Effects of Ranolazine on Cloned Cardiac Kv4.3 Potassium Channels

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ABSTRACT

The effects of ranolazine, an antianginal drug, on potassium channel Kv4.3 were examined by using the whole-cell patch-clamp technique. Ranolazine inhibited the peak amplitude of Kv4.3 in a reversible, concentration-dependent manner with an IC50 of 128.31 μM. The activation kinetics were not significantly affected by ranolazine at concentrations up to 100 μM. Applications of 10 and 30 μM ranolazine had no effect on the fast and slow inactivation of Kv4.3. However, at concentrations of 100 and 300 μM ranolazine caused a significant decrease in the rate of fast inactivation, and at a concentration of 300 μM it caused a significant decrease in the rate of slow inactivation, resulting in a crossover of the current traces during depolarization. The Kv4.3 inhibition by ranolazine increased steeply between −20 and +20 mV. In the full activation voltage range, however, no voltage-dependent inhibition was found. Ranolazine shifted the voltage dependence of the steady-state inactivation of Kv4.3 in the hyperpolarizing direction in a concentration-dependent manner. The apparent dissociation constant (K) for ranolazine for interacting with the inactivated state of Kv4.3 was calculated to be 0.32 μM. Ranolazine produced little use-dependent inhibition at frequencies of 1 and 2 Hz. Ranolazine did not affect the time course of recovery from the inactivation of Kv4.3. The results indicated that ranolazine inhibited Kv4.3 and exhibited a low affinity for Kv4.3 channels in the closed state but a much higher affinity for Kv4.3 channels in the inactivated state.

Introduction

Ranolazine, a piperazine derivative, is a compound with a structure similar to that of local anesthetics that share an N-diethyl group as a common structure (Wang et al., 2008). Various local anesthetics are known to be effective inhibitors of Kv channels. Lidocaine suppresses transient outward K+ currents in rat inferior colliculus neurons, inward-rectifying K+ currents in chick ventricular myocytes, and cloned cardiac Kv4.2 channel currents expressed in Xenopus laevis oocytes (Josephson, 1988; Rolf et al., 2000; Yu and Chen, 2008). In addition, ropivacaine and bupivacaine concentration-dependently inhibit cloned cardiac Kv4.3 currents stably expressed in CHO cells (Friederich and Solth, 2004; Solth et al., 2005). Ranolazine is widely used for the treatment of angina, but its mechanism of action is not well understood (Pepine and Wolff, 1999; Chaitman, 2006). The proposed mechanism of action for ranolazine is the inhibition of the late Na+ currents in cardiac myocytes (Wang et al., 1997; Pepine and Wolff, 1999). Similar to other local anesthetics, ranolazine displays a broad inhibitory effect on several different cardiac ion channels including Na+ and Ca2+ channels (Antzelevitch et al., 2004a; Schram et al., 2004; Rajamani et al., 2009). Although most studies of ranolazine have focused on the Na+ channels, ranolazine has also been reported to inhibit HERG as well as rapidly activating delayed rectifier and slowly activating delayed rectifier K+ currents in different cell types (Schram et al., 2004; Rajamani et al., 2008). However, ranolazine had little or no effect on the transient outward K+ currents in canine ventricular myocardial cells (Antzelevitch et al., 2004a). Ranolazine is expected to exert complex electrophysiological effects in the heart, because Kv channels play an important role in determining the duration and shape of the action potential there (Snyders, 1999). Kv4.3 is a member of the Shal family of Kv channels and generates a rapidly activating and rapidly inactivating current, contributing to the transient outward K+ current in the heart (Dixon et al., 1996; Ohya et al., 1997). This channel may be an important therapeutic target for certain antiarrhythmic drugs (Wulff et al., 2009). However, only limited

ABBREVIATIONS: Kv, voltage-gated K+ channel; CHO, Chinese hamster ovary; HERG, human ether-a-go-go related gene.
information about the effect of ranolazine on the Kv4.3 channel is available. Therefore, the present study was performed to investigate in detail the effects of ranolazine on cloned cardiac Kv4.3 channels.

Materials and Methods

Kv4.3 cDNA was subcloned into a mammalian expression vector, pcR3.1 (Invitrogen, Carlsbad, CA), as described previously (Ohya et al., 1997; Ahn et al., 2006). CHO cells (American Type Culture Collection, Manassas, VA) were maintained in Iscoves' modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine, and 0.01 mM thymidine in a humidified 5% CO2 incubator at 37°C. Kv4.3 constructs were stably transfected into CHO cells by using Lipofectamine reagent (Invitrogen). The transfected CHO cells in Iscoves' modified Dulbecco’s medium containing 1 mg/ml G418 (Geneticin; Invitrogen) were incubated for 48 h under a humidified 5% CO2 incubator at 37°C. Whole-cell Kv4.3 currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) at room temperature (22–24°C). Patch pipettes were pulled from glass capillary tubing (PG10165-4; World Precision Instruments, Inc., Sarasota, FL) by using a puller (model P-97; Sutter Instrument Company, Novato, CA). The tip resistances of the recording pipettes in the bath solution were 2 to 3 MΩ. Whole-cell capacitive currents were compensated with analog compensation, and series resistance compensation (80%) was used if the current exceeded 1 nA. Data acquisition and analysis were performed with an IBM (White Plains, NY) Pentium computer, using pClamp 10.0 software (Molecular Devices). The bath solution contained 140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES and was adjusted to pH 7.3 by using NaOH. The internal pipette solution contained 140 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM EGTA and was adjusted to pH 7.3 by using KOH. The measured osmolality of the solutions was 300 to 340 mOsm. Ranolazine (Sigma, St. Louis, MO) was dissolved in distilled water to make a stock solution and then appropriately diluted with a bath solution. The Origin 8.0 software program (OriginLab Corp., Northampton, MA) was used for the analysis. The concentration-response data were fitted to the Hill equation: $y = I/I_{\text{max}} = 1/[1 + (D/IC_{50})^n]$, where IC_{50} is the concentration of ranolazine required to produce 50% inhibition, [D] is the concentration of ranolazine, and n is the Hill coefficient. The voltage dependence for steady-state inactivation was investigated by using a two-pulse voltage protocol; currents were measured by 500-ms depolarizing pulses to +40 mV, while 1-s preconditioning pulses were varied from −110 to 0 mV in 10-mV steps at 10-s intervals in the absence and presence of the drug. The resulting steady-state inactivation data were fitted to the Boltzmann equation: $(I - I_c)/(I_{\text{max}} - I_c) = 1/[1 + \exp(V - V_{1/2})/k]$, in which $I_{\text{max}}$ represents the current measured at the most hyperpolarized preconditioning pulse, $I_c$ is the nonzero current that was not inactivated at the most depolarized preconditioning pulse, k is the slope factor, V is the test potential, and $V_{1/2}$ is the potential at which the conductance was half-maximal. We eliminated the nonzero residual current by subtracting it from the actual value. Data are expressed as the mean ± S.E. A one-way analysis of variance, followed by a Dunnett’s test, was used to evaluate the statistical significance of the observed differences. A value of $P < 0.05$ was considered to be statistically significant.

Results

Concentration-Dependent Inhibition of Kv4.3. Representative Kv4.3 current tracings elicited by 500-ms depolarizing pulses to +40 mV in the absence and presence of ranolazine are shown in Fig. 1A. Under control conditions, the Kv4.3 current rose rapidly and was then rapidly inactivated as described previously (Ohya et al., 1997; Ahn et al., 2006; Kim et al., 2011). Ranolazine significantly decreased the peak amplitude of Kv4.3 in a concentration-dependent manner. The concentration-response curve for ranolazine on Kv4.3 at +40 mV is shown in Fig. 1B. The normalized inhibition was plotted as a function of drug concentration and fit to a Hill equation, giving an IC_{50} of 128.31 ± 8.54 μM and a Hill coefficient of 1.18 ± 0.012 (n = 7). The time-dependent kinetics of Kv4.3 was determined in the presence of ranolazine (Fig. 2). The time constants of activation were calculated by fitting a single exponential to the final 50% of activation (Snyders et al., 1993; Snyders and Yeola, 1995). Under control conditions, the time constant of activation was 0.55 ± 0.09 ms (n = 6). In the presence of ranolazine, the time constants of activation were 0.58 ± 0.15, 0.51 ± 0.13, and 0.61 ± 0.12 ms (n = 6) for 10, 30, and 100 μM, respectively, indicating that the kinetics of activation were not significantly affected by ranolazine. The time course of inactivation of Kv4.3 at +40 mV under control conditions was fitted to a double exponential function, with a fast time constant (τ_f) of 23.60 ± 1.87 ms and a slow time constant (τ_s) of 119.60 ± 5.13 ms (n = 6). At concentrations of 10 and 30 μM, ranolazine had no effect on the fast and slow inactivation of Kv4.3. However, at concentrations of 100 and 300 μM ranolazine caused a significant decrease in the rate of fast inactivation, and at a concentration of 300 μM it caused a significant decrease in the rate of slow inactivation, as shown in Fig. 2B. In the present study, the addition of high concentrations (100
and 300 µM of ranolazine caused an inhibition of the peak amplitude of Kv4.3 and decreased the rates for current inactivation, which resulted in a crossover of the current traces during depolarization (Fig. 2A).

**Voltage-Dependent Inhibition of Kv4.3.** Figure 3A shows the representative Kv4.3 currents evoked by a series of 500-ms depolarizing pulses between −70 and +60 mV from a holding potential of −80 mV before and after application of ranolazine. Current-voltage relationships in the absence and the presence of ranolazine for peak currents are represented in Fig. 3B. To quantify the voltage dependence of inhibition, the relative current inhibition was plotted as a function of potential (Fig. 3C). The Kv4.3 currents began to activate at −30 mV, and the conductance was fully saturated at +20 mV. In the presence of ranolazine, the inhibition was increased between −20 and +20 mV, which corresponded to the voltage range of channel activation. However, inhibition in the voltage range between +20 and +60 mV, where the channels are fully activated, was not voltage-dependent.

**Shift of the Steady-State Inactivation Curves of Kv4.3.** The voltage dependence of steady-state inactivation in the presence of ranolazine was evaluated by using a conventional two-pulse protocol (Fig. 4A). Under control conditions, the half inactivation ($V_{1/2}$) and $k$ values were $-48.75 ± 3.13$ and $4.82 ± 0.31$ mV ($n = 6$), respectively (Fig. 4B). Ranolazine significantly shifted the inactivation curve ($V_{1/2}$) by $-10.45 ± 1.23$ mV ($n = 6$, $P < 0.05$). The dotted line represents the activation curve of Kv4.3 channels under control conditions.

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**Fig. 2.** Effects of ranolazine on activation and inactivation kinetics. A, superimposed Kv4.3 currents under control conditions and in the presence of ranolazine. Ranolazine decreased the peak amplitude of Kv4.3 and slowed the inactivation kinetics, resulting in a crossover of currents. The dotted lines indicate the zero current level. B, the activation time constants under control conditions and in the presence of ranolazine were calculated from single exponential fits of the currents during depolarization at +40 mV. The inactivation time constants of the fast and slow components were obtained from double exponential fits ($n = 6$; $*, P < 0.05$). Data are expressed as the mean ± S.E.

**Fig. 3.** Effect of ranolazine on current-voltage relationships. A, the representative whole-cell Kv4.3 current traces under control conditions and in the presence of ranolazine. B, current-voltage curves for ranolazine-induced Kv4.3 currents. The data were taken from the peak amplitude of Kv4.3 currents under control conditions and in the presence of ranolazine. C, the voltage-dependent inhibition of Kv4.3 by ranolazine. In the voltage range for channel activation between −20 and +20 mV, inhibition of Kv4.3 increased and was significantly different from inhibition at −20 mV ($n = 6$, $*, P < 0.05$). The dotted line represents the activation curve of Kv4.3 channels under control conditions. Data are expressed as the mean ± S.E.
to a hyperpolarized potential in a concentration-dependent manner (−70.88 ± 2.54 mV at 100 μM and −79.23 ± 2.73 mV at 300 μM; n = 6; P < 0.05). However, no significant change in k for the curve in the presence of ranolazine was found (4.92 ± 0.38 mV at 100 μM and 4.52 ± 0.27 mV at 300 μM; n = 6). The affinity of ranolazine for the inactivated state (K_i) of Kv4.3 was calculated on the basis of the shift of V_1/2 (Bean et al., 1983): ΔV = k ln [(1 + D/K_i)/(1 + D/K_R)], where D is the ranolazine concentration responsible for the steady-state inactivation shift and K_R is the affinity for the closed state (IC_50) taken from the concentration-response curves (Lenkowski et al., 2004). At concentrations of 100 and 300 μM the theoretical values of K_R were 0.50 ± 0.01 and 0.13 ± 0.02 μM (n = 6), respectively. The average value for K_R was 0.32 ± 0.03 (n = 12).

Use-Dependent Inhibition of Kv4.3. Figure 5A shows the effects of 1- and 2-Hz trains of 10 depolarizing pulses under control conditions and after the addition of ranolazine. Currents were normalized to the first pulse, and the results are plotted in Fig. 5B. Under control conditions, the peak amplitude of Kv4.3 currents displayed a small decline at 1 Hz (4.8 ± 1.4%; n = 6). This percentage increased to 18.5 ± 2.5% (n = 6) at 2 Hz. In the presence of 100 μM ranolazine, the peak amplitude of Kv4.3 currents declined rapidly after the first pulse and reached a steady-state inhibition. The extents of the steady-state inhibition at frequencies of 1 and 2 Hz were 9.7 ± 2.4 and 22.4 ± 4.6%, respectively. These results suggest that ranolazine exhibited little use-dependent inhibition of Kv4.3.

Recovery Time Course from Inactivation of Kv4.3. The effect of ranolazine on the kinetics of Kv4.3 recovery from inactivation was studied by using a two-pulse protocol (Fig. 6A). Under control conditions, the recovery time course from the inactivation of Kv4.3 was best fit to a single exponential function with a time constant of 249.5 ± 21.6 ms (n = 6) (Fig. 6B). The recovery time course in the presence of 100 μM ranolazine was also best fit to a single exponential function with a time constant of 245.3 ± 24.7 ms (n = 6), indicating that ranolazine has no effect on recovery from the inactivation of Kv4.3 currents.

Reversible Inhibition of Kv4.3. To examine the reversibility of the effect of the drug, a time course for the alteration of steady-state currents produced by the drug and the ensuing drug washout was analyzed (Fig. 7A). The inhibitory effect of ranolazine was rapid, and the steady-state inhibition of Kv4.3 was reached within 30 s (Fig. 7B). After washout, the current recovered to 96.2 ± 3.1% (n = 6), indicating that the effect of ranolazine is completely reversible.

Discussion

The results reported here show that ranolazine inhibited the peak amplitude of Kv4.3 currents stably expressed in CHO cells in a reversible, concentration-dependent manner. The inhibitory effects of ranolazine, both a reduction in the peak Kv4.3 currents and a simple scale-down of these currents in general, can be defined as the inhibition of the closed state (Jacobs and DeCoursey, 1990). At concentrations of 10 and 30 μM, ranolazine had no effect on either theactivation or inactivation kinetics of the Kv4.3 channel. However, at concentrations of 100 and 300 μM, ranolazine decreased the rate of current inactivation, resulting in a crossover of the current traces obtained under the control conditions and after the addition of ranolazine (Fig. 2A). A possible explanation for this phenomenon is that ranolazine was released from the closed state of the channel during the late phase of the depolarizing pulse and the channel thus re-entered the open state. After the dissociation of the drug from the binding site of the Kv4.3 channel, Kv4.3 became inactivated at a normal rate and increased during the late phase of the depolarizing pulse, which resulted in the crossover phenomenon. Similar results have been reported for 4-aminopyrine on K^+ currents in squid giant axons (Yeh et al., 1976; Kirsch et al., 1986) and for riluzole on the Kv4.3 channel (Ahn et al., 2006). However, bupivacaine and ropivacaine accelerated the decline of the macroscopic current of Kv4.3 channels, which is a characteristic feature of open channel block, but increased the slow time constants of inactivation, resulting in the crossover of the currents (Friederich and Solth, 2004; Solth et al., 2005).

The present study found that ranolazine caused a concentration-dependent hyperpolarizing shift of the steady-state inactivation curves. This effect on the steady-state inactivation curves suggests that ranolazine bound to and stabilized...
the Kv4.3 channel in its inactivated state (Wang et al., 1995). Likewise, ranolazine interacted with the cardiac Na+/H+ currents in the inactivated state in canine myocytes and human embryonic kidney 293 cells and shifted the steady-state inactivation curve in a hyperpolarizing direction (Valdivia et al., 2005; Rajamani et al., 2009). In addition, ranolazine inhibited Kv4.3 more effectively over the channel opening voltage range. This result also reflects the voltage dependence of channel inactivation and results from the preferential interaction of the drug with the inactivated state of Kv4.3 (Caballero et al., 2003). Generally, use-dependent inhibition is interpreted as the interaction of the drug with the inactivated state of channel and reflects a fast binding rate during depolarization and a slow unbinding rate upon repolarization (Courtney, 1975; Butterworth and Strichartz, 1990; Slawsky and Castle, 1994). Ranolazine exhibited only little use-dependent inhibition, suggesting that ranolazine binds slowly to and unbinds rapidly from the inactivated state of Kv4.3 channels. Consistent with this view, ranolazine did not affect the time course of recovery from the inactivation of Kv4.3. Likewise, ranolazine did not show use-dependent block of HERG, suggesting that the drug may dissociate rapidly from the channel (Rajamani et al., 2008). On the other hand, ranolazine causes the use-dependent inhibition of the Na+ current and slows the recovery from inactivation (Rajamani et al., 2009). In the present study, repetitive depolarization resulted in little use-dependent inhibition of Kv4.3. One possible explanation is that the unblocking observed during depolarization was superimposed on the use-dependent inhibition induced by repeated depolarization, thus weakening this inhibition. However, the interaction of ranolazine with the Kv4.3 channel was voltage-independent between +20 and +60 mV, where the channels are fully activated. Ranolazine has a pK_a value of 7.2 and exists in both the charged and uncharged forms at physiological pH (Rajamani et al., 2008). Because interaction of the charged form with the binding site suggests an open-channel block mechanism (Snyders
Ranolazine, a piperazine derivative, is a compound with a structure similar to lidocaine, a local anesthetic, and is used for the treatment of angina (Pepine and Wolff, 1999). The underlying mechanism of the antianginal action of ranolazine is thought to be the selective inhibition of late Na⁺ currents (Wang et al., 1997; Pepine and Wolff, 1999; Rajamani et al., 2009). Thus, the inhibitory effect on Kv4.3 is not relevant to the pharmacological action of ranolazine for angina. In addition to its antianginal effects, an antiarrhythmic effect has been suggested (Eckhardt et al., 2008). Ranolazine reportedly inhibits several different types of K⁺ currents in addition to late Na⁺ currents. Ranolazine inhibits HERG, rapidly activating delayed rectifier, and slowly activating delayed rectifier K⁺ currents in canine atrial myocytes (Schram et al., 2004). However, ranolazine produces little or no inhibition of transient outward K⁺ currents and inward rectifier K⁺ currents in isolated canine ventricular myocytes (Antzelevitch et al., 2004a). Kv4.3 constitutes the α subunit that underlies the transient outward K⁺ currents in the human heart, an important determinant of cardiac action potentials (Dixon et al., 1996). Action potential duration depends on the balance between the inward and outward currents through the membrane in cardiac myocytes (Snyders, 1999). Whereas the inhibition of late Na⁺ currents would be expected to decrease the action potential duration, inhibition of Kv4.3 both prolonged the action potential duration and increased the refractory period in cardiac myocytes. Because ranolazine is not a selective inhibitor of Kv4.3, but also inhibits other ionic currents, such as Na⁺ and Ca²⁺ currents (Wang et al., 1997; Antzelevitch et al., 2004a; Schram et al., 2004), the overall impact of ranolazine on the action potential duration probably would result from the relative potencies of the effect on the ionic currents that are involved in cardiac action potentials. Although drugs that increase the ventricular action potential duration and the QT interval can produce significant ventricular proarrhythmias (Kass and Cabo, 2000), ranolazine is not reported to exert any significant early after depolarization or ventricular tachycardia, despite prolonging the duration of the action potential (Antzelevitch et al., 2004b; Wu et al., 2004). Functional knockout of the Kv4 α subunit leads to a marked increase in action potential duration in ventricular myocytes and a prolongation of the QT interval (Barry et al., 1998). It is noteworthy that these mice did not seem to develop arrhythmias. Kv4.3 protein is expressed in both the human atrium and ventricle, but the density of Kv4.3 is greater in the atrium (Gaboir et al., 2007). Although selective blocking of repolarizing K⁺ currents in the atrium is an ideal strategy for the treatment of atrial arrhythmias, blocking of Kv4.3 is also beneficial for obtaining a desired antiarrhythmic effect (Brendel and Peukert, 2003). Thus, the inhibitory effect of ranolazine on Kv4.3 may help to explain the mechanism by which this drug displays such a lack of arrhythmogenicity. The therapeutic blood concentration of ranolazine as an anti-ischemic agent is in the range of 2 to 6 μM (Wang et al., 2008). The IC₅₀ values of ranolazine for inhibiting cardiac ion currents (Na⁺, HERG, rapid and slow components of the delayed rectifier K⁺ current) are higher than the therapeutic concentrations (Schram et al., 2004; Rajamani et al., 2009). In the present study, ranolazine exhibited a low affinity for Kv4.3 channels in the closed state, but a much higher affinity in the inactivated state. Kv4.3 channel, a member of the Shal-type of Kᵥ channel, underlies rapidly inactivating K⁺ currents found in the nervous system as A-type K⁺ currents and in the heart as transient outward K⁺ currents (Birnbaum et al., 2004). In many neurons, significant fractions of A-type K⁺ currents are inactivated at resting membrane potential (Connor and Stevens, 1971; Winkelmann et al., 2005). In contrast, transient outward K⁺ currents are in a closed state at resting

![Fig. 7. Reversible inhibition of Kv4.3 by ranolazine.](image)
membrane potential in cardiac myocytes. Thus, ranolazine had little or no effect on normal myocytes because Kv4.3 channels are in the closed states at resting membrane potential in cardiac myocytes (Nerbonne, 2000). However, some pathologic conditions, such as cardiac ischemia, depolarized membrane potentials of myocytes (Kodama et al., 1984) and possess a pathologically higher fraction of Kv4.3 channels in the inactivated state. Thus, ranolazine inhibits Kv4.3 channels in ischemic myocytes to a much greater degree than in normal cells.

In conclusion, our results indicate that ranolazine inhibits Kv4.3 channels and exhibits a low affinity for Kv4.3 channels in the closed state but a much higher affinity for Kv4.3 channels in the inactivated state.

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Authorship Contributions

Participated in research design: B. H. Choi and Hahn.
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References


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