Full Paper

Effect of Azelastine on Cardiac Repolarization of Guinea-Pig Cardiomyocytes, hERG K⁺ Channel, and Human L-type and T-type Ca²⁺ Channel

Mi-Hyeong Park¹, Seung Ho Lee², Dae Hyun Chu³, Kwang Hee Won³, Bok Hee Choi^{2,*a}, Han Choe^{3,*b}, and Su-Hyun Jo^{1,*c}

¹Department of Physiology, Institute of Bioscience and Biotechnology, Kangwon National University College of Medicine, Chuncheon 200-701, Korea

²Department of Pharmacology, Institute for Medical Sciences, Chonbuk National University Medical School, Jeonju 561-180, Korea

³Department of Physiology, Research Institute for Biomacromolecules, University of Ulsan College of Medicine, Seoul 138-736, Korea

Received October 18, 2012; Accepted July 17, 2013

Abstract. Azelastine is a second generation histamine H₁-receptor antagonist used as an anti-asthmatic and anti-allergic drug that can induce QT prolongation and torsades de pointes. We investigated the acute effects of azelastine on human *ether-a-go-go*-related gene (*hERG*) channels, action potential duration (APD), and L-type (I_{Ca,L}) and T-type Ca²⁺ current (I_{Ca,T}) to determine the electrophysiological basis for its proarrhythmic potential. Azelastine increased the APD at 90% of repolarization concentration dependently, with an IC₅₀ of 1.08 nM in guinea-pig ventricular myocytes. We examined the effects of azelastine on the hERG channels expressed in *Xenopus* oocytes and HEK293 cells using two-microelectrode voltage-clamp and patch-clamp techniques. Azelastine induced a concentration-dependent decrease of the hERG current amplitude at the end of the voltage steps and tail currents. The IC₅₀ for the azelastine-induced block of the hERG currents expressed in HEK293 cells was 11.43 nM, while the drug inhibited I_{Ca,L} and I_{Ca,T} with IC₅₀ values of 7.60 and 26.21 μM, respectively. The S6 domain mutations, Y652A partially attenuated and F656A abolished hERG current block. These results suggest that azelastine is a potent blocker of hERG channels rather than I_{Ca,L} or I_{Ca,T}, providing molecular mechanisms for the arrhythmogenic side effects during the clinical administration of azelastine.

Keywords: action potential duration, antipsychotic drug, azelastine, hERG channel, rapidly-activating delayed rectifier K⁺ channel

Introduction

Azelastine, a 2nd-generation antihistamine, is a phthalazinone compound that possesses not only potent and selective histamine H₁-receptor antagonist activity but also inhibits histamine release from mast cells (1), which is useful for the effective relief of asthma-

associated symptoms (2, 3). Azelastine is pharmacologically active and is metabolized by CYP3A4 and 2D6 isozymes to its major, active metabolite, desmethylazelastine (4, 5). The most common adverse events related to azelastine were alterations in taste perception, nasal burning, headaches, and dry mouth (6). In addition, this agent has been shown to modify the electrical activity in several types of tissues. For example, azelastine reduced the basal cytosolic free Ca²⁺ content in airway smooth muscle and inhibited voltage-dependent Ca²⁺ currents in single tracheal smooth muscle cells, which may contribute to the anti-allergic actions of azelastine

Corresponding authors. *^abhchoi@jbnu.ac.kr,
*^bhchoe@amc.seoul.kr, *^csuhyunjo@kangwon.ac.kr
Published online in J-STAGE on September 3, 2013
doi: 10.1254/jphs.12239FP

in airways (7, 8). Nakamura et al. (9) showed that azelastine inhibits PAF-acether and *N*-formylmethionyl-leucyl-phenylalanine-mediated mobilization of intracellular Ca^{2+} in guinea-pig peritoneal macrophages. In human airway epithelia, azelastine decreased Cl^- transport and thus may have a detrimental effect on airway surface liquid homeostasis (10). Azelastine depressed electrical and mechanical activities in the study with guinea-pig papillary muscles using conventional microelectrodes (11, 12). However, azelastine increased the cytosolic free Ca^{2+} content in rat heart myoblast cells H_9C_2 (7).

Some second-generation antihistamines, notably terfenadine and astemizole, have been associated with prolongation of the QT interval and the development of torsades de pointes. Together with the azelastine-induced electrical changes of heart mentioned above, azelastine may induce lengthening of the QT interval (13). Also, to our knowledge, there are reports which have shown that azelastine has been associated with arrhythmia (19 cases) and ventricular tachycardia (8 cases) in the Adverse Event Reporting System from the FDA (http://www.druginformer.com/search/side_effect/azelastine%20hydrochloride). On the other hand, it has been shown that azelastine does not cause QT prolongation or torsades de pointes (14–16). One of the most likely candidates for the cause of drug-induced arrhythmia is the rapid component of the delayed rectifier K^+ channels (I_{Kr}). I_{Kr} determines the shape of the repolarization phase (phase 3) of the ventricular action potential (17), thereby resulting in prolongation of the QT interval on electrocardiograms (ECG). The pore-forming subunit of the channel underlying I_{Kr} is encoded by the human *ether-a-go-go*-related gene (*hERG*) (18), and Tyr-652 and Phe-656 in the S6 transmembrane domain are vulnerable to diverse drugs (19). Therefore, azelastine could increase QT interval possibly by inhibition of the hERG channel and prolongation of the action potential duration (APD).

This study examined the possible azelastine block of the hERG channels expressed in *Xenopus* oocytes and HEK cells. Furthermore, we studied the inhibitory mechanisms and molecular determinants for the hERG channel block of azelastine using two mutant hERG channels, Y652A and F656A. We also performed patch-clamp electrophysiology for the measurement of voltage-dependent L-type ($\text{I}_{\text{Ca,L}}$) and T-type Ca^{2+} current ($\text{I}_{\text{Ca,T}}$) expressed in HEK cells to explore the drug's possible role in intracellular Ca^{2+} regulation. Finally, a virtual docking simulation was carried out to understand the blocking mode of the hERG channel by the drug using the KvAP channel structure as a template.

Materials and Methods

Ventricular myocyte isolation

Single ventricular myocytes were isolated from each guinea-pig heart using a method described previously (20). Guinea pigs (300–500 g) were anesthetized with pentobarbital (up to 50 mg/kg, i.p.) and the heart was quickly excised. The heart was retrogradely perfused at 37°C with solution A containing 750 μM Ca^{2+} and Ca^{2+} -free solution A followed by an enzyme solution. The enzyme solution contained solution A, 150 μM Ca^{2+} , collagenase type II, and protease type XIV. Solution A contained 130 mM NaCl, 4.5 mM KCl, 21 mM glucose, 2.5 mM MgCl_2 , 1 mM NaH_2PO_4 , 20 mM taurine, 5 mM creatine, and 23 mM HEPES (pH 7.2). The heart was then flushed with a 150 μM Ca^{2+} solution. The ventricles were removed and chopped into small pieces, which were then shaken in a flask containing a 150 μM Ca^{2+} solution. The cell suspension was left to sediment. The supernatant was replaced with a 500 μM Ca^{2+} solution. The cells were kept at room temperature. This study was performed according to the Research Guidelines of Kangwon National University IACUC.

Solutions and action potential recordings from myocytes

Myocytes in the experimental chamber were continuously superfused at room temperature (24°C–26°C) with Tyrode solution containing 10 mM glucose, 5 mM HEPES, 140 mM NaCl, 4 mM KCl, 1 mM MgCl_2 , and 1.8 mM CaCl_2 (adjusted to pH 7.4 with NaOH). The experimental chamber had a volume of 150 μl and the flow rate of the Tyrode solution was 2 ml/min. Miniature solenoid valves (LFAA1201618H; Lee Products, Bucks, UK) selected the solution entering the chamber, and the superfusate within the chamber could be changed within 5 s. The solution level in the chamber was controlled with a suction system. The chamber and solenoid valves were mounted on the sliding stage of a microscope (Diaphot; Nikon, Tokyo) that sat on an antivibration table (Newport, Irvine, CA, USA).

Protease type XIV, dimethyl sulfoxide (DMSO), tetraethylammonium chloride (TEA-Cl) (Sigma, St. Louis, MO, USA), and collagenase type 1 (Worthington Biochemical, Freehold, NJ, USA) were used in the form of stock solutions or test solutions. The other reagents were purchased from Sigma except azelastine (Enzo Life Sciences, New York, NY, USA). Azelastine (Enzo Life Sciences) was dissolved in distilled water to make a 30 mM stock solution and added to the external solutions at suitable concentrations shortly before each experiment.

Membrane potential was measured with conventional microelectrodes pulled from filamented thin wall glass tubing with a 1.5-mm outer diameter and a 1.2-mm inner

diameter (World Precision Instruments, Sarasota, FL, USA). They were filled with filtered 300 mM KCl and had a resistance of between 25 and 40 M Ω . Membrane potential was measured with an Axoclamp 900A amplifier (Axon Instruments, Foster City, CA, USA). Action potentials were elicited at 0.33 Hz by 2-ms depolarizing current pulses passed through the microelectrode. We selected rod shaped myocytes with clear cross striations and rejected records that showed noisy or drifting baselines. Data acquisition was performed with a digital computer, analogue data acquisition equipment (National Instruments, Austin, TX, USA), and the software WCP (written and supplied by Dr. John Dempster of Strathclyde University, Glasgow, UK) online.

Expression of hERG in oocytes

hERG (accession no. U04270) cRNA was synthesized by in vitro transcription from 1 μ g of linearized cDNA using T7 message machine kits (Ambion, Austin, TX, USA) and stored in 10 mM Tris-HCl (pH 7.4) at -80°C . The amino acid mutations were generated by the polymerase chain reaction (PCR) with synthetic mutant oligonucleotide primers. The mutations Y652A and F656A were verified by sequencing (ABI3100). Stage V–VI oocytes were surgically removed from female *Xenopus laevis* (Nasco, Modesto, CA, USA) anesthetized with 0.17% tricane methanesulphonate (Sigma). Using fine forceps, the theca and follicle layers were manually removed from the oocytes, and each oocyte was injected with 40 nl of cRNA (0.1–0.5 $\mu\text{g}/\mu\text{l}$). The injected oocytes were maintained in a modified Barth's Solution. The modified Barth's Solution contained: 88 mM NaCl, 1 mM KCl, 0.4 mM CaCl_2 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 1 mM MgSO_4 , 2.4 mM NaHCO_3 , 10 mM HEPES (pH 7.4), and 50 $\mu\text{g}/\text{ml}$ gentamicin sulphonate. Currents were studied 2–7 days after injection.

Solutions and voltage clamp recordings from oocytes

Normal Ringer's Solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES (adjusted to pH 7.4 with NaOH). Solutions were applied to oocytes by continuous perfusion of the chamber while recording. Solution exchanges were completed within three min, and the hERG currents were recorded 5 min after the solution exchange. Currents were measured at room temperature (20°C – 23°C) with a two-microelectrode voltage clamp amplifier (Warner Instruments, Hamden, CT, USA). Electrodes were filled with 3 M KCl and had a resistance of 2–4 M Ω for voltage-recording electrodes and 0.6–1 M Ω for current-passing electrodes. Stimulation and data acquisition were controlled with an AD-DA converter (Digidata 1200, Axon Instruments) and pCLAMP software (v5.1, Axon Instruments).

HEK cell culture and whole-cell patch recording

HEK293 cells stably expressing hERG channels, a kind gift from Dr. C. January (21), were used for electrophysiological recordings. The cultures were passaged every 4–5 days with a brief trypsin–EDTA treatment followed by seeding onto glass coverslips (diameter: 12 mm; Fisher Scientific, Pittsburgh, PA, USA) in a Petri dish. After 12–24 h, the cell-attached coverslips were used for electrophysiological recordings.

hERG currents were recorded from HEK293 cells, with the whole-cell patch-clamp technique (22) at room temperature (22°C – 23°C). The micropipettes fabricated from glass capillary tubing (PG10165-4; World Precision Instruments) with a double-stage vertical puller (PC-10; Narishige, Tokyo) had a tip resistance of 2–3 M Ω when filled with the pipette solution. Whole-cell currents were amplified with the Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA, USA), digitized with the Digidata 1200A (Molecular Devices) at 5 kHz, and low-pass filtered with a four-pole Bessel filter at 2 kHz. Capacitive currents were canceled and series resistance was compensated at 80% with the amplifier, while leak subtraction was not used. The generation of voltage commands and acquisition of data were controlled with pClamp 6.05 software (Molecular Devices) running on an IBM-compatible Pentium computer. The recording chamber (RC-13; Warner Instrument Corporation, Hamden, CT, USA) was continuously perfused with a bath solution (see below for composition) at a rate of 1 ml/min.

The external solution contained 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose, and 10 mM HEPES (adjusted to pH 7.4 with NaOH). The intracellular solution contained 130 mM KCl, 1 mM MgCl_2 , 5 mM EGTA, 5 mM MgATP, and 10 mM HEPES (adjusted to pH 7.4 with KOH).

The method for transiently expressing the Ca^{2+} channels in HEK293 cells is briefly described as follows: Human T-type Ca^{2+} channel $\alpha 1$ subunit (CACNA1H) cDNA and L-type Ca^{2+} channel α subunit (CACNA1C) cDNA were transferred to the plasmid expression vector pEF5/FRT/V5-DEST (Invitrogen, Carlsbad, CA, USA). HEK293 cells were transiently transfected with the Ca^{2+} channel using lipofectamine (Invitrogen). The transfected cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino-acid solution, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate. The cells were seeded onto glass coverslips (diameter: 12 mm, 5 mm; Fisher Scientific, Pittsburgh, PA, USA) in a Petri dish. After 12–24 h, the cell-attached coverslips were used for electrophysiological recordings.

For T-type Ca^{2+} channel current recordings, the external solution contained 120 mM NaCl, 5 mM CsCl, 10 mM BaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 1 μM tetrodotoxin, and 10 mM glucose (adjusted to pH 7.3 with NaOH). The internal solution contained 120 mM CsCl, 1 mM MgCl_2 , 10 mM HEPES, 10 mM EGTA, and 2 mM MgATP, (adjusted to pH 7.3 with CsOH).

For L-type Ca^{2+} channel current recordings, the external solution contained 10 mM BaCl_2 , 125 mM CsCl, and 10 HEPES (pH adjusted to 7.40 with CsOH). The internal solution contained 114 mM CsCl, 10 EGTA, 5 Mg ATP (pH adjusted to 7.20 with CsOH).

Homology modeling

A homology model of the hERG potassium channel was built on the basis of the 2.9 Å crystal structure (PDB ID code: 2A79) of the rat Kv1.2 channel (23) using the homology modeling program, MODELLER v9.1 (24). Only the amino acids from F513 to A671, comprising the fourth, fifth, and sixth transmembrane domains with the P-loop, were selected for the model because the corresponding region of the template was a continuous chain in the crystal structure and the region is believed to be mainly responsible for the drug inhibition of the hERG channel. Sequence alignment between the hERG channel and the Kv1.2 channel was carried out by the Vector-NTI program version 10.1.1 (Invitrogen). A stretch of amino acids from L589 to T613, located at the third extracellular loop of the hERG channel, was not included for the model because there was no corresponding region in the template structure and does not appear to be involved in the drug-induced inhibition of the hERG channel. The homotetrameric structure was restrained to maintain symmetry during homology modeling. Hydrogen atoms were added to the homology model of the hERG channel using Maestro (Schrödinger LLC, New York, NY, USA).

Virtual docking

The homo-tetramer was energy-minimized using OPLS-AA force field of MacroModel version 9.1 (Schrödinger LLC) until the average RMS deviation of the non-hydrogen atoms fell below 0.3 Å. The Y652 and F656 residues of each of the four monomers were selected for the grid center for docking simulation. The grid box size was set to 15 Å from the grid center. The 2D structure of azelastine was produced by Chemdraw (Cambridgesoft, Cambridge, MA, USA) based on its IUPAC name. Using LigPrep version 2.0 (Schrödinger LLC), we generated a 3D energy-minimized and hydrogenated conformation of azelastine. In the LigPrep operation, not generating stereoisomers, ionization was done targeting pH from 7.0 to 7.4. Docking simulation was

performed using GLIDE version 4.0 (Schrödinger LLC) in extra precision (XP) mode.

Statistical evaluations

All data are expressed as the mean $\pm$ S.E.M. Comparisons of the value of the half-maximal activation ($V_{1/2}$) effect for each concentration of azelastine and that in the absence of the drug were performed using ANOVA followed by Tukey HSD post hoc testing to determine significant differences among data groups. A P value of < 0.05 was considered to be statistically significant.

Results

Effects of the azelastine on action potentials in guinea pig ventricular myocytes

This study examined the effects of azelastine on the action potentials in guinea-pig ventricular myocytes. The mean control action potential duration at 90% (APD_{90}) of myocytes was 759 ± 77 ms ($n = 8$). Figure 1A shows

Fig. 1. The effect of azelastine on action potentials in isolated guinea-pig ventricular myocytes. A) Superimposed action potentials recorded before and after exposure to various concentrations of azelastine. B) Concentration-dependent prolongation of action potential duration at 90% (APD_{90}). APD_{90} was normalized to the control and plotted as a function of azelastine concentration. The data are fitted with the equation $y = [A_1 - A_2 / 1 + (x/x_0)^p] + A_2$, where A_1 is maximum value, A_2 is minimum value, x_0 is a half-maximal inhibitory concentration (IC_{50}), and p is slope, giving an IC_{50} of 1.08 ± 7.14 nM.

superimposed traces of the action potentials recorded before and during exposure to different concentrations of azelastine. The APD_{90} increased with increasing azelastine concentration from 0.3 – 1000 nM (Fig. 1B). The IC_{50} for the azelastine-induced prolongation of the APD_{90} in the guinea-pig ventricular myocytes was 1.08 ± 7.14 nM, as shown in Fig. 1B.

Concentration-dependence of WT hERG channel block by azelastine in Xenopus oocytes

Next, to examine whether the azelastine-induced prolongation of APD_{90} could be due to the inhibition of I_{Kr} , we examined the effect of azelastine on hERG currents using a *Xenopus* oocyte expression system. Throughout these experiments, the holding potential was maintained at -70 mV and tail currents (I_{tail}) were recorded at -60 mV after depolarizing pulses from -50 to $+40$ mV. Figure 2A gives an example of a voltage-clamp recording from a *Xenopus* oocyte and the representative current traces under control conditions and after exposure to $1 \mu\text{M}$ azelastine. The amplitude

of the outward currents measured at the end of the pulse (I_{HERG}) increased with increasing positive voltage steps, reaching a maximum at -10 mV. The amplitude of I_{HERG} was normalized to the maximum amplitude of the I_{HERG} obtained under control conditions and was plotted against the potential of the step depolarization ($I_{HERG,nor}$, Fig. 2B). The amplitude of $I_{HERG,nor}$ showed a concentration-dependent decrease with increasing azelastine concentration.

After the depolarizing steps, repolarization to -60 mV induced an outward I_{tail} , which had an amplitude even greater than that of I_{HERG} during depolarization, which is due to a rapid recovery from inactivation and a slow deactivation mechanism (21). When $1 \mu\text{M}$ azelastine was added to the perfusate, both I_{HERG} and I_{tail} were reduced (Fig. 2A, bottom panel). The amplitude of I_{tail} was normalized to the peak amplitude obtained under control conditions at the maximum depolarization and was plotted against the potential of the step depolarization (Fig. 2C). The data obtained under control conditions were well-fitted by the Boltzmann equation with a

Fig. 2. The effect of azelastine on *human-ether-a-go-go-related gene* (*hERG*) currents (I_{HERG}) elicited by depolarizing voltage pulses. A) Superimposed current traces elicited by depolarizing voltage pulses (4 s) in 10-mV steps (upper panel) from a holding potential of -70 mV in the absence of azelastine (control, center panel) and in the presence of $1 \mu\text{M}$ azelastine (lower panel). B) Plot of the normalized hERG current measured at the end of depolarizing pulses ($I_{HERG,nor}$) against the pulse potential in the control and azelastine conditions. The maximal amplitude of the I_{HERG} in the control was given a value of 1. C) Plot of the normalized tail current measured at its peak just after repolarization. The peak amplitude of the tail current in the absence of the drug was set as 1. Control data were fitted to the Boltzmann Equation, $y = 1 / \{1 + \exp[(-V + V_{1/2}) / dx]\}$, with $V_{1/2}$ of -13.0 mV. D) Activation curves with values normalized to the respective maximum value at each concentration of azelastine. Symbols with error bars represent the mean $\pm$ S.E.M. ($n = 6 - 9$).

half-maximal activation ($V_{1/2}$) at -13.0 mV. The peak I_{tail} amplitude decreased with increasing azelastine concentration, which indicates that the maximum conductance of the hERG channels is decreased by azelastine. In addition, in the presence of azelastine, I_{tail} does not reach the steady-state level but decreases at more positive potentials, indicating that the blockade is more pronounced at the positive potentials.

The values shown in Fig. 2C were normalized to the respective maximum values at each concentration to determine if azelastine shifts the activation curve (Fig. 2D). The activation curves representing the higher concentrations of the drug ($0.3 - 50 \mu\text{M}$) were shifted downward and leftward. The $V_{1/2}$ calculations are consistent with this finding, yielding values of -13.0 ± 0.21 , -15.7 ± 0.93 , -21.6 ± 2.66 , -24.8 ± 3.01 , -22.9 ± 4.05 , and -21.6 ± 3.09 mV in the control and 0.3, 1, 3, 10, and 50 μM azelastine-treated groups, respectively ($n = 6 - 9$, $P < 0.05$). Therefore, the dual effect of azelastine on hERG tail current may be explained by the drug both inhibiting hERG currents and producing a leftward shift in voltage-dependent activation of the channel.

Inhibition of WT hERG currents expressed in HEK cells by azelastine

The IC_{50} values of many hERG-channel blockers have been shown to differ depending on whether the hERG channels are expressed in *Xenopus* oocytes or mammalian cells, an effect probably due to the sequestration of blockers in the large ooplasm of oocytes (20). We therefore tested the effects of azelastine in HEK293 cells expressing hERG channels, using the whole-cell patch-clamp technique (Fig. 3). As shown in Fig. 3A (upper trace), whole-cell currents were elicited with 4-s depolarization to $+20$ mV from a holding potential of -80 mV, and the tail current was recorded at -60 mV for 6 s in HEK293 cells expressing hERG channels. Bath-applied azelastine reduced the I_{HERG} in a concentration-dependent manner (Fig. 3A). As shown in Fig. 3B, dose dependency of the steady-state currents measured at the end-pulse of $+20$ mV or peak tail currents was analyzed quantitatively. A nonlinear least-squares fit of dose-response plots with the Hill equation yielded an IC_{50} value of 11.08 ± 1.26 nM and a Hill coefficient of 0.97 ± 0.10 ($n = 5$) for the steady-state currents and an IC_{50} value of 11.43 ± 0.91 nM and a Hill coefficient of 0.98 ± 0.07 ($n = 5$) for the peak tail currents. These results indicate that the azelastine-induced inhibition of hERG channels stably expressed in HEK293 cells occurred at a concentration approximately 40 times lower than that required by hERG channels expressed in *Xenopus* oocytes.

Fig. 3. Concentration dependence of azelastine-induced inhibition of I_{HERG} . A) Superimposed I_{HERG} traces were elicited with 4-s depolarizations to $+20$ mV from a holding potential of -80 mV, and the tail current was recorded at -60 mV for 6 s in the absence or presence of 1, 3, 10, 30, and 100 nM azelastine, as indicated. The protocol was applied every 15 s. The dotted line represents zero current. B) Concentration-dependent curve of inhibition by azelastine for steady-state currents (open triangle) measured at the end of the depolarizing pulse to $+20$ mV or peak tail currents (closed square). The respective normalized currents were plotted against various concentrations of azelastine. The solid lines are fitted to the data points by the Hill equation.

Effects of azelastine on L-type Ca^{2+} current expressed in HEK cells

We examined the effects of azelastine on $I_{\text{Ca,L}}$ using the whole-cell patch-clamp technique. As shown in Fig. 4A (upper trace), whole-cell currents were elicited with 200-ms depolarizations to 0 mV from a holding potential of -80 mV in the absence or presence of 3, 10, 30, and 100 μM azelastine, as indicated. The protocol was applied every 10 s. Bath-applied azelastine reduced $I_{\text{Ca,L}}$ in a concentration-dependent manner (Fig. 4A). As shown in Fig. 4B, dose dependency of the steady-state currents measured at the peak amplitude of 0 mV was

Fig. 4. Concentration-dependent inhibition of $I_{Ca,L}$ by azelastine. A) Superimposed $I_{Ca,L}$ traces were elicited with 200-ms depolarizations to 0 mV from a holding potential of -80 mV in the absence or presence of 1, 3, 10, and 30 μ M azelastine, as indicated. The protocol was applied every 10 s. The dotted line represents zero current. B) Concentration-dependent curve of inhibition by azelastine for the peak current amplitudes. The normalized inhibitions were plotted against various concentrations of azelastine. The solid line is fitted to the data points by the Hill equation. Data are expressed as the mean $\pm$ S.E.M.

analyzed quantitatively. A nonlinear least-squares fit of dose-response plots with the Hill equation yielded an IC_{50} value of $7.60 \pm 4.26 \mu$ M and a Hill coefficient of 0.76 ± 0.35 ($n = 3$).

Effects of azelastine on T-type Ca^{2+} current expressed in HEK cells

We examined the effects of azelastine on $I_{Ca,T}$ using the whole-cell patch-clamp technique. As shown in Fig. 5A (upper trace), whole-cell currents were elicited with 200-ms depolarizations to 0 mV from a holding potential of -80 mV in the absence and presence of 3, 10, 30, and 100 μ M azelastine, as indicated. The protocol was applied every 10 s. Bath-applied azelastine reduced $I_{Ca,T}$ in a concentration-dependent manner (Fig. 5A). As shown in Fig. 5B, dose dependency of the steady-state

Fig. 5. Concentration-dependent inhibition of $I_{Ca,T}$ by azelastine. A) Superimposed $I_{Ca,T}$ traces were elicited with 200-ms depolarizations to 0 mV from a holding potential of -80 mV in the absence or presence of 3, 10, 30, and 100 μ M azelastine, as indicated. The protocol was applied every 10 s. The dotted line represents zero current. B) Concentration-dependent curve of inhibition by azelastine for the peak current amplitudes. The normalized inhibitions were plotted against various concentrations of azelastine. The solid line is fitted to the data points by the Hill equation. Data are expressed as the mean $\pm$ S.E.M.

currents measured at the peak amplitude of 0 mV was analyzed quantitatively. A nonlinear least-squares fit of dose-response plots with the Hill equation yielded an IC_{50} value of $26.21 \pm 2.17 \mu$ M and a Hill coefficient of 1.33 ± 0.14 ($n = 5$).

Azelastine block of WT and mutant hERG channels expressed in oocytes

Previous studies reported that two aromatic residues, Tyr-652 and Phe-656, which are located in S6 domain and face the pore cavity of the channel, are important components of the binding site for a number of compounds (19). The potency of a channel block for the wild type and two mutant hERG channels (Y652A and F656A) were compared in order to determine if these key residues are also important in the azelastine-induced

Fig. 6. Concentration-dependent inhibition of WT and mutant hERG channels expressed in oocytes. A – C) Representative traces for WT and mutant hERG channel currents in the presence or absence of indicated concentrations of azelastine. The effect of the drug on WT, Y652A, and F656A was quantified during 4-s activating pulses to 0 mV from a holding potential of -70 mV. D) The concentration–response curves were fitted with a logistic dose–response equation to obtain the IC_{50} values of $0.6 \pm 0.1 \mu\text{M}$, $17.8 \pm 3.7 \mu\text{M}$, and $977.9 \pm 5.7 \mu\text{M}$ in WT, Y652A, and F656A hERG channels, respectively. Data were expressed as the mean $\pm$ S.E.M. ($n = 4 - 9$). The potency of azelastine block was reduced in the mutant channels.

blocking of the hERG channel. The effect of azelastine on wild-type channels was quantified during a 4-s activating pulse to 0 mV from a holding potential of -70 mV (Fig. 6: A, B, and C). As shown in Fig. 6, the inhibitory effect of azelastine ($100 \mu\text{M}$) was partially attenuated by the Y652A mutation (panel A, B) or abolished by the F656A mutation (panel A, C). The wild-type hERG channel current was blocked by azelastine with an IC_{50} of $0.6 \pm 0.1 \mu\text{M}$ ($n = 7 - 9$), while the IC_{50} values were $17.8 \pm 3.7 \mu\text{M}$ ($n = 4 - 6$) and $977.9 \pm 5.7 \mu\text{M}$ ($n = 5 - 8$) for the Y652A and F656A hERG mutants, respectively. This indicates that a mutation of Phe-656 located in the S6 domain of the hERG channel reduced the potency of the channel block by azelastine more than a mutation of Tyr-652 in the same region.

Virtual docking simulation for the binding of azelastine to the hERG channel

We carried out a virtual docking simulation to study the binding mode of azelastine to the hERG channel (Fig. 7). The homology model of the Kv1.2 channel, an open potassium channel, was generated and used for the docking simulation. Y652 and F656 were selected as active sites for the ligand. Results with high GLIDE score were selected for further evaluation. One of the

results showed that the hydrophobic moiety of azelastine made a hydrophobic interaction with F656 in segments A, B, and C. A protonated nitrogen of the ligand formed a close interaction with the carbonyl oxygen of T623 and hydroxyl oxygen of the side chain of S624 in segment C even though the distance between them is slightly longer than the usual length of hydrogen bonds (dashed red lines indicate bond lengths $3.9 \text{ \AA}$ and $3.8 \text{ \AA}$, respectively). The aromatic ring of azelastine interacts with A653(A), A653(D), F656(D), and T652(D).

Discussion

These results suggest that azelastine inhibits the hERG channels stably expressed in the HEK cells and increases the APD_{90} of guinea-pig ventricular myocytes with IC_{50} values of 11.43 and 1.08 nM , respectively. The IC_{50} value of azelastine was $0.6 \mu\text{M}$ for the hERG channels expressed heterologously in *Xenopus* oocytes (Fig. 6). A higher extracellular concentration of the drug was required to block the hERG channels due to the properties of the *Xenopus* oocyte–expression system. For example, the block of hERG by trifluoperazine (20) resulted in IC_{50} values 10 times higher when the drug was applied to *Xenopus* oocytes rather than to mammalian cells. This

Fig. 7. Binding mode of the azelastine to the hERG channel is shown downward a little obliquely. The letter in parenthesis indicates the segment of the homotetrameric subunit involved in the interaction. Sky blue colored residues displayed as a stick in the hERG channel indicate residues that seem to interact with the azelastine. Aromatic ring of the azelastine interacts with A653(D), T652(D), A653(A), and F656(D). A hydrophobic moiety of azelastine participates in an hydrophobic interaction with F656 in segment A, B, and C. The dashed red line shows the interaction between the protonated nitrogen of the ligand and the carbonyl oxygen of T623(C) and between the protonated nitrogen of the ligand and the hydroxyl oxygen of the side chain of S624(C).

might be due to a decrease in drug concentration at the cell membrane caused by the vitelline membrane and egg yolk. Other antihistamines have been reported to block hERG channels. In *Xenopus* oocytes, the 1st generation antihistamines chlorpheniramine and diphenhydramine blocked the channel with a lower potency than the 2nd generation antihistamines terfenadine and astemizole (25). Katchman et al. (26) showed that loratadine, a nonsedating 2nd generation antihistamine, blocks hERG current in HEK cells with an IC_{50} value of 4 μ M, which is lower than that of chlorpheniramine (IC_{50} value = 13 μ M). Therefore, our results indicate that azelastine has a relatively high potency for blockage of hERG channels compared with other 2nd generation antihistamines.

Azelastine is an anti-allergic and anti-asthmatic drug, which is used as a first-line treatment for allergic and non-allergic vasomotor rhinitis. Intranasally administered, azelastine reaches a peak plasma concentration in 2 – 3 h (27), and the elimination half-life is estimated to be 22 – 36 h (28). The therapeutically relevant plasma concentration of azelastine ranges from 10 to 100 nM (29, 30). The free plasma concentration of azelastine might range from 2.2 to 22 nM when the rate of protein binding reaches 78% (28). Also, it was reported that in heart tissues, azelastine can reach a plasma concentration up to 3 times higher (31), indicating that the accumulated concentration may be assumed to be in the range of 6.6 – 66 nM in the tissue. Our results show that azelastine

inhibited the hERG channels in the HEK cells with an IC_{50} value of 11.43 nM, and the drug increased the APD_{90} of guinea pig ventricular myocytes with the value of 1.08 nM. Therefore, the concentration range for the hERG channel block and the prolongation of APD_{90} is similar to the range of clinically relevant cardiac tissue concentrations. The possibility of azelastine-induced prolongation of the QT interval has been suggested (13). Also, to our knowledge, there are reports showing that azelastine was associated with arrhythmia (19 cases) and ventricular tachycardia (8 cases) in the Adverse Event Reporting System from the FDA (http://www.druginformer.com/search/side_effect/azelastine%20hydrochloride). In addition, the pharmacokinetics of orally administered azelastine has been studied in the elderly in whom elimination is decreased, leading to a doubling of plasma concentrations (32). Also, the elimination half-life of azelastine is increased in the elderly and in patients with hepatic dysfunction (32). Therefore, in patients with hepatic disease, overdoses may increase the possibility of blocking of the I_{Kr} current in the heart, which could induce proarrhythmic effects such as torsades de pointes and the prolongations of the APD_{90} (Fig. 1) and the QT interval.

Azelastine inhibited L-type Ca^{2+} channels in tracheal smooth muscle cells and decreased the cytosolic free Ca^{2+} content in cultured airway smooth muscle (7, 8). These Ca^{2+} -antagonistic actions can contribute to the anti-allergic activity of azelastine as a bronchodilator.

Also, azelastine at concentrations higher than 10 μM inhibited voltage-dependent K^+ currents and Ca^{2+} -activated oscillatory K^+ currents in tracheal smooth muscle cells, although at a relatively mild degree in comparison to its Ca^{2+} antagonistic effects (8). These dual actions of azelastine for $\text{I}_{\text{Ca,L}}$ and the K^+ currents could cause the compromise of the membrane potential in tracheal smooth muscle cells (8, 33). As for cardiac tissues, azelastine reduced the electrical and mechanical activity by inhibiting the slow Ca^{2+} channels and the fast Na^+ channels in guinea-pig papillary muscles at the concentration range of approximately 10 μM (11). Our results with L-type Ca^{2+} channels stably expressed in HEK293 cells also showed that azelastine inhibited $\text{I}_{\text{Ca,L}}$ with an IC_{50} of 7.60 μM . Li et al. (12) showed that azelastine reduced the force of contraction, shortened APD, and depressed maximum upstroke velocity in guinea pig papillary muscle by blocking L-type Ca^{2+} channels of guinea-pig ventricular myocytes with an IC_{50} of 20.2 μM because the drug shortened the slow inactivation time constant of the channels. Also, our computer simulation of action potential of the guinea-pig ventricular myocyte model (34) using Matlab ver. 7.10 (Mathworks, Natick, MA, USA) shows that azelastine would increase the APD_{90} at 0.4 – 53 μM approximately, however, the drug at > 53 μM would shorten the APD_{90} (data not shown). Therefore, the depressive action of azelastine on cardiac contraction, APD, and L-type Ca^{2+} channels (12) does not seem to occur in vivo as the possible therapeutic concentrations in cardiac tissue range from 6.6 to 66 nM as aforementioned. Also, azelastine does not seem to block the inward rectifier K^+ channels, which is mainly involved in the resting membrane potential as azelastine failed to change the resting membrane potential in tracheal smooth muscle cells (33) and in cardiac ventricular myocytes (Fig. 1). Finally, the dual actions of azelastine on L-type Ca^{2+} channels and the hERG K^+ channels at supra-therapeutic concentrations could compromise APD change induced by the drug, possibly with the result that azelastine does not cause QT prolongation or torsades de pointes (14 – 16).

It is possible that azelastine could cause the modification of the cardiac electrical activity by a direct non-specific drug–membrane interaction, such as perturbation of the membrane lipid–protein interaction. This is because the assessment of the lytic potential of marketed concentrations of azelastine revealed significant membrane perturbation of human conjunctival mast cells and human corneal epithelial cells as indexed by LDH release (35). Also, the action of azelastine could be related to an increase in intracellular cAMP level (36). However, previous reports showed that agents that increase intra-

cellular cAMP levels, such as isoproterenol and norepinephrine, increased $\text{I}_{\text{Ca,L}}$ and the contraction of cardiomyocytes (37). Therefore, it is unlikely that an increase in cAMP is involved in the inhibitory action of azelastine on $\text{I}_{\text{Ca,L}}$ and the contractions of the heart (11, 12). Instead, the direct inhibition of hERG channels by azelastine would be a major cause of APD prolongation because the pore mutations Y652A and F656A of the channel protein significantly attenuate the drug-induced hERG block (Fig. 6).

The molecular modeling and docking results are consistent with the previous blocking mechanism of most hERG channel inhibitors (38). The molecular modeling and docking itself could not directly prove the blocking mode but suggests the possibility of the mode. The molecular charge of a compound is important to the inhibition of hERG channels. Usually a positively charged compound more strongly inhibits hERG channels than a neutral or negatively charged compound. At neutral pH, azelastine tends to have a positive charge at its azepine nitrogen. In the virtual docking, this azepine nitrogen interacts with the carbonyl oxygen of T623 and hydroxyl oxygen of S624 (Fig. 7). The aromatic moiety of azelastine may have π - π stacking with the aromatic side chains of Y652 and F656.

In conclusion, the H_1 antihistamine azelastine increases the APD_{90} in guinea-pig ventricular myocytes and blocks the hERG channels expressed in HEK cells within the therapeutic concentration range. Also, azelastine inhibited $\text{I}_{\text{Ca,L}}$ expressed in HEK cells at the supra-therapeutic concentration level. The Tyr-652 and Phe-656 in the S6 domain of the hERG channel are important molecular determinants for azelastine-induced blocking of the channel. These results suggest a potential mechanism by which azelastine may increase the possibility of cardiac arrhythmia in drug-treated patients.

Acknowledgments

This study was supported by a grant (09172KFDA670) from the Korea Food & Drug Administration, a grant (A100096) of the Korea Healthcare technology R&D Project, Ministry of Health & Welfare, and a grant (2011-0013171) from the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.

References

- 1 Davies RJ, Vardi-Visy K, Clarke M, Stradling JR. Identification of sleep disruption and sleep disordered breathing from the systolic blood pressure profile. *Thorax*. 1993;48:1242–1247.
- 2 Tinkelman DG, Bucholtz GA, Kemp JP, Koepke JW, Repsher LH, Spector SL, et al. Evaluation of the safety and efficacy of multiple doses of azelastine to adult patients with bronchial asthma over time. *Am Rev Respir Dis*. 1990;141:569–574.

- 3 Pichat P, Angel I, Arbillia S. Anti-inflammatory properties of mizolastine after oral administration on arachidonic acid-induced cutaneous reaction in the rat. *Arzneimittelforschung*. 1998;48:173–178.
- 4 Imai T, Taketani M, Suzu T, Kusube K, Otagiri M. In vitro identification of the human cytochrome P-450 enzymes involved in the N-demethylation of azelastine. *Drug Metab Dispos*. 1999;27:942–946.
- 5 Nakajima M, Nakamura S, Tokudome S, Shimada N, Yamazaki H, Yokoi T. Inhibitory effects of azelastine and its metabolites on drug oxidation catalyzed by human cytochrome P-450 enzymes. *Drug Metab Dispos*. 1999;27:792–797.
- 6 LaForce C, Dockhorn RJ, Prenner BM, Chu TJ, Kraemer MJ, Wildtitz MD, et al. Safety and efficacy of azelastine nasal spray (Astelin NS) for seasonal allergic rhinitis: a 4-week comparative multicenter trial. *Ann Allergy Asthma Immunol*. 1996;76:181–188.
- 7 Senn N, Jeanclos E, Garay R. Action of azelastine on intracellular Ca^{2+} in cultured airway smooth muscle. *Eur J Pharmacol*. 1991;205:29–34.
- 8 Hazama H, Nakajima T, Hisada T, Hamada E, Omata M, Kurachi Y. Effects of azelastine on membrane currents in tracheal smooth muscle cells isolated from the guinea-pig. *Eur J Pharmacol*. 1994;259:143–150.
- 9 Nakamura T, Nishizawa Y, Sato T, Yamato C. Effect of azelastine on the intracellular Ca^{2+} mobilization in guinea pig peritoneal macrophages. *Eur J Pharmacol*. 1988;148:35–41.
- 10 Jepsen M, Graham S, Karp PH, Zabner J. Effect of topical nasal pharmaceuticals on sodium and chloride transport by human airway epithelia. *Am J Rhinol*. 2000;14:405–409.
- 11 Molyvdas PA, James FW, Sperelakis N. Azelastine effects on electrical and mechanical activities of guinea pig papillary muscles. *Eur J Pharmacol*. 1989;164:547–553.
- 12 Li S, Blaschke M, Heubach JF, Wettwer E, Ravens U. Effects of azelastine on contractility, action potentials and L-type Ca^{2+} current in guinea pig cardiac preparations. *Eur J Pharmacol*. 2001;418:7–14.
- 13 Moneret-Vautrin DA, de Chillou C, Codreanu A. Long QT syndrome in a patient with allergic rhinoconjunctivitis and auto-immune diabetes: focus on the choice of anti-H1 drugs. *Eur Ann Allergy Clin Immunol*. 2006;38:347–350.
- 14 DuBuske LM. Second-generation antihistamines: the risk of ventricular arrhythmias. *Clin Ther*. 1999;21:281–295.
- 15 Slater JW, Zechnich AD, Haxby DG. Second-generation antihistamines: a comparative review. *Drugs*. 1999;57:31–47.
- 16 Ten Eick AP, Blumer JL, Reed MD. Safety of antihistamines in children. *Drug Saf*. 2001;24:119–147.
- 17 Tie H, Walker BD, Valenzuela SM, Breit SN, Campbell TJ. The heart of psychotropic drug therapy. *Lancet*. 2000;355:1825.
- 18 Warmke JW, Ganetzky B. A family of potassium channel genes related to eag in *Drosophila* and mammals. *Proc Natl Acad Sci U S A*. 1994;91:3438–3442.
- 19 Mitcheson JS, Chen J, Lin M, Culbertson C, Sanguinetti MC. A structural basis for drug-induced long QT syndrome. *Proc Natl Acad Sci U S A*. 2000;97:12329–12333.
- 20 Choi SY, Koh YS, Jo SH. Inhibition of human ether-a-go-go-related gene K^{+} channel and IKr of guinea pig cardiomyocytes by antipsychotic drug trifluoperazine. *J Pharmacol Exp Ther*. 2005;313:888–895.
- 21 Zhou Z, Gong Q, Ye B, Fan Z, Makielski JC, Robertson GA, et al. Properties of hERG channels stably expressed in HEK 293 cells studied at physiological temperature. *Biophys J*. 1998;74:230–241.
- 22 Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch*. 1981;391:85–100.
- 23 Long SB, Campbell EB, Mackinnon R. Crystal structure of a mammalian voltage-dependent Shaker family K^{+} channel. *Science*. 2005;309:897–903.
- 24 Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol*. 1993;234:779–815.
- 25 Suessbrich H, Waldegger S, Lang F, Busch AE. Blockade of hERG channels expressed in *Xenopus* oocytes by the histamine receptor antagonists terfenadine and astemizole. *FEBS Lett*. 1996;385:77–80.
- 26 Katchman AN, Koerner J, Tosaka T, Woosley RL, Ebert SN. Comparative evaluation of hERG currents and QT intervals following challenge with suspected torsadogenic and nontorsadogenic drugs. *J Pharmacol Exp Ther*. 2006;316:1098–106.
- 27 Bernstein JA. Azelastine hydrochloride: a review of pharmacology, pharmacokinetics, clinical efficacy and tolerability. *Curr Med Res Opin*. 2007;23:2441–2452.
- 28 Ten Eick AP, Blumer JL, Reed MD. Safety of antihistamines in children. *Drug Saf*. 2001;24:119–147.
- 29 Tatsumi K, Ou T, Yamada H, Yoshimura H. Studies of metabolic fate of a new antiallergic agent, azelastine (4-(p-chlorobenzyl)-2-[N-methylperhydroazepinyl-(4)]-1-(2H)-phthalazinone hydrochloride). *Jpn J Pharmacol*. 1980;30:37–48.
- 30 McNeely W, Wiseman LR. Intranasal azelastine. A review of its efficacy in the management of allergic rhinitis. *Drugs*. 1998;56:91–114.
- 31 Wong KK, Yang JT, Kucharczyk N, Sofia RD. Tissue distribution and lung localization of [^{14}C]azelastine in guinea pigs. *Drug Metab Dispos*. 1992;20:525–529.
- 32 Simons FE, Simons KJ. Clinical pharmacology of new histamine H1 receptor antagonists. *Clin Pharmacokinet*. 1999;36:329–352.
- 33 Lee HK, Sperelakis N. Azelastine inhibits agonist-induced electromechanical activity in canine tracheal muscle. *Chest*. 1989;96:665–669.
- 34 Faber GM, Silva J, Livshitz L, Rudy Y. Kinetic properties of the cardiac L-type Ca^{2+} channel and its role in myocyte electrophysiology: a theoretical investigation. *Biophysical Journal*. 2007;92:1522–1543.
- 35 Brockman HL, Momsen MM, Knudtson JR, Miller ST, Graff G, Yanni JM. Interactions of olopatadine and selected antihistamines with model and natural membranes. *Ocul Immunol Inflamm*. 2003;11:247–268.
- 36 Katayama S, Tsunoda H, Sakuma Y, Kai H, Tanaka I, Katayama K. Effect of azelastine on the release and action of leukotriene C4 and D4. *Int Arch Allergy Appl Immunol*. 1987;83:284–289.
- 37 Leblais V, Jo SH, Chakir K, Maltsev V, Zheng M, Crow MT, et al. Phosphatidylinositol 3-kinase offsets cAMP-mediated positive inotropic effect via inhibiting Ca^{2+} influx in cardiomyocytes. *Circ Res*. 2004;95:1183–1190.
- 38 Choe H, Nah KH, Lee SN, Lee HS, Jo SH, Leem CH, et al. A novel hypothesis for the binding mode of hERG channel blockers. *Biochem Biophys Res Commun*. 2006;344:72–78.