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Novel electropharmacological activity of amiodarone on human HCN channels heterologously expressed in the *Xenopus* oocytes

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ABSTRACT

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels underlie the pacemaker currents (I_f) in cardiac cells. The objectives of this study were to investigate the electropharmacological activity of amiodarone on human HCN channels heterologously expressed in *Xenopus laevis* oocytes. hHCN channels were expressed in oocytes and studied using the standard two-electrode voltage-clamp techniques. The results show that amiodarone blocks hHCN channels heterologously expressed in the *Xenopus* oocytes in a concentration- and use-dependent manner, but doesn't modify the voltage dependence of activation and reversal potentials. And the removal of blockage of HCN channels by amiodarone was favored by inward current flow, not by hyperpolarizing potential. Characteristics of blockage on hHCN channels were consistent with those of amiodarone as "trapped" drugs on human ether-a-go-go-related gene (HERG) channels. These results will be useful for elucidating the potentially antiarrhythmic mechanism of amiodarone.

1. Introduction

The hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels, responsible for the cardiac pacemaker current (I_f), are widely expressed on cardiac myocytes, where they fulfill important physiological functions. The HCN mediated currents play a key role in the generation and regulation of diastolic depolarization which controls the spontaneous rate in sinoatrial node myocytes (DiFrancesco, 1993). Recently, four mammalian HCN isoforms, respectively termed HCN1–4, have been cloned (from human, rat, rabbit and mouse) (Kaupp and Seifert, 2001; Ludwig et al., 1998). Among them, three isoforms (HCN1, 2 and 4) have been identified in cardiac tissues, with HCN4 being main subtype present in sinoatrial node (Ishii et al., 1999; Ludwig et al., 1998; Shi et al., 1999). In addition, the expression of I_f in nonpacemaker cardiac myocytes has been demonstrated in ventricles and in atria in the past several years, including HCN2 and 4 as the dominant subtypes (Fernandez-Velasco et al., 2003; Hoppe and Beuckelmann, 1998; Ludwig et al., 1999; Moosmang et al., 2001). It was speculated that I_f could play a role in causing ectopic automaticity, particularly during several pathological conditions such as heart failure (Cerbai et al., 1997; Hoppe et al., 1998; Stillitano et al., 2008; Zorn-Pauly et al., 2004).

Since 1970s, amiodarone has been widely believed to be the most effective antiarrhythmic drug, and it has been proven to be the only antiarrhythmic agent to reduce arrhythmic death in patients with frequent ventricular ectopy postmyocardial infarction (Cairns et al., 1997) and it has appeared to have superior efficacy to other antiarrhythmic drugs for sinus rhythm maintenance in patients with atrial fibrillation (Nattel, 1995). According to early researches on the cellular actions of amiodarone, it was classified in Class III antiarrhythmic agent by Vaughan Williams' classification (Larsen et al., 2000). However, amiodarone, as a multi-ion channels blocker, has complex electropharmacological profiles, such as significant blockade on delayed rectifier potassium current (I_K), including fast component (I_{Kr}) and slow component (I_{Ks}), and nonselective blocking effects on other potassium currents, including transient outward current (I_{to}), inward rectifier potassium current (I_{K1}) and acetylcholine-sensitive muscarinic potassium current ($I_{K,ACh}$) (Kodama et al., 1997). In addition, this drug also can inhibit both inward sodium current (I_{Na}) and calcium current (I_{Ca}) enhanced in a use- and voltage-dependent manner (Kodama et al., 1997). In an electropharmocological research, the properties of amiodarone on human ether-a-go-go-related gene (HERG) channels, with which the HCN channels share several structural similarities (Baruscotti et al., 2005) has been certified, with amiodarone binding to inner channel, being trapped during channel closure and dissociating from the open channel state (Stork et al., 2007).

Lately, Tamura et al. reported that amiodarone had inhibitory effects on rabbit HCN4 mediated currents expressed in HEK 293 cell,

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Tamura et al. (2009) and Li HX et al. demonstrated the current density of I_f was decreased by this drug in the neonatal rat ventricular myocytes (Li et al., 2007). Although these results indicated that amiodarone might affect the function of human HCN channels, the mechanism of its action has not been elucidated. In this study, we were interested in investigating whether amiodarone also affects human HCN1, 2 and 4 mediated currents, present in cardiac tissue, heterologously expressed in *Xenopus* oocytes, and exploring the mechanism behind it. By doing so, we hoped to provide novel insights into the possible antiarrhythmic mechanisms of amiodarone.

2. Materials and methods

2.1. Molecular biology

This study was approved by the Animal Research Committee of the First Clinic College of Wuhan University.

Wild-type (WT) human HCN1, 2 and 4 (hHCN1, 2 and 4) cDNA inserted into the pcDNA3 vector were kindly provided by Professor Ludwig A and Stieber J (Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany). Complementary RNAs (cRNAs) for injection into oocytes were prepared with mMESSAGE mMACHINE® T7 Kit (Ambion, Austin, TX, USA) after linearization of the expression construct with XbaI (Takara, Kyoto, Japan). RNA quality was checked by gel electrophoresis, and its concentration was quantified by UV spectroscopy (UV-2201, SHIMADZU, Japan).

2.2. Voltage clamp of Xenopus oocytes

Xenopus frogs were anesthetized by cooling on crushed ice for 30–40 min. Ovarian lobes were digested with 1.5 mg/ml type IA collagenase (Sigma Chemical, St Louis, MO, USA) in Ca^{2+}-free ND96 solution for 1 h to remove follicle cells. Stage IV and V *Xenopus* oocytes were injected with 30 nl (1 μg/μl) of hHCN1, 2 and 4 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 before being used in voltage clamp experiments. ND96 solution contained (in mM):96 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES, titrated to pH 7.4 with NaOH.

Recordings were performed 2–12 days after oocytes injection. A standard two-microelectrode voltage-clamp technique was used to record currents at 21–23 °C. The glass microelectrodes were filled with 3 M KCl to obtain a resistance of 1–3 MΩ. Oocytes were clamped with a standard and advanced two-microelectrode voltage-clamp amplifier (DAGAN CA-1B, Minneapolis, MN, USA) and pCLAMP software (Axon Instruments, Foster City, CA, USA). Oocytes were superfused with ND96 solution at a rate of 2.0 ml/min. Control currents were recorded repeatedly at 1 min intervals, with drugs being applied until the control currents achieved a stable level. In low (30 mM) Na^+ solution, Na^+ was replaced by an equimolar amount of choline chloride; and in high (20 mM) K^+ solution, K^+ was elevated to 20 mM in ND96.

2.3. Drugs

Amiodarone, CsCl, zatebradine and HEPES were purchased from Sigma Chemical (Saint Louis, Missouri, USA). Amiodarone was dissolved in absolute ethanol at a concentration of 100 mM and then added to the bath solution containing bovine serum albumin (0.1%). Zatebradine was prepared as 50 mM solution in distilled water. The stock solution was diluted with cell external solution to reach the desired final concentration before experiments and the drug was applied in cumulative doses during experiments. In order to keep the concentrations of the various types of ions and drugs constant, we strictly controlled the perfusion rate by using the perfusion device BPS-4 (ALA Scientific Instruments, Inc., Westbury, NY, USA) and a constant-flow pump.

2.4. Data analysis

All data were stored on the computer hard disk and analyzed off-line using Clampfit 10.0 (Axon Instruments,USA) and Origin 7.0 software (Origin Laboratory, Northampton, MA, USA).

The amplitude of HCN mediated currents was defined as the time-dependent component (I_{step}) at the end of hyperpolarizing pulses in various durations (1000–20000 ms) or peak current (I_{tail}) at the beginning of depolarizing pulses. To construct I–V relationships, currents were measured before drug and the plotted as a function of test potential (V_t). The voltage dependent of HCN current activation was determined by analysis of I_{tail} measured at depolarizing potential. All tail current amplitudes from an individual oocyte were normalized to its own maximum (I_{max}), plotted as a function of V_t, and fitted again with a Boltzmann function: $I/I_{max} = 1/[1+\exp((V_t - V_{1/2})/k)]$ to determine the values of the half-point ($V_{1/2}$) and the slope (k). The time constants for HCN currents activation or deactivation ($\tau_{activation}$ or $\tau_{deactivation}$) at different V_t were determined using the 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 by using Hill equation in the form, $f = 1/[1+(IC_{50}/D)^h]$, where f was the decrease in HCN currents, expressed as percentage change compared with the control values, IC_{50} was the concentration of amiodarone for half-maximum blockade, D was the concentration of amiodarone, and h was the Hill coefficient.

Data were presented as means ± S.D. Student's t-test was used for statistical analysis of the paired observations, and an analysis of variance (ANOVA) was performed to test the difference among the groups. A P value <0.05 was considered statistically significant.

3. Results

3.1. Electrophysiological properties of hHCN channels expressed in the Xenopus oocytes

Heterologous expression of hHCN cRNAs in the *Xenopus* oocytes resulted in the formation of functional homomeric ion channels, which display the slowly activating inward currents upon hyperpolarization of the cell membrane. Three kinds of membrane currents were elicited by hyperpolarizing pulses of 4000 ms from a holding potential of −30 mV to −140 mV in 10 mV decrements at 0.1 Hz and then clamp back to 0 mV for 1000 ms (Fig. 1). The amplitudes of HCN1, 2 and 4 currents were 1.4 ± 0.5 μA (n = 13), 1.1 ± 0.4 μA (n = 13) and 0.5 ± 0.2 μA (n = 8) in the oocytes. Thereafter, we examined effects of nonselective and selective f-channel blockers, CsCl and zatebradine, on the HCN currents. The HCN1 (n = 4), 2 (n = 4) and 4 (n = 3) currents were mainly and readily blocked by 5 mM CsCl. In addition, 10 μM zatebradine, potently inhibited the HCN1, 2 and 4 currents by 74.7 ± 12.1% (n = 4), 69.2 ± 16.3% (n = 4) and 87.6 ± 8.3% (n = 3), as shown in Fig. 1.

The activation curves of HCN currents were plotted by amplitudes of tail current at depolarizing potential to 0 mV. From HCN1 to 4, the activation curves were shifted toward negative voltages (Fig. 1D), the $V_{1/2}$ for the HCN1, 2 and 4 currents being −53.2 ± 6.5 mV (n = 10), −76.9 ± 7.1 mV (n = 10) and −100.9 ± 5.8 mV (n = 6), respectively. When the oocytes were clamped to hyperpolarizing potential from −70 mV to −140 mV, the activation of HCN currents was speeded. The $\tau_{activation}$ of HCN1, 2 and 4 currents ranged from 583.6 ± 54.9 ms to 55.6 ± 4.8 ms (n = 10), 2257.8 ± 250.8 ms to 378.8 ± 42.1 ms (n = 10) and 9901.8 ± 681.1 ms to 836.6 ± 69.7 ms (n = 6), respectively (Fig. 2D), indicating that HCN1 was the fastest isoform followed by HCN2 and HCN4 in the activation kinetics.

In aspect of the deactivation kinetics, a double-pulse mode was applied, including conditioning pre-pulse of 4000 ms in width at