Tanshinone IIA Selectively Enhances Hyperpolarization-Activated Cyclic Nucleotide–Modulated (HCN) Channel Instantaneous Current

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Abstract. Tanshinone IIA, one of the main active components from the Chinese herb \textit{Danshen}, is widely used to treat cardiovascular diseases in Asian countries, especially in China. To further elucidate its heart rate–reducing and anti-ischemic mechanisms, here we investigated the effects of tanshinone IIA on hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels expressed in \textit{Xenopus} oocytes using two-electrode voltage clamp techniques. When applied to the extracellular solution, 100 µM tanshinone IIA caused a slowing of activation and deactivation and an increase of minimum open probabilities (from 0.06 ± 0.01 to 0.29 ± 0.03, \(P<0.05\)) in HCN2 channels without shifting the voltage dependence of channel activation. Tanshinone IIA potently enhanced the amplitude of voltage-independent current (instantaneous current) of HCN2 at −90 mV in a concentration-dependent manner with an \(EC_{50}\) of 107 µM. Similar but 2.3-fold less sensitivity to tanshinone IIA was observed in the HCN1 subtype. More significant effect on HCN2 and MiRP1 co-expression was observed. In conclusion, tanshinone IIA changed HCN channel gating by selectively enhancing the instantaneous current (one population of HCN channels), which resulted in the corresponding increment of minimum open probabilities, slowing channel activation and deactivation processes with little effect on the voltage-dependent current (another population of HCN channels).

Keywords: hyperpolarization-activated cyclic nucleotide–modulated (HCN) channel, instantaneous current, tanshinone IIA

Introduction

Hyperpolarization-activated cyclic nucleotide–modulated (HCN) channels are activated by membrane hyperpolarization and conduct an inward cation current that contributes to spontaneous activity of pacemaker cells in the heart (1, 2). The HCN current is abbreviated \(I_f\) (“funny”) in cardiac cells. Activation of HCN current during diastole is the main process underlying generation of the diastolic depolarization and spontaneous activity of cardiac pacemaker cells. The molecular identity of this current in mammals was first identified by Santoro et al. (3), and the channels are now known to be formed by homomeric or heteromultimeric co-assembly of four HCN channel subunits (4). There are four mammalian HCN isoforms, HCN1 – HCN4, which combine to form tetrameric channels in the heart and the nervous system. HCN-channel currents contain two components: the instantaneous current (voltage-independent) that is fully activated within a few milliseconds and the slowly developing component which reaches its steady-state level within one to several seconds under fully activating conditions (voltage-dependent) (5, 6). HCN channels are also regulated by voltage-gated potassium channel auxiliary subunits such as MinK-related protein 1 (MiRP1 or KCNE2) (7).

\textit{Danshen} (extracted from root of \textit{Salvia miltiorrhiza}) is a traditional Chinese medicinal herb. Tanshinone IIA is one of the main active components from \textit{Danshen} and has been widely used to treat ischemic heart disease and ischemic stroke in Asian countries, especially in China. Tanshinone IIA exhibits a variety of cardiovascular effects including slowing sinus rhythm, protection against ischemia-reperfusion injury, and anti-
arrhythmia (8, 9). In vitro, tanshinone IIA was found to suppress rabbit sinoatrial node automaticity (10), shorten action potential duration in rat heart, inhibit L-type \( \text{Ca}^{2+} \) current in guinea-pig ventricular myocytes (11, 12), inhibit \( I_{\text{KCa}} \) currents in rat ventricular myocytes (13), activate KCNQ1/KCNE1 (\( I_{\text{Ks}} \)) potassium current (14), as well as depress cardiac hypertrophy (15). Although tanshinone IIA has been extensively studied, the mechanisms for its heart rate–reducing and anti-ischemia actions have not been completely elucidated. The pacemaker activity involves several ionic currents that influence the spontaneous diastolic depolarization of the sino-atrial node, including HCN-channel current, calcium currents \( I_{\text{Ca-L}} \) (long-lasting), and \( I_{\text{Ca-T}} \) (transient). Besides its inhibition of L-type calcium current, the effect of tanshinone IIA on HCN channels has never been characterized. Moreover, currently, the HCN channels have become pharmacological targets of anti-ischemia. Here, we addressed the mode of action of tanshinone IIA on cloned HCN channels heterologously expressed in Xenopus oocytes and outlined the therapeutic implications of the existing research data on this drug. Our findings suggested that tanshinone IIA changed HCN2-channel gating by selectively enhancing the instantaneous current rather than the voltage-dependent current.

Materials and Methods

Molecular biology

This study was approved by the Animal Research Committee of Tongji Medical College of Huazhong University of Science and Technology. Wild-type (WT) \( mHCN2 \) cDNA was cloned from Marathon-Ready (Clontech, Mountain View, CA, USA) mouse brain cDNA and inserted into the pSP64T oocyte expression vector. WT human \( HCN1 \) (\( hHCN1 \)) and MiRP1 were kindly provided by D. Krafte (Icagen, Inc., Durham, NC, USA). cRNA for injection into oocytes was prepared with SP6 Capscribe (Roche, Indianapolis, IN, USA). cRNA was dissolved in distilled water. An aliquot of the stock solution was dissolved in the modified ND96 external solution immediately before use to obtain the final desired drug concentrations.

Voltage clamp of oocytes

Isolation and maintenance of \( X. laevis \) oocytes and cRNA injections were performed as described (16). In brief, stage IV and V \( X. laevis \) oocytes were injected with 10–50 ng of cRNA encoding HCN channels. For HCN2 and MiRP1 co-expression, oocytes were co-injected with nearly equal molar ratio of HCN2 and MiRP1 cRNAs. After injection with cRNA, the oocytes were cultured at 18°C for 1 to 3 days in Barth’s solution containing 88 mM NaCl, 1 mM KCl, 1 mM MgSO\(_4\), 0.41 mM CaCl\(_2\), 2.4 mM NaHCO\(_3\), 10 mM HEPES, 0.33 mM Ca(NO\(_3\))\(_2\), 50 \( \mu \)g/ml gentamicin, and 1 mM pyruvate, pH 7.4. For voltage-clamp experiments, oocytes were bathed in a modified ND96 external solution containing 96 mM NaCl, 4 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 5 mM HEPES, pH 7.6 at a perfusion rate of 1.5 ml/min. Currents were recorded at room temperature (22°C – 24°C) with a GeneClamp 500 amplifier (Molecular Devices, Union City, CA, USA) using standard two-microelectrode voltage-clamp techniques (17).

The effect of tanshinone IIA on the voltage dependence of HCN channels was determined using 3-s pulses to a wide range of test voltage (\( V_t \)), which was ranged from −130 to −40 mV, applied in 10-mV steps from a holding potential of −30 mV. Each \( V_t \) was followed by a second pulse to a potential of +30 mV. Pulses were applied once every 21 s.

Drugs

Tanshinone IIA (sulfotanshinone sodium), abbreviated as TSN in figures, was purchased from Shanghai No. 1 Biochemical & Pharmaceutical Co., Ltd. (purity of 99%, Lot No: 030701; Shanghai, China). CsCl was purchased from Sigma (St. Louis, MO, USA). ZD7288 (Tocris Bioscience, Ellsvile, MO, USA) was prepared as a 50 mM stock solution in distilled water. An aliquot of the stock solution was dissolved in the modified ND96 external solution immediately before use to obtain the final desired drug concentrations.

Data analyses

Voltage clamp data were stored on the computer hard disk and analyzed off-line using pClamp 8 (Molecular Devices) and Origin 7.5 (OriginLab, Northampton, MA, USA) software.

The instantaneous component was defined as the difference between the holding current at −30 mV and the initial current level where the time-dependent current component began. Peak current was defined as the time-dependent component at the end of a 3-s hyperpolarizing pulse. To construct I – V relationships, instantaneous and peak currents were normalized to its own maximum current measured before drug and then plotted as a function of \( V_t \).

The voltage dependence of HCN-current activation was determined by analysis of peak tail currents (\( I_{\text{tail}} \)) measured at +30 mV. All tail current amplitudes from an individual oocyte were normalized to its own \( I_{\text{max}} \), plotted as a function of \( V_t \), and fitted again with a Boltzmann function: \( I / I_{\text{max}} = 1 / [1 + \exp((V_t - V_{1/2}) / k)] \).
to determine the values of the half-point ($V_{1/2}$) and the slope factor ($k$). The minimal open probability (min-Po) was defined as the minimal value of the relative tail current.

The time constants for HCN2 current activation ($\tau_{\text{activation}}$) at different $V_i$ were determined using the standard exponential curve fitting. Time-dependent activating currents ($I_{\text{act}}$) were fitted to a single exponential function: $I_{\text{act}}(t) = Ae^{-t/\tau_{\text{activation}}} + C$. The time constants for deactivation ($\tau_{\text{deactivation}}$) were determined at $+30 \text{ mV}$ after each test pulse.

Concentration–effect data were fit to the Hill equation: $f = 1 / \{1 + (\text{EC}_{50} / [D])^h\}$, where $f$ was the increase in instantaneous current at $-90 \text{ mV}$, expressed as percentage change compared with the control values, $\text{EC}_{50}$ was the drug concentration required for 50% of the average percentage change in instantaneous current at $-90 \text{ mV}$, $[D]$ was the concentration of tanshinone IIA, and $h$ was the Hill coefficient.

Data were each presented as the mean ± S.E.M. ($n =$ number of oocytes), and statistical comparisons between experimental groups were performed using Student’s $t$-test. Differences were considered significant at $P<0.05$.

**Results**

**HCN2 channel biophysical properties**

HCN2-channel currents were elicited with 3-s hyperpolarizing pulses applied from a holding potential of $-30 \text{ mV}$, a voltage where the open probability of HCN2 channels is at its minimum. Pulses to test potentials ranging from $-130$ to $-40 \text{ mV}$ were applied in 10-mV increments. Currents activated by hyperpolarization consisted of two components, an initial instantaneous component (after the capacitive transient but before the voltage-dependent current began to activate) and a much larger, time-dependent component that developed faster.
at more negative potentials (Fig. 1A).

HCN channel biophysical properties were confirmed by HCN blockers (30 μM ZD7288 and 20 mM CsCl) (Fig. 1C) (18–20). Similar $V_{1/2}$, $k$, and min-\(P_o\) values were found when compared to those reported in the literature (20, 21).

**Tanshinone IIA increased the instantaneous current amplitude and slowed kinetics of HCN2-channel currents**

The effect of tanshinone IIA on mHCN2 channels expressed in oocytes was first determined at a concentration of 100 μM. All current amplitudes from an individual oocyte were normalized to its own maximum current before drug and then plotted as a function of $V_t$. The averaged and normalized peak current I–V relationship for HCN2 channels is shown in Fig. 1D. At a concentration of 100 μM, tanshinone IIA did not change the amplitude of peak current at all test potentials (Fig. 1: A, B, and D).

The amplitudes of tail currents measured at +30 mV were normalized to the peak value, plotted as a function of test voltage, and fitted with a Boltzmann function to obtain the isochronal voltage dependence of HCN2-channel activation. Tanshinone IIA did not cause a shift in $V_{1/2}$ (Fig. 1E). The average value for $V_{1/2}$ was $-70.6 \pm 4.4$ mV under control conditions and $-72.9 \pm 2.8$ mV after addition of tanshinone IIA (n = 4, $P>0.05$). The slope factor ($k$) of the activation curve was decreased from $15.6 \pm 2.4$ to $14.9 \pm 2.5$ mV in the presence of tanshinone IIA (n = 4, $P>0.05$) (Fig. 1E). Tanshinone IIA partially disrupted channel closure, resulting in an increase in min-\(P_o\) from 0.06 ± 0.01 to 0.29 ± 0.03 (n = 4, $P<0.05$) (Fig. 1: A, B, and E).

However, tanshinone IIA significantly enhanced initial instantaneous current magnitude at test potentials ranging from $-130$ to $-90$ mV (n = 4, $P<0.05$) (Fig. 1: A, B, and F). The increase was more pronounced at more hyperpolarized voltages (Fig. 1F).

At test potentials from $-130$ to $-90$ mV, the onset of current activation was single exponential (Fig. 1: A and B, Fig. 2B). The slowing activation kinetics induced by 100 μM tanshinone IIA was more pronounced at hyperpolarized voltages (Fig. 2B). Tanshinone IIA increased the time constant of activation at a test potential of $-130$ mV from 223 ± 8 to 365 ± 37 ms (n = 4, $P<0.05$). At 100 μM, tanshinone IIA slowed the apparent rate of current deactivation at +30 mV from different $V_t$ (Fig. 1: A and B, Fig. 2C). The time constant for current deactivation at +30 mV corresponding to a test potential of $-130$ mV was 200 ± 32 ms in the control and 343 ± 17 ms after 100 μM tanshinone IIA (n = 4, $P<0.05$, Fig. 2C).

**Differential sensitivity of instantaneous current in HCN1 and HCN2 channels to tanshinone IIA**

The effect of 100 μM tanshinone IIA was further determined on WT HCN1 channels. HCN1-channel current was activated at more positive potentials and faster than WT HCN2, as shown in Figs. 1A and 3A. Similar to WT HCN2 channels, tanshinone IIA at 100 μM caused no shift in the voltage dependence of activation, no change in the slope factor, along with no difference in the averaged and normalized peak current I–V relationship at all test potentials (Fig. 3: A, B, and D). The voltage-dependent increment of instantaneous current by tanshinone IIA was readily apparent from $-30$ to $-80$ mV in the averaged and normalized I–V plot (Fig. 3E). In contrast to WT HCN2 channels, 100 μM tanshinone IIA only slightly slowed the kinetics of activation or deactivation of WT HCN1 channels (Fig. 3: A, B, and C).
The effects of tanshinone IIA on the increment of instantaneous current at \(-90\) mV was concentration-dependent. The percentage change compared with control values was determined at four concentrations, ranging from 10 to 300 \(\mu M\). The EC\(_{50}\) for this effect was 250 \(\pm\) 31 \(\mu M\) for HCN1 and 107 \(\pm\) 6 \(\mu M\) for HCN2 (\(n=5\) for each channel type). Comparing to HCN1 channels, HCN2 channels were about 2.3-fold more sensitive to tanshinone IIA at \(-90\) mV (Fig. 2A, and Fig. 3: C and F). In other words, unlike the effects on WT HCN2 channels, tanshinone IIA had minor effects on the WT HCN1 channels. Thus, the difference in sensitivity to tanshinone IIA between HCN1 and HCN2 changes could be largely accounted for by alignment differences.

**Effect of tanshinone IIA was magnified by co-expression of HCN2 and MiRP1**

MiRP1 is purported to be a \(\beta\) subunit for several voltage-gated potassium channels, including HCN channel. Co-immunoprecipitation experiments involving HA-tagged MiRP1 and HCN1 demonstrated that the two proteins interacted when co-expressed in oocytes (7). MiRP1 and HCN2 functionally co-assembled in cardiac myocytes. Some studies reported that co-expression of MiRP1 with HCN2 increased the instantaneous current and decreased the voltage-dependent current (5). We found that co-expression of MiRP1 with HCN2 not only increased peak current magnitude of HCN2 current at voltages from \(-130\) to \(-50\) mV (\(n=6\), \(P<0.05\)), but also affected the instantaneous component (min-Po for HCN2 channels was 0.06 \(\pm\) 0.01 and min-Po for HCN2 and MiRP1 co-expression was 0.11 \(\pm\) 0.01, \(n=6\), \(P<0.05\)). However, co-expression of MiRP1 did not affect the voltage dependence of HCN2-channel activation (Fig. 1E and Fig. 4C) \((V^{1/2}\) for HCN2 channels was \(-74.8\) \(\pm\) 1.8 and \(V^{1/2}\) for HCN2 and MiRP1 co-expression was \(-73.0\) \(\pm\) 1.2, \(n=6\), \(P>0.05\)). The slope factor \(k\) for the relationship did not change either. Co-expression of MiRP1 accelerated the activation kinetics of HCN2 channels at voltages from \(-130\) to \(-80\) mV (\(n=6\), \(P<0.05\)). Our results were very similar to the findings in ventricular myocytes (7).
Because co-expressing MiRP1 with HCN2 increased instantaneous current, we reasoned that the effect of tanshinone IIA would be amplified. To explore this possibility, we used exactly the same tanshinone IIA concentration and same recording conditions to determine the response of co-expressed MiRP1 and HCN2 channels (Fig. 4: A and B). A notable observation was found consequently. Tanshinone IIA shifted the $V_{1/2}$ for HCN2 and MiRP1 channels from $-72.7 \pm 3.1$ to $-64.2 \pm 4.6$ mV ($n = 4$, $P < 0.05$, Fig. 4C). In addition to the shift in activation, tanshinone IIA increased min-$P_o$ from $0.11 \pm 0.01$ to $0.34 \pm 0.06$ ($n = 4$, $P < 0.05$). However, $k$ for the relationship did not change significantly (control: $18.3 \pm 3.6$ mV, tanshinone IIA: $20.9 \pm 5.6$ mV, $n = 4$, $P > 0.05$). The increment of instantaneous and peak current was found at a wide potential (e.g., up to 1.85-fold increase at $-130$ mV in instantaneous current) (Fig. 4: D and E).

**Discussion**

The present study for the first time demonstrated that tanshinone IIA potently enhanced instantaneous current, increased the channel minimum open probabilities, and decelerated the activation and deactivation in cloned HCN2 channels heterologously expressed in *Xenopus* oocytes; the activation curve was shifted to positive potential when co-expressed with MiRP1. These results suggested that tanshinone IIA changed the gating of HCN channels.

The two components of HCN-channel currents, the instantaneous current and the slowly developing component, are supposed to flow through two distinct channel populations (22). The instantaneous current is voltage-independent and accompanies expression of HCN2 and HCN1 channels. The voltage-independent current could play a very important role in determining the excitability of cells where HCN channels are expressed, yet many mechanistic questions remain about how these voltage-dependent channels could also produce a voltage-
independent current. This current is still thought to be produced by HCN channels because it depends on surface expression of the channels. It has a reversal potential similar to that of $I_f$, and its amplitude is correlated with that of $I_f$. The instantaneous current was selectively enhanced by tanshinone IIA in the present study, supporting that it was produced by a separate population of HCN channels that was not in rapid equilibrium with the main population of voltage-dependent channels.

Interestingly, the voltage sensors and the gate molecularly failed to couple in instantaneous current–producing HCN channels (22). Figure 1C showed that in addition to the blockade of the voltage-dependent current, ZD7288 (one of the HCN-channel blockers) also blocked the inward instantaneous current at hyperpolarized potentials. Alanine mutagenesis results suggested that ZD7288 binds in the pore of either HCN2 or HCN1 channels (20) from the intracellular side of the membrane (23). Different from ZD7288, tanshinone IIA selectively enhanced instantaneous current. It suggested that tanshinone IIA could either bind to the pore or separately bind to voltage sensors resulting in the shift of voltage dependence in HCN2 and MiRP1 co-expression experiments.

Despite the differing responses to tanshinone IIA among HCN1, HCN2, HCN2 and MiRP1, the three shared both an increase of instantaneous current (corresponding to increment of min-Po) and slowed channel activation and deactivation processes. The selective enhancement of instantaneous current by tanshinone IIA was more pronounced in channels displaying slow kinetics (HCN2, HCN2 and MiRP1 co-expression) than fast channels (HCN1), as shown in Figs. 1–3. Since instantaneous current reflected one population of HCN channels that did not fully deactivate (19), the current amplitude largely depended on channel numbers at the state in which channels recover from the deactivation state (inter-pulse interval). Therefore, instantaneous current change in channels displaying slow kinetics by tanshinone IIA might result from incomplete channel deactivation. That was why the min-Po of the channel increased correspondingly. To rule out the possibility of channel accumulation caused by the pulse interval (21 s) used in our experiment, a much longer pulse interval (300 s) was used in both the presence and absence of tanshinone IIA. However, similar results were observed (data not shown). It suggested that instantaneous current represents an intrinsic slow deactivation kinetics–channel population and the pulse interval used in our experiment was proper.

Besides acting as a pacemaker, HCN instantaneous current also functions as a regulator of resting potential and membrane resistance (22). It stabilizes the resting membrane potential because a small hyperpolarization activates HCN channels, whose inward current depolarizes the cell. This depolarization deactivates HCN channels, preventing further departure from the resting potential. Compared with populations of voltage-dependent currents, instantaneous current only accounts for a small part in the total HCN channel populations. For example, the instantaneous current in the spHCN isoform (HCN homolog from sea urchin) averages only about 4% of the maximum HCN conductance (24). In the HCN2 isoform, instantaneous current is about 2% of the maximal current (22). So, even if there was an enhancement of HCN channel instantaneous current in the presence of tanshinone IIA, its effect was not sufficient to increase the heart rate in vivo and the rate of spontaneous diastolic depolarization in vitro (8, 10), but it could prevent the excessive reduction of heart rate by its blockade of L-type calcium current (11, 12).

Since the physiological significance of instantaneous current and corresponding channel structure are still poorly understood, the binding site of tanshinone IIA can not be probed in our study. That is the limitation of our study. In the present study, only HCN2 and HCN1 channels were used as the model for cardiac $I_f$. However, different subunits of HCN channels and ancillary subunits are heterologously expressed in mammalian cardiac myocytes. In reference to its clinically relevant plasma concentration (about 280 $\mu$M) (9), the EC$_{50}$ (64.5 $\mu$M) of tanshinone IIA on $I_{Ks}$ in HEK293 cells was much lower than that on HCN2 channels in oocytes (107 $\mu$M), which is mainly from the differences between oocytes and mammalian cell line (HEK293) (14).

In summary, the present study provided the novel finding that tanshinone IIA directly and specifically changes HCN channel gating by selectively enhancing HCN instantaneous current.

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