Berberine attenuates spontaneous action potentials in sinoatrial node cells and the currents of human HCN4 channels expressed in *Xenopus laevis* oocytes

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Abstract. The present study investigated the electropharmacological effects of a traditional Chinese herbal drug, berberine, on the spontaneous activity of sinoatrial nodes (SANs) of the rabbit heart and on human hyperpolarization-activated cyclic nucleotide-gated 4 (hHCN4) channels, which are heterologously expressed in xenopus oocytes, and which contribute to pacemaker currents (Ifs). A standard microelectrode technique and standard two-electrode voltage-clamp recordings were employed to examine the properties of transmembrane potentials and cloned hHCN4 subunit currents, respectively, under control conditions and berberine administration. Berberine decreased the rate of pacemaker firing and the rate of diastolic depolarization, and modified the action potential parameters. In addition, berberine suppressed the hHCN4 channel currents in a concentration- (1-300 μM) and use-dependent manner, and simultaneously decreased the activation and deactivation kinetics of the hHCN4 channels. The ability of berberine to modulate the If of cardiac pacemaker cells may contribute to its antiarrhythmic action.

Introduction

In mammals, the cardiac pacemaking current (If) naturally originates in the sinoatrial node (SAN) due to its spontaneous firing of action potentials (APs). The cardiac If is one of the most noteworthy features of the SAN myocytes, as it has an important role in generating and modulating cardiac rhythmic activity through slow diastolic depolarization (1-4). The cardiac If has an unusual characteristic in that it is activated by membrane hyperpolarization within a voltage rage, which is also why it is known as the 'funny' current. The If is a mixed-cation current that is carried by both Na^+ and K^+, and is controlled by the direct binding of intracellular cyclic adenosine monophosphate, which accounts for the activation and inhibition of the cardiac If by β-adrenergic and muscarinic M2 receptor stimulation, respectively (5). Four hyperpolarization-activated cyclic nucleotide-gated (HCN) channel members, HCN1-4, have been detected in the heart and are members of a superfamily of voltage-dependent K^+ and cyclic nucleotide gated (CNG) channels, and combine to form tetrameric channels. The earliest studies demonstrated that HCN4 is the major isoform for the mediation of the sympathetic stimulation of pacemaker activity that exists in the SAN (6-9). In addition, a previous study demonstrated that the 'funny' current in non-pacemaker cardiomyocytes may affect membrane excitability and predispose the human heart to atrial and ventricular arrhythmias (10-12). These findings suggested that the If may have a role in causing ectopic automaticity, particularly in several pathological conditions. However, the mechanisms underlying its action are yet to be fully elucidated.

Berberine (Fig. 1), a benzodioxoloquinolozine alkaloid occurring in numerous plants of the genera Berberis and Coptis, has been used in Traditional Chinese Medicine for many centuries. The chemical name of berberine is 5,6-dihydro-9, 10-dimethoxy-benzo[g]-1, 3-benzodioxolo [5,6-α] quinolizinium (13). It has been demonstrated that berberine exerts a protective effect against cardiac arrhythmias and has positive inotropic actions. Previous studies have also revealed that berberine decreases the maximal velocity of depolarization (Vmax) and prolongs the AP duration (APD) and effective refractory period (ERP) in cardiac myocytes and Purkinje fibers (14,15). It has been suggested that berberine exerts class III antiarrhythmic effects in the cardiac muscle of mammals *in vitro*. However, several experiments on cellular electrophysiology have demonstrated that berberine is a multi-ion channel blocker, with blockade actions on numerous currents, including the cardiac ATP-sensitive K^+ (K_{ATP}), the delayed rectifier K^+ current (I_K), inward rectifier K^+ current (I_{K1}),

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L-type Ca^{2+} ($I_{Ca\text{-}L}$) and the Na^{+}-Ca^{2+} exchange current (16-20). To the best of our knowledge, to date there have been no studies investigating the action of berberine on cardiac If. Therefore, the present study aimed to investigate the effects of berberine on the SAN of rabbits and hHCN4-mediated currents that are present in cardiac tissue. The heterologously expressed hHCN4 currents in xenopus oocytes were examines, and it was sought to examine the mechanisms underlying these effects. The present study provided insight into the ionic mechanisms responsible for the possible antiarrhythmic effects of berberine.

Materials and methods

Ethical considerations. Animals used in the present study were treated in accordance with the Guide for the Care and Use of Laboratory Animals regulated by the Administrative Regulation of Laboratory Animals of Hubei Province and all experimental methods were approved by the Animal Research Committee of the First Clinic College of Wuhan University (Wuhan, Hubei, China).

Preparation of SAN tissues and AP recordings. Healthy rabbits of both sexes, weighing 1.5-2.5 kg and ~6-8 weeks old, were anaesthetized with 30 mg/kg sodium pentobarbital intravenously. Following exsanguination, the hearts were rapidly removed and immersed in cold (0-4˚C) oxygenated Ca^{2+}-free tyrode solution containing 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2x6H_2O$, 0.33 mM NaH_2PO_4, 10 mM hydroxyethyl piperazineethanesulfonic acid (HEPES) and 10 mM glucose (4˚C; pH 7.4). The SA node preparations, bounded by the crista terminalis, the superior and inferior vena cava and the interatrial septum, were carefully dissected out to be pinned in the experimental chamber. The preparations were continuously superfused with modified tyrode solution at a rate of 10 ml/min and a temperature of 37±0.5˚C until ~1 h prior to the recordings. The composition of modified tyrode solution was as follows: 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2x6H_2O$, 5 mM HEPES and 10 mM glucose (pH 7.4).

Following 1 h of recovery, the transmembrane potentials were recorded with glass microelectrodes filled with 3 mM KCl (10-15 MΩ) connected to a high input impedance amplifier (Dua 773; World Precision Instruments, Sarasota, FL, USA). The signal was digitalized and collected using specific software (AcqKnowledge 4.1; BIOPAC Systems, Inc., Norfolk, UK). Spontaneous APs from the pacemaker cells were recorded for 20-30 min in control conditions. In this experiment, three concentrations of berberine (0.3, 3 and 30 μM) were added and the spontaneous AP firing rate was measured every 5 min for 1-2 h. In addition, changes in the AP amplitude and duration (APA, APD_{50} and APD_{90}), the spontaneous firing frequency, the maximal diastolic potential (MDP) and the diastolic depolarization rate (DDR) were determined at the end of the drug exposure period.

HCN channels expressed in xenopus oocytes

In vitro transcription and functional expression in xenopus oocytes. Wild-type human HCN4 (hHCN4) cDNA inserted into the pcDNA3 vector were provided by Professor A. Ludwig and J. Stieber (Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany). Complementary RNAs (cRNAs) which were used for injection into oocytes were prepared with the mMESSAGEmMACHINE® T7 kit (Ambion, Austin, TX, USA) following linearization of the expression constructs with XbaI (Takara, Kyoto, Japan). The RNA quality was examined by gel electrophoresis and the RNA concentration was quantified by ultraviolet spectroscopy (UV-2201; Shimadzu Corporation, Kyoto, Japan).

Voltage clamp assay of xenopus oocytes. Xenopus frogs were anesthetized by cooling on crushed ice for 30-40 min. Ovarian lobes were digested with 1 mg/ml type IA collagenase (Sigma Chemicals, St. Louis, MO, USA) in Ca^{2+}-free ND96 solution for 30 min to remove follicle cells. Stage IV and V xenopus oocytes were injected with 30 nl (1 μg/μl) of hHCN4 cRNAs per oocyte using a Nanoject microdispenser (Nanoliter 2000; World Precision Instruments, Sarasota, FL, USA) and then cultured in ND96 solution supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 2.5 mM pyruvate at 17˚C for 2-3 days prior to their use in the voltage clamp experiments. The ND96 solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES, titrated to pH 7.40 with NaOH. The recordings were performed 2-9 days following injection. A standard two-microelectrode voltage-clamp technique was used to record the currents at 21-23˚C. The glass microelectrodes were filled with 3 M KCl solution to obtain a resistance of 1-3 MΩ. The oocytes were clamped with a standard two-microelectrode voltage-clamp amplifier (DAGAN CA-1B; Dagan Corporation, Minneapolis, MN, USA) and PLAMP software (Axon Instruments, Foster City, CA, USA). Oocytes injected with hHCN4 cRNA were superfused with ND96 solution at a rate of 2.0 ml/min. The control currents were recorded repeatedly at 1 min intervals, with drug application continuing until the control currents achieved a stable level.

Drugs and reagents. Collagenase type I, zaterbradine, CsCl, HEPES and 4-aminopyridine were purchased from Sigma Chemicals. Pronase E was obtained from Roche (Basel, Switzerland). Berberine hydrochloride was obtained from the Yichang Humanwell Pharmaceutical Co., Ltd (Yichang, Hubei, China) as base powders and dissolved in distilled water. To maintain the drug and ion concentrations constant, the perfusion rate was strictly controlled using the perfusion device BPS-4 (ALA Scientific Instruments, Inc, Westbury, NY, USA) and a constant-flow pump.

Data acquisition and statistical analysis. All data were stored on a computer hard disk and analyzed off-line using Clampfit 10.0 (Axon Instruments) and Origin 8.0 software (Origin Laboratory, Northampton, MA, USA). The amplitudes of HCN-mediated currents were defined as the time-dependent components (Istep) at the end of the hyperpolarizing pulses or peak tail currents (Itail) at the beginning of the depolarizing pulses. To construct the I-V correlations, the currents were normalized to their own maximum current measured prior to drug treatment and then plotted as a function of the test potential (Vt). The voltage dependency of the HCN current activation was determined by analysis of the Itail measured at depolarizing potentials. All tail current amplitudes from

Table I. Effects of berberine on spontaneous AP characteristics in rabbit sinoatrial node preparations.

	n	MDP (mV)	APA (mV)	DDR (mV/s)	APD_{50} (ms)	APD_{90} (ms)	RPF (bpm)
Control	6	-54.4±4.1	59.5±8.1	27.3±5.2	111.6±16.8	153.0±14.9	112.8±10.5
3 μM	6	-32.5±3.4[b]	43.4±4.4[a]	18.3±2.5[b]	126.9±34.6	161.9±41.8	95.1±14.7[a]
30 μM	6	-21.0±1.7[b]	39.3±2.4[b]	13.6±1.2[b]	133.9±14.5	180.0±18.6[a]	86.4±9.8[b]

Spontaneous APs recorded at the beginning of the experiments (control) and at the end of exposure to berberine. Values are represented as the mean ± standard deviation. [a]P≤0.05, [b]P≤0.01, vs. the control. APs, action potentials; APA, action potential amplitude; MDP, maximal diastolic potential; DDR, diastolic depolarization rate; APD_{50}, action potential duration at 50% of total repolarization; APD_{90}, action potential duration at 90% of total repolarization; RPF, rate of pacemaker firing.

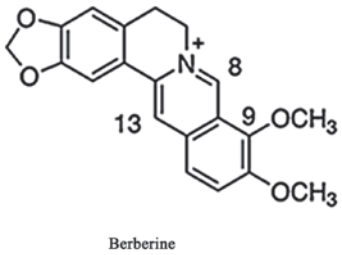
each individual oocyte were normalized to their own Imax, plotted as a function of Vt and fitted with a Boltzmann function: $I/Imax=1/[1+\exp(Vt-V_{1/2})/k)]$ to determine the values of the half-point ($V_{1/2}$) and the slope (k). The time constants for HCN current activation or deactivation (τactivation or τdeactivation) at different Vt were determined using standard exponential curve fitting. Activating or deactivating currents were fitted to a single exponential function: $I(t)=Ae^{-t/\tau}+C$. The concentration-effect curves were fitted using the Hill equation in the form $f=1/[1+(IC_{50}/D)n]$, where f represents the increase in HCN currents, expressed as a percentage change from the control values, IC_{50} was the half-maximum inhibitory concentration of berberine, D was the concentration of berberine and n was the Hill coefficient.

The data are presented as the mean ± standard deviation. The Student's t-test was used for statistical analysis of the paired observations, and an analysis of variance was performed to test the difference among the groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of berberine on the spontaneous APs of rabbit SNA tissues. To examine the effects of berberine on the spontaneous APs in rabbit SAN tissues, transmembrane potentials were recorded by a standard microelectrode technique. Berberine application had depressant effects on spontaneous activity, as demonstrated in Fig. 2. The effects of berberine (3 or 30 μM) on the AP parameters in the normal SA node pacemaker cells are shown in Table I. Compared with the control group, berberine (3 or 30 μM) significantly decreased the DDR and rate of pacemaker firing (RPF), and the changes in the RPF induced by berberine paralleled those in the DDR. Meanwhile, the amplitude of the AP and the maximal diastolic potential decreased as a result of berberine treatment. The above effects occurred following 5-10 min of berberine superfusion and reached their peak within 15-20 min.

Electrophysiological properties of hHCN4 channels heterologously expressed in xenopus oocytes. Xenopus oocytes were utilized as a heterologous expression system and the actions of berberine on the expression of hHCN4 were analyzed. For the voltage-clamp recordings, the hHCN4 current was elicited by hyperpolarization pulses of 4,000 ms from a holding potential of -60 to -150 mV in 10 mV decrements at 0.1 Hz and then



Berberine

Figure 1. Chemical structure of berberine.

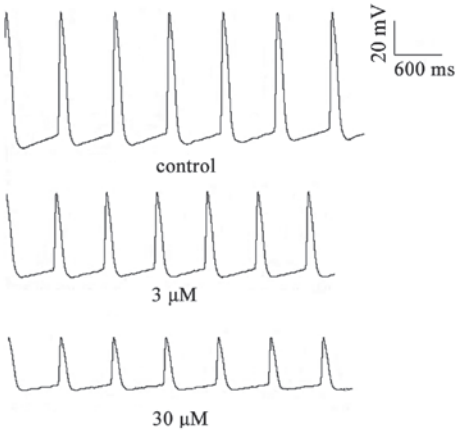


Figure 2. Representative traces of the spontaneous action potential represented prior to (control), and following 3 or 30 μM berberine in rabbit sinoatrial node pacemaker cells.

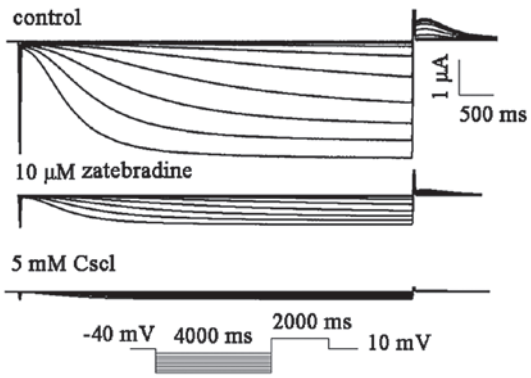


Figure 3. Heterologous expression of hHCN4 channels in the xenopus oocytes, representative current traces of HCN4 prior to (control) and following 10 μM zatebradine or 5 mM CsCl as indicated, respectively. The voltage protocol is shown at the bottom of the figure.

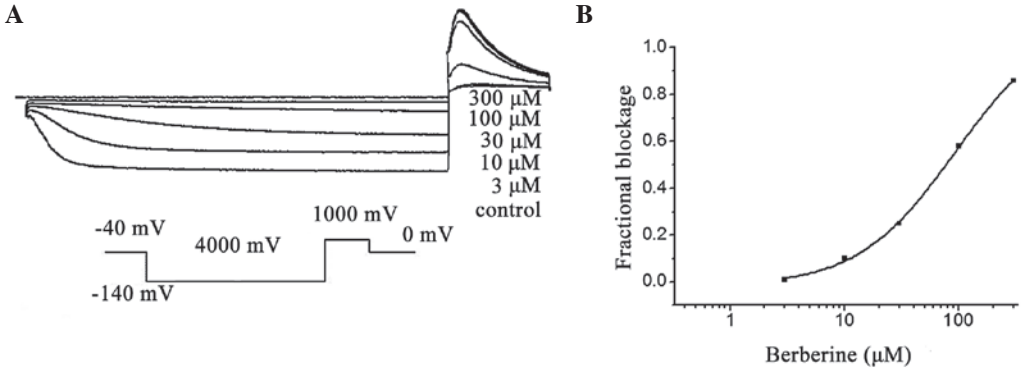


Figure 4. Concentration-dependent blockage of berberine on hHCN4 currents in xenopus oocytes. (A) Original current tracing of the f-channel was superimposed prior to (control) and following superfusion of berberine (1 to 300 μM). (B) Concentration-response curve was plotted based on data from panel A and fitted by the Hill equation, and the IC_{50} values were calculated.

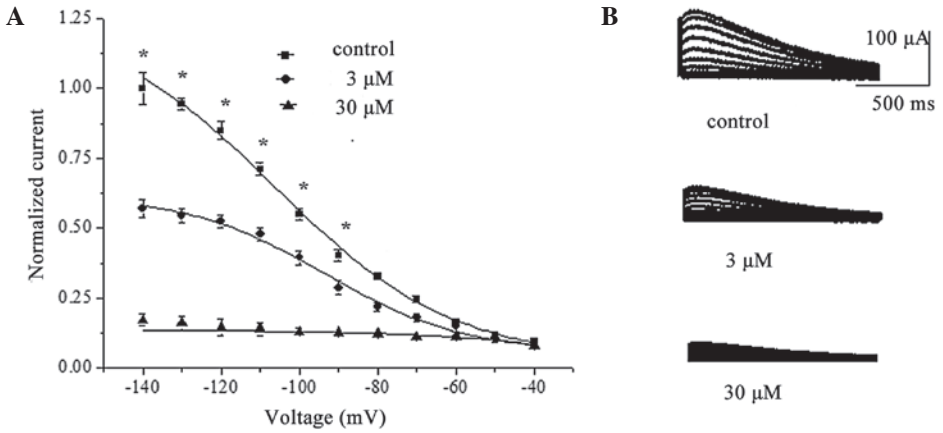


Figure 5. Effects of berberine on voltage dependence of activation of hHCN4 currents. (A) Normalized activation curves of HCN4 currents expressed in the oocytes were plotted in the ND96 solution and in the presence of 3 or 30 μM berberine. Tail currents were normalized to the peak tail currents in the control condition for each oocyte and the data were fitted with a Boltzmann function. (B) Current traces of hHCN4 channels expressed in the xenopus oocytes were superimposed prior to (control) and following superfusion of berberine (3 or 30 μM). *P<0.05, compared with the control.

clamped back to 10 mV for 2,000 ms (Fig. 2). Thereafter, selective and non-selective f-channel blockers, zatebradine and CsCl, were utilized to confirm the biophysical properties of the HCN channel. The HCN4 (n=4) currents were readily and completely blocked by 5 mM CsCl. By contrast, 10 μM zatebradine markedly inhibited the hHCN4 currents by 82.1±8.4% (n=4; Fig. 3).

Concentration-dependent blockage of hHCN currents by berberine. In the present study, it was identified that hHCN4 currents were inhibited by berberine in a concentration-dependent manner at the investigated test potential of -110 mV (Fig. 4A). Superfusion of berberine (1 to 300 μM) reduced the normalized Istep at voltages ranging from -100 to -140 mV, with the most pronounced effects observed at the more hyperpolarized voltages (n=8; P<0.05). Fig. 4 also depicts the correlation between the decreased fraction of the hHCN4 current and the concentration of berberine at -120 mV, with an IC_{50} value of 32.3±2.1 μM and a Hill coefficient of 1.5±0.2 (n=8; Fig. 4B).

Berberine increases the HCN4 current values of τactivation and τdeactivation and slows the kinetics of the hHCN4 channel. In addition to the inhibitory effect of berberine on current amplitude in HCN4 channels demonstrated above, berberine also modulated HCN4 channel current kinetics. The representative traces of the hHCN4 current and the expanded traces of the outward tail current prior to (control) and following 3 or 30 μM berberine treatment are illustrated in Fig. 5A, and the amplitudes of the measured tail currents 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 HCN4-channel activation. Superfusion of berberine (3 or 30 μM) reduced the normalized Itail at voltages ranging from -90 to -140 mV, with more pronounced effects at the more hyperpolarized voltages (n=8; P<0.05; Fig. 5B). Furthermore, the average value for $V_{1/2}$ was -102.7±1.9 mV under the control conditions and -93.8±1.7 mV (n=8; P<0.05) or -80.1±2.4 mV (n=8; P<0.05) following the addition of berberine (3 or 30 μM). The slope factor (k) of the activation curve was decreased from 19.0±1.9 to 14.7±1.8 mV and 12.5±2.3 mV in the presence of berberine at 3 and 30 μM, respectively.

In accordance with the more negative Vt caused by berberine treatment, the activation of HCN4 channels was significantly easier. In the presence of berberine, τactivation

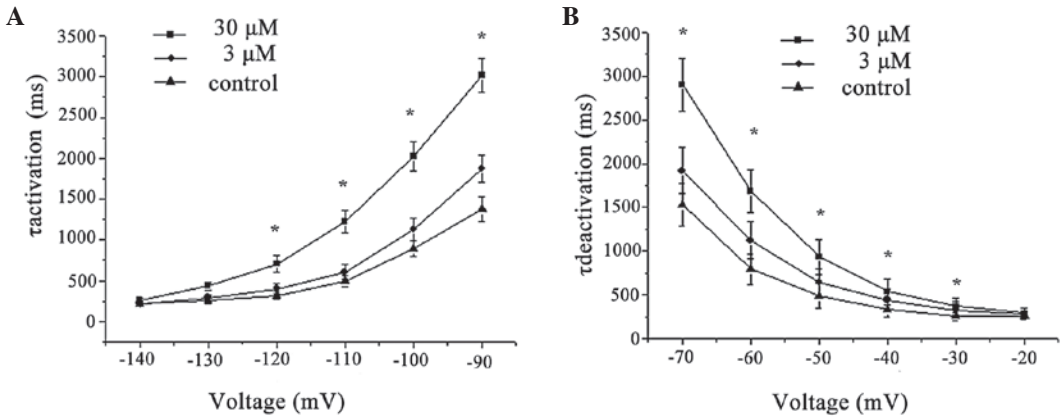


Figure 6. Berberine accelerates the activation and deactivation kinetics of hHCN4 currents in xenopus oocytes. (A) Time constants for activation of hHCN4 currents at Vt were assessed between -140 and -90 mV. (B) Time constants for deactivation of hHCN4 currents corresponding to Vt were assessed between -70 and -20 mV. *P<0.05, compared with the control. Vt, test potential; τ, time constant.

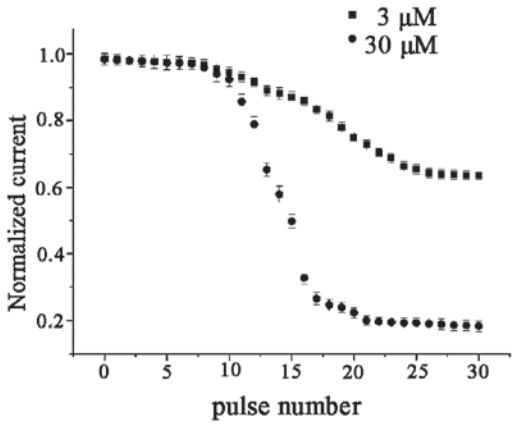


Figure 7. Blockage of hHCN4 channels by berberine was use-dependent. The superimposed tracings of HCN4 in the presence of 3 or 30 μM berberine are shown. The normalized currents of HCN4 were plotted against each pulse number.

was significantly increased in the potential channel from -140 mV to -90 mV, and with Vt becoming more negative, this change was more evident. The values of τactivation were markedly increased by berberine at 3 and 30 μM, from 504.6±39.8 ms (n=8) to 588.4±21.7 ms (3 μM, n=8, P<0.05) and 1176.4±57.3 ms (30 μM, n=8, P<0.05), respectively, at a Vt of -110 mV. The time constant for the current deactivation at 10 mV corresponding to a test potential of -120 mV was 504.6±39.8 ms (n=8) in the control and 588.4±21.7 ms (3 μM, n=8, P>0.05) and 1176.4±57.3 ms (30 μM, n=8, P<0.05) in the presence of berberine at 3 and 30 μM, respectively (Fig. 6).

Use-dependent blockage of hHCN4 currents by berberine in xenopus oocytes. To study the use-dependent blockage of the hHCN4 current by berberine, a standard activation/deactivation protocol of 2,000 ms pulse from a holding potential of -30 mV to -100 mV followed by a 1,000 ms depolarizing pulse to 0 mV at a rate of 0.25 Hz was used. The HCN currents, measured at the end of -100 mV, were plotted as a function of the pulse number (Fig. 7). During the train pulse stimulation there was no decline in the amplitude of the hHCN4 currents in the absence of berberine. In the absence of berberine, hHCN4 currents generated by 30 pulse burst stimulations were essentially identical. Further studies demonstrated that hHCN4 currents appeared to exhibit a gradual decline following superfusion with berberine (1-300 μM), until the amplitude of HCN currents reached a steady state. Furthermore, compared with the control conditions, superfusion with 30 μM berberine may have no marked blocking effect on the amplitude of hHCN4 currents at the first pulse stimulation, which may be considered as the control pulse. Following berberine application at the holding potential, a significant decline in the amplitudes of the hHCN4 currents occurred. From the results, it was identified that the higher concentration of the drug (30 μM) had faster kinetics of blockage than the lower concentration (3 μM).

Discussion

In the present study, it was identified that the partial depression of If by berberine was parallel to the decrease of the slope of DDR and the reduction of the firing rate of the SAN cells by the intracellular microelectrode technique. This indicated that the negative chronotropic effect of berberine was mainly through its inhibition of the If. Next, the effects of berberine on the hHCN4 channels expressed in xenopus oocytes, which were able to generate the If of the SAN, were characterized by the standard two-microelectrode voltage-clamp technique. The results were as follows: i) Berberine decreased the rate of spontaneous RPF and the DDR of the SAN pacemaker cells; ii) hHCN4 channel blockage by this drug was concentration-dependent; iii) berberine markedly shifted the activation and deactivation curve of the hHCN4 currents towards more negative potentials and markedly slowed the kinetics of the activation and deactivation of hHCN4 channels; iv) berberine blocked the hHCN4 channel current in a use-dependent manner.

Previous studies have indicated that berberine decreases the frequency of the spontaneous contractions of rabbit sinoatrial cells and guinea pig right atria in a concentration-dependent manner (15,21), Riccioppo (15) hypothesized that this decrease in the spontaneous contraction frequency was accompanied by a depression of the phase 4 depolarization, without significant changes in the other parameters of the nodal AP. However, the present study identified that berberine potently decreased

the spontaneous firing and increased the AP duration of SAN pacemaker cells in rabbits. Next, the study focused on the action of berberine on the most prevalent members of the HCN family in cardiac SA node cells, the hHCN4 subunits, which were heterologously expressed in xenopus oocytes. Individual HCN subunits have six transmembrane segments (S1-S6). The highly positively charged S4 domain is the putative voltage sensor, and the P domain between the S5 and S6 domains acts as the ion conducting pore and selectivity filter C (22-25). The allosteric hypothesis proposed by Altomare *et al* (26) suggested that the probability of a channel opening increased every time one voltage sensor switched to the activated state. The results of the present study demonstrated that berberine principally affected the activation of the HCN4 channel, which decreased the probability of channel opening. This may be one reason why berberine is able to inhibit the HCN channel current. By contrast, the fully activated current relation of native HCN channels was linear and reversed at potentials compatible with permeability to both Na^+ and K^+, with a preference for a P_{Na}/P_K ratio ranging from 0.25 to 0.41 (5). Therefore, K^+ current inhibition by berberine may lead to the reduction of the HCN channel current.

In addition, it was hypothesized that the effect of berberine on the hHCN4 currents in xenopus oocytes may occur in a use-dependent manner. By the application of a train voltage pulse stimulation, it was observed that the inhibitory action of berberine on the HCN current was progressively strengthened, until reaching a steady state. There was incomplete blockage during the first pulse and incomplete recovery during the interval between the pulses until a steady state was reached. This demonstrated that through repeated stimulation, inhibition of the HCN4 current by berberine increased, i.e., it reduced the number of channel openings per unit of time. The repeated stimulation led to the channels combining with the drug in an inactive state.

In recent studies, it has been well established through the cardiac-specific and inducible knockout model of HCN4 and HCN4 channel mutation models, that the HCN4 current provides a fundamental contribution to basal heart rate maintenance and modulation, as its removal led to basal bradycardia and a markedly reduced response to sympathetic stimulation. Furthermore, HCN4 ablation in the model by Baruscotti *et al* (25) caused progressive development of deep bradycardia (~50% of the original rate), as recorded by telemetry, eventually leading to an atrioventricular (AV) block and heart arrest in ~5 days. These data revealed that the expression of HCN4 in the SAN is a direct determinant of the heart rate and that removal of cardiac HCN4 channels from pacing tissue is lethal. Clinical trials and animal studies have suggested a number of beneficial effects of berberine on cardiovascular performance. In one study, berberine prevented ischemia-induced ventricular tachyarrhythmias, enhanced the force of cardiac contractions and decreased peripheral vascular resistance and blood pressure (27). Previously, it has been noted that paroxysmal fibrillation may be triggered by ectopic firing foci located in the pulmonary veins and that slow diastolic depolarization and HCN4 proteins have been observed in the pulmonary veins (28,29). Other studies observed that, in cardiac hypertrophy and heart failure, HCN4 is upregulated in the atrial and ventricular myocardium and may therefore contribute to ectopic beat formation and enhanced electrical activity (10). Therefore, the overexpression of If may be an important trigger of arrhythmogenic activity in the hypertrophied heart (30-33). Therefore, inhibition of the pacemaker current by berberine in these extranodal areas may contribute to its well known antiarrhythmic actions.

Acknowledgements

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