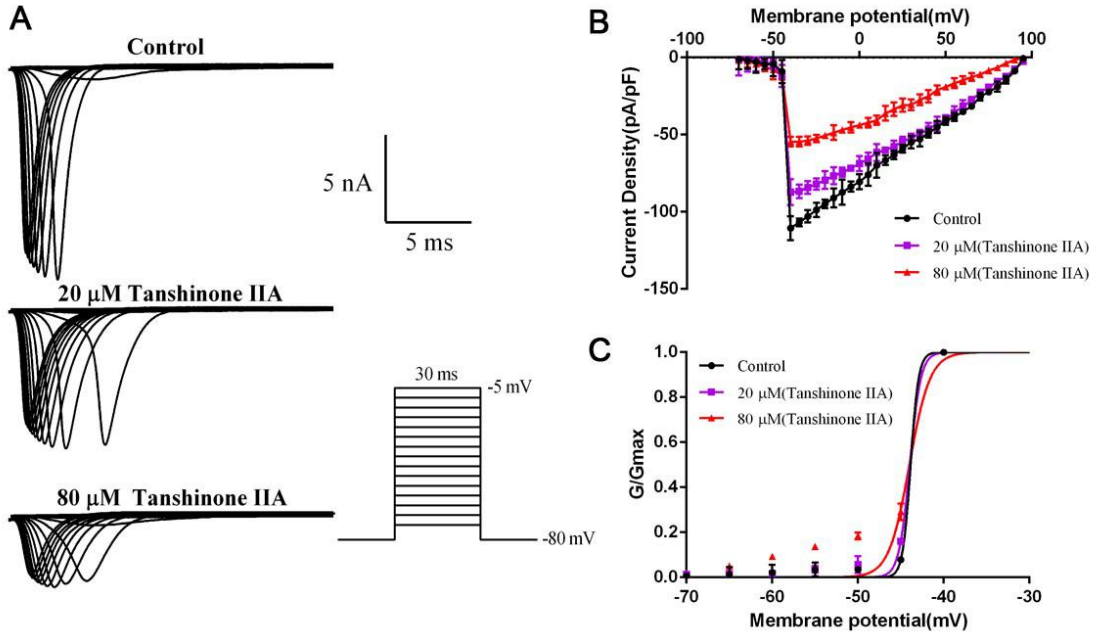

Figure 2. Effects of tanshinone IIA on the activation process of I_{Na}. A. The effect of 20 and 80 μM tanshinone IIA on the primary activation current of I_{Na}. B. The effect of 20 and 80 μM tanshinone IIA on the I-V curve of I_{Na}, compared with control group, P <0.01, n= 6. C. The effect of 20 and 80 μM tanshinone IIA on the steady-state activation curve of I_{Na}, compared with control group, P >0.05, n= 6.

The original data of the steady-state activation curve of I_{Na} were transformed from part of the data of I-V curve, and the steady-state activation curve was obtained according to the fitting method of appendix 2.3(Fig. 2C). As shown in the figure, tanshinone IIA had no significant effect on the steady-state activation curve of I_{Na}. $V_{1/2\text{-}ac}$ changed from (-43.85±8.07)mV to (-43.95±2.44)mV and (-43.99±0.79)mV.

Effects of tanshinone IIA on the inactivation process of I_{Na}.

The steady-state inactivation current of I_{Na} in the presence or absence of tanshinone IIA was recorded by the appendix 2.4 stimulus (Fig. 3A). And the steady-state inactivation curve was obtained by fitting the equation(Fig. 3B). It can be seen from the figure that tanshinone IIA can make the steady-state inactivation curve of I_{Na} shift significantly to the left, that is, to the direction of hyperpolarization. 20 and 80 μM tanshinone IIA made $V_{1/2\text{-}in}$ from (-52.15 ± 0.96)mV to (-64.87 ± 0.99)mV and (-75.71±0.75)mV(P<0.05, n=6), and the value of k changed from (6.41±0.85) to (7.92±0.88) and (10.92±0.66)(P <0.05, n= 6). Therefore, tanshinone IIA can obviously change the inactivation kinetics of I_{Na} and accelerate the inactivation of sodium channel.

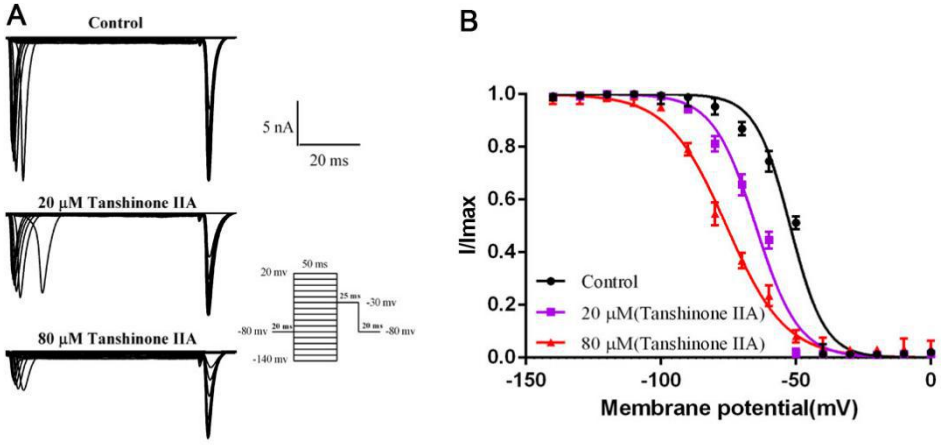

Figure 3. Effects of tanshinone IIA on the inactivation process of I_{Na}. A. The effect of 20 and 80 μM tanshinone IIA on the primary inactivation current of I_{Na}. B. The effect of 20 and 80 μM tanshinone IIA on the steady-state inactivation curve of I_{Na}, compared with control group, $P<0.05$, n= 6.

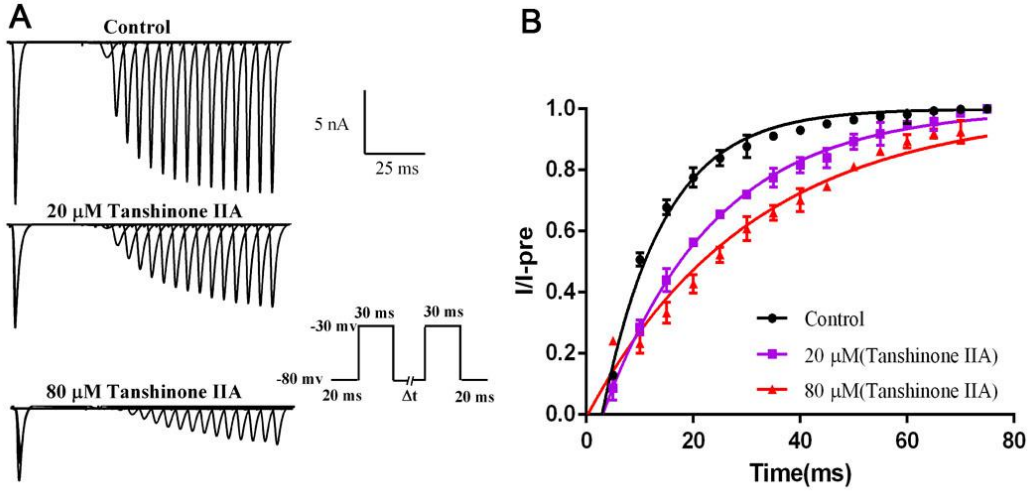

Figure 4. Effects of tanshinone IIA on the recovery process of I_{Na}. A. The effect of 20 and 80 μM tanshinone IIA on the primary recovery current of I_{Na}. B. The effect of 20 and 80 μM tanshinone IIA on the recovery curve of I_{Na}, compared with control group, $P<0.05$, n= 6.

Effects of tanshinone IIA on the recovery process of I_{Na}.

The recovery currents of the channel in the presence or absence of tanshinone IIA were recorded according to the appendix 2.5 stimulus(Fig. 4A), and the recovery curve of I_{Na} was obtained by fitting the equation(Fig. 4B). As shown in the figure that tanshinone IIA can make the recovery curve of I_{Na} shift to the right, 20 and 80 μM tanshinone IIA made τ from (11.52 ± 1.18)ms to (20.84 ± 0.99)ms and (30.81 ± 1.01)ms ($P<0.05$, n=6). It is concluded that tanshinone IIA can change the recovery dynamic characteristics of the sodium channel after inactivation and prolong the recovery time of I_{Na} from inactivation state to activation state.

Discussion

An effective way to explore the function of ion channels is the patch clamp technique, which was founded by Neher and Sakmann in the Mapper Biophysical Laboratory in Germany in 1976 [20]. It is a technique that reflects the activity of a single (or multiple) ion channel molecule on a cell membrane, based on the principle that pre-treated glass microelectrodes are used to contact the cell and form a blocking state of the kilomega ohm(GΩ) or more impedance. At this time, the small area of the membrane in contact with the tip of the electrode is electrically separated from the other structures around it, so the ionic channel current on the membrane can be recorded on the basis of the fixed membrane potential [21]. With the development of this technology, the characteristics and physiological meaning of various ion channels in cells have been

recognized and understood. For example, the sodium ion current in cardiomyocytes is an inward current, which is mainly involved in the depolarization process of the cell AP 0 phase, which affects the Rm of the cell, and then affects the excitability of the cardiomyocytes and the ECG waveform [2].

Sodium channels are channels that selectively allow sodium ions to through the cell membrane, including VGSC (Na_v, also known as voltage-dependent sodium channels), ENaC, ASIC, and Nax channels, and Na_v is the most typical [22]. Na_v is subdivided into nine sub-types according to the functional α subunit of channel, $Na_v1.1$-1.9, among them, $Na_v1.5$ is mainly distributed on myocardial cell membrane, which has important significance for the normal exertion of cardiomyocyte and cardiac physiological function. Therefore, it is a new research direction to explore the therapeutic effect of drugs on heart disease for $Na_v1.5$ target in recent years.

Therefore, the aim of this study was to explore the possible mechanism of anti-arrhythmic effect of tanshinone IIA, an effective component of traditional Chinese medicine Danshen, at the ion channel level. In introduction section, we learned that tanshinone IIA can affect LTCC, $K_v1.5$, I_{Ks} and $I_{Kr.}$ In addition, Ao Ri-Ge-le[23] et.al found tanshinone IIA attenuated painful diabetic neuropathy by effecting VGSCs activities and expressions in dorsal root ganglion. Thus, in order to observe the effects of tanshinone IIA on I_{Na} in myocytes, we selected isolated rat ventricular myocytes as the research object, because the physiological characteristics of atrial and ventricular myocytes are different [17]. The whole-cell patch clamp technique was used to record I_{Na}, and by collecting and analysing the data, we can further understand the changes of the related kinetic processes of sodium channels under the influence of tanshinone IIA. We found that tanshinone IIA inhibited I_{Na} in SD rat ventricular myocytes in a dose-dependent manner. As for channel dynamics, tanshinone IIA could delay the activation, accelerate the inactivation, and slow down the recovery after inactivation of channels.

However, this study only focused on the cell level in vitro. The further research on the electrophysiological characteristics of isolated heart and even in vivo, the characteristics of each channel and the safety of drugs need to be improved. Besides $Na_v1.5$, there are many other ion channels in ventricular myocytes, such as LTCC, K_v, etc, and the interaction between channels and the expression of channel proteins should also be taken into account [12]. Moreover, Tetrodotoxin (TTX) is recognized as a sodium channel blocker, However, the blocking effect and intensity of TTX are different due to the large number of sodium channel types and the different structural and functional characteristics of different sub-types. As $Na_v1.7$, which is mainly distributed in peripheral nerves, is highly sensitive to TTX [24], while $Na_v1.5$ is certainly resistant to it [22]. Therefore, searching for a more suitable blocker for this experiment should be included in the next research plan.

There is still a lot of work to be done. It is believed that the effects of tanshinone IIA in the treatment of arrhythmia will be well explained at the molecular and electrophysiological level, and further explore the possible mechanism of Salvia miltiorrhiza to exert antiarrhythmic effect.

Conflict of interest

None.

Acknowledgements

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