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Original Article

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Background: Pilsicainide, classified as a relatively selective Na⁺ channel blocker, also has an inhibitory action on the rapidly-activating delayed-rectifier K⁺ current (I_{Kr}) through human ether-a-go-go-related gene (hERG) channels. We studied the effects of chronic exposure to pilsicainide on the expression of wild-type (WT) hERG proteins and WT-hERG channel currents, as well as on the expression of mutant hERG proteins, in a heterologous expression system.

Methods: HEK293 cells stably expressing WT or mutant hERG proteins were subjected to Western blotting, immunofluorescence microscopy and patch-clamp experiments.

Results: Acute exposure to pilsicainide at 0.03–10 μM influenced neither the expression of WT-hERG proteins nor WT-hERG channel currents. Chronic treatment with 0.03–10 μM pilsicainide for 48 h, however, increased the expression of WT-hERG proteins and channel currents in a concentration-dependent manner. Chronic treatment with 3 μM pilsicainide for 48 h delayed degradation of WT-hERG proteins and increased the channels expressed on the plasma membrane. A cell membrane-impermeant pilsicainide derivative did not influence the expression of WT-hERG, indicating that pilsicainide stabilized the protein inside the cell. Pilsicainide did not influence phosphorylation of Akt (protein kinase B) or expression of heat shock protein families such as HSF-1, hsp70 and hsp90. E4031, a chemical chaperone for hERG, abolished the pilsicainide effect on hERG. Chronic treatment with pilsicainide could also increase the protein expression of trafficking-defective mutant hERG, G601S and R752W.

Conclusions: Pilsicainide penetrates the plasma membrane, stabilizes WT-hERG proteins by acting as a chemical chaperone, and enhances WT-hERG channel currents. This mechanism could also be applicable to modulations of certain mutant-hERG proteins.

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1. Introduction

Human ether-a-go-go-related gene (hERG) encodes the alpha subunit of the rapidly-activating delayed-rectifier K⁺ channel, which plays a pivotal role in repolarization of cardiac action potentials (APs) [1]. Most of mutant hERG proteins causing the type 2 long QT syndrome (LQT2) fail to mature in the endoplasmic reticulum (ER) and Golgi apparatus [2]. Because of their instability, they are degraded through the ubiquitin proteasome system (UPS), resulting in reductions of hERG protein expression on the cell membrane and channel currents [3].

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A possible way to rescue mutant hERG proteins is the use of a chemical chaperone, which is defined as a small molecule that can bind to a protein and stabilize it [4]. Several agents have been reported to bind to hERG proteins inside the cell and restore their stability, including hERG activators [5] and hERG blockers [6,7]. hERG channel blockers, such as E4031, fexofenadine and astemizole, facilitated maturation of hERG proteins by acting as chemical chaperones [8]; however, these agents cannot be used clinically for enhancing hERG channel currents, because they acutely block the rapidly-activating delayed-rectifier K+ channel current (I_{Kr}).

Pilsicainide (C_{12}H_{24}N_{4}O) is a widely used antarrhythmic agent, especially for treating atrial fibrillation [9]. It belongs to the class Ic antarrhythmic agent (Na+ channel blocker) as classified by Vaughan Williams [10], reducing the maximum rate of AP upstroke in atria, ventricles and Purkinje fibers [11]. The effects of pilsicainide on AP repolarization are controversial: Pilsicainide did not influence AP repolarization in rat ventricular myocytes [12]. However, pilsicainide has been reported to block hERG channel currents expressed in HEK293 cells [13], implying the reduction of I_{Kr} and prolongation of AP duration (APD) by the agent. In contrast, pilsicainide shortened APDs in canine Purkinje fibers [11] and in guinea pig ventricular myocardia [14], which might reflect the enhancement of I_{Kr} via stabilization of hERG proteins. However, it remains unknown whether pilsicainide actually stabilizes hERG proteins. In the present study, we studied the effects of acute and chronic exposures to pilsicainide on the expression of hERG proteins. In the present study, we studied the effects of acute and chronic exposures to pilsicainide on the expression of hERG proteins with a mutation of G601S or R752W, both of which are reported to bind to hERG proteins inside the cell and restore their stability, including hERG activators [5] and hERG blockers [6,7].

### 2. Materials and methods

#### 2.1. Cell culture and establishment of HEK293 cell lines stably expressing WT-hERG and mutant hERG

cDNA encoding the wild-type hERG protein tagged with the FLAG octapeptide epitope (WT-hERG-FLAG) was cloned in a mammalian expression vector, pcDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA). Individual expression plasmids were transfected into HEK293 cells using Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. To establish the cells stably expressing WT-hERG-FLAG, they were cultured in the presence of 1 mg/ml Geneticin (G418) and cell clones were then harvested. The expression of WT-hERG-FLAG was confirmed by Western blotting.

Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Nichirei Biosciences, Tokyo, Japan) and 0.5% penicillin-streptomycin G (Wako, Osaka, Japan) at 37 °C in a 5% CO2 incubator in the presence and absence of pilsicainide at 0.03–10 μM.

We also used HEK293 cells stably expressing mutant hERG proteins with a mutation of G601S or R752W, both of which are trafficking-defective. G601S locates in the pore region of hERG channels, while R752W in the intracellular domain, as described elsewhere [15].

#### 2.2. Western blotting

Cells were scraped into lysis buffer (PBS/1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml amphotin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride) and lysed by sonication; insoluble materials were then removed by centrifugation. Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA, USA). Ten μg of proteins was separated on 7.5% SDS-PAGE and electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA). Membranes were probed with antibodies against FLAG (1:1000; Agilent Technologies, Santa Clara, CA, USA), β-actin (1:5000; Abcam, Tokyo, Japan), total and phosphorylated Akt (protein kinase B) (1:1000; Enzo Life Sciences, NY, USA), and heat shock protein (hsp) families of the heat shock factor 1 (HSF-1), hsp70 and hsp90 (1:1000; Enzo Life Sciences, NY, USA), and were developed using an enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ, USA). Band intensities were quantified using Image J software (NIH, Bethesda, MD, USA).

To determine the half-maximal effective concentration (EC_{50}) of pilsicainide for the increase of hERG proteins, concentration dependence data were fitted by the following equation:

$$D = \frac{(D_{\text{max}} - 100) \times [C]/(C + EC_{50}) + 100}{100},$$

where D (%) represents the normalized hERG protein density at a given concentration ([C]). D_{max} (%) denotes the maximum attainable density.

#### 2.3. Chase assay

HEK293 cells stably expressing WT-hERG-FLAG were seeded into 6-well plates in the presence or absence of pilsicainide at 3 μM. After the addition of the protein synthesis inhibitor cycloheximide (60 μg/ml), protein extracts were prepared at 2–24 h and subjected to anti-FLAG Western blotting. Band intensity was quantified using the Image J software. The decay rate constant (k) for the density of expressed WT-hERG-FLAG proteins was determined using OriginPro 9.1 (Origin Lab, Northampton, MA, USA). The half-life (t_{1/2}) of the protein was calculated using the formula t_{1/2} = 0.693/k.

#### 2.4. Immunofluorescence

HEK293 cells stably expressing WT-hERG-FLAG were seeded on gelatin-coated coverslips and transfected with pDsRed2-E2 (Clontech, Mountain View, CA, USA), pDsRed-Monomer-Golgi (Clontech) or pPM-mKeima-Red (BML, Tokyo, Japan). Twenty-four hour later, they were fixed with 4% paraformaldehyde/PBS and then permealzed with 0.5% Triton X-100. After blocking in 3% albumin solution diluted with PBS containing 0.5% Triton X-100, they were incubated for 1 h with anti-FLAG antibody (1:200; Agilent Technologies, Santa Clara, CA, USA). Bound antibodies were visualized with Alexa Fluor 488-conjugated mouse secondary antibody (1:2000; Invitrogen, Eugene, OR, USA) and images were obtained by using a Bio-Rad MRC 1024 confocal microscope (Hercules, CA, USA). All the staining procedures were conducted at room temperature in the presence or absence of pilsicainide at 3 μM.

#### 2.5. Electrophysiological recordings

WT-hERG channel currents were recorded by the whole-cell patch-clamp technique. The extracellular solution had the following composition (mM): NaCl 140, KCl 4, MgCl2 1.8, MgSO4 0.53, NaH2PO4 0.33, glucose 5.5, HEPES 5, with pH adjusted to 7.4 by NaOH. The internal pipette solution contained (mM) K-aspartate 100, KCl 20, CaCl2 1, Mg-ATP 5, EGTA 5, HEPES 5, and creatine phosphate dipotassium salt 5 (pH 7.2 with KOH). Patch pipettes had a resistance of 2–4 MΩ when filled with the pipette solution. After rupture of the cell membrane, whole-cell membrane currents were recorded at 37 °C with a holding potential (HP) of –50 mV. Series resistance (R_s) was determined by fitting a single exponential function to the capacitive current decay to estimate its time constant (τ) and membrane capacitance (C_m); R_s calculated with the equation $R_s = \tau/C_m$ during the capacitive current.
cancellation averaged 2.2 ± 0.4 MΩ with τ = 98 ± 8 μs and
Cm = 46 ± 2 pF (n = 5). After 50–60% compensation of Rm, voltage
errors arising from the Rm were estimated to be less than 5 mV. The
membrane potential was not corrected for the liquid junction
potential, which was estimated to be < 10 mV. Currents were
elicited by 300-ms depolarizing test pulses ranging from −50 to
+50 mV (in 10 mV increments). To isolate hERG channel currents,
E4031, a blocker selective for hERG channels, was added at 10 μM
to the external solution; E4031-sensitive currents were deter-
dined by digital subtraction of the currents recorded in the pre-
sence of 10 μM E4031 from those recorded without E4031. The peak
currents during the depolarizing test pulses were measured
and plotted as functions of the test potentials.

2.6. Synthesis of membrane-impermeant pilsicainide

Commercial pilsicainide (Sigma Aldrich, Tokyo, Japan) was
provided as a hydrochloric salt (pilsicainide-HCl); this form is
immediately converted to the non-protonic form (pilsicainide)
commercially named as pilsicainide (N-Me-PIL-MeSO4) which has the same struc-
ture, and thus turned our attention to the methylsulfate of N-
methylpilsicainide (N-Me-PIL-MeSO4) which has the same struc-
tural motif as pilsicainide. This analogue cannot be converted to the
original molecule (pilsicainide) even if it is treated with a
strong base, although the counter anion could be changeable
(Supplemental Fig. S1, bottom). Therefore, it is expected that
pilsicainide-HCl and N-Me-PIL-MeSO4 should show completely
different cell membrane permeant properties: The former can pass
through the cell membrane freely; the latter cannot pass, because a
positively-charged compound should be trapped by the
negatively-charged lipid phosphate moiety in the cell mem-
brane. Thus, we decided to prepare N-Me-PIL-MeSO4 as a model com-
 pound for the membrane-impermeant pilsicainide analogue in
this study. For the details of the synthesis, see Supporting
Information.

2.7. Qualitative RT-PCR

Total RNAs were extracted from HEK293 cells using an RNeasy
Plus mini kit (QIAGEN, Tokyo, Japan) and were then subjected to
RT-PCR assays using PrimeScrips RT-PCR Kit (Takara, Kusatsu,
Japan). RNA samples were treated with DNase I (Promega, Tokyo,
Japan). RNA samples were treated with DNase I (Promega, Tokyo,
Japan). Primers used were as follows: hERG
forward primer, GGGCTCTCATGAGATCCT; hERG reverse primer,
AGGCCCTGCATACGGTCA; GAPDH forward primer, TGAACC-
GAAGCTCACGTGG; GAPDH reverse primer, TCCACCACCTGTGCT
GTA.

2.8. Statistical analysis

All data were presented as mean ± SEM, and were analyzed
using Student t-test to compare treated and untreated groups. The differences between the groups were considered significant at
P < 0.05.

3. Results

3.1. Acute effects of pilsicainide on the expression of WT-HERG
proteins and channel currents

Fig. 1A shows the acute effects of pilsicainide on the hERG
protein expression in HEK293 cells stably expressing WT-hERG.
Treatment with pilsicainide at 0.03–10 μM for 10 min influenced
neither the expression level of the mature form (155 kDa) nor that
of the immature form (135 kDa). We also examined the effects of
10 min perfusions of pilsicainide at 3 μM on WT-hERG channel
currents in HEK293 cells stably expressing WT-hERG (Fig. 1B).
Acute exposure to 3 μM pilsicainide did not significantly influence
the peak amplitude of hERG currents.

3.2. Chronic effects of pilsicainide on the expression of WT-hERG
proteins and channel currents

Fig. 2A shows effects of the chronic exposure to pilsicainide for
48 h on the expression of WT-hERG proteins in HEK293 cells
stably expressing WT-hERG. Pilsicainide at 0.03–10 μM increased
the mature form (155 kDa) of WT-HERG proteins as well as the
immature form (135 kDa) in a concentration-dependent manner.
The EC50 value for the pilsicainide-induced increase of the
immature form of hERG was 0.33 μM. As shown in Supplemental
Fig. S2, pilsicainide at 3 μM did not influence the expression of
hERG mRNA.

We further studied the effects of chronic treatment with pil-
sicainide at 3 μM on the E4031-sensitive WT-hERG current
(Fig. 2B). Chronic treatment with 3 μM pilsicainide for 48 h sig-
nificantly increased E4031-sensitive currents. The current-voltage
relationships for E4031-sensitive peak currents indicate that the
chronic treatment with 3 μM pilsicainide significantly increased
the peak amplitude of WT-hERG channel currents at the test
potentials ranging from 0 mV to +40 mV.

3.3. Chronic effects of pilsicainide on degradation of WT-HERG
proteins

To clarify effects of chronic treatment with pilsicainide on the
stability of WT-hERG proteins, we examined WT-hERG protein
degradation by the chase experiments. In the absence of pilsicai-
nide, the immature form of WT-HERG proteins was degraded with
the half-life of 4.9 ± 0.8 h, as shown in Fig. 3A (left) and B. Treatment
with 3 μM pilsicainide for 48 h significantly slowed the
degradation process (Fig. 3A, right), prolonging the half-life of the
immature form to 8.9 ± 0.8 h (Fig. 3B).

3.4. Chronic effects of pilsicainide on the intracellular localization
of WT-HERG proteins

Fig. 4 shows the intracellular localization of WT-hERG proteins
in the absence and presence of pilsicainide at 3 μM. Immuno-
fluorescence demonstrated that the signals of WT-hERG-FLAG co-
localized with those of the ER, Golgi apparatus and plasma
membrane (PM), as depicted by DsRed2-ER (#1–3), DsRed-
Monomer-Golgi (#7–9), and PM-mKeima-Red (#13–15), respec-
tively. In the presence of pilsicainide, the signal of WT-HERG-FLAG
significantly increased in the ER (#4–6), Golgi apparatus (#10–12)
and PM (#16–18).

3.5. Failure of a membrane-impermeant pilsicainide derivative to
stabilize WT-HERG

Fig. 5 shows the effects of the cell membrane-impermeant form
of pilsicainide (N-Me-PIL-MeSO4) on the expression of WT-hERG.
Chronic treatment with this form of pilsicainide failed to increase the protein level of WT-hERG.

3.6. Pilsicainide influenced neither phosphorylation of Akt nor the expression levels of hsp family proteins

Since it has been reported that antiarrhythmic agents could modulate ion channel activity via activation of the Akt signal, we tested whether pilsicainide could influence phosphorylation of Akt. As shown in Fig. 6A, LY294002, a PI3-kinase inhibitor, abolished phosphorylation of Akt without changing the total expression of Akt, but did not affect the expression of WT-hERG. Pilsicainide at 3 μM, not influencing phosphorylation of Akt, increased the protein level of WT-hERG even in the presence of LY294002.

It is also known that HSF-1, hsp70 and hsp90 could stabilize breaking-defective mutant hERG proteins observed in LQT2 patients. As shown in Fig. 8, therefore, we examined the effects of pilsicainide on the expressions of two trafficking-defective mutant hERG proteins, G601S and R752W. Pilsicainide at 1 and 3 μM obviously increased the immature form (135 kDa) of G601S and also slightly increased the mature form (155 kDa). By contrast, pilsicainide at 3 and 10 μM increased the immature form of R752W without changes in its mature form.

3.7. Chronic effects of pilsicainide on WT-hERG expressions in the presence of E4031

It has been reported that E4031 increases hERG expression levels via an action as a chemical chaperone [7]. To determine whether pilsicainide could also enhance the expression of WT-hERG by acting as a chemical chaperone, we examined the effects of pilsicainide on WT-hERG expression levels in the presence of E4031. As shown in Fig. 7, 10 μM E4031 as well as 3 μM pilsicainide increased WT-hERG expression levels, as expected. However, in the presence of E4031, pilsicainide could not further enhance WT-hERG expressions.

3.8. Chronic effects of pilsicainide on the trafficking-defective mutant hERG expression

It is important to test whether pilsicainide can stabilize trafficking-defective mutant hERG proteins observed in LQT2 patients. As shown in Fig. 8, therefore, we examined the effects of pilsicainide on the expressions of two trafficking-defective mutant hERG proteins, G601S and R752W. Pilsicainide at 1 and 3 μM obviously increased the immature form (135 kDa) of G601S and also slightly increased the mature form (155 kDa). By contrast, pilsicainide at 3 and 10 μM increased the immature form of R752W without changes in its mature form.

4. Discussion

In the present study, chronic treatment with pilsicainide stabilized WT-hERG proteins, increased WT-hERG channels in the plasma membrane, and thereby enhanced the hERG channel current $i_{K_r}$. Pilsicainide exerted these effects inside the cell via an action as a chemical chaperone, independently of phosphorylation of Akt and enhanced expression of HSF-1, hsp70 or hsp90. Pilsicainide is a class 1c antiarrhythmic agent that blocks Na+ channels and reduces the maximum rate of AP upstroke. Pilsicainide occasionally caused QT prolongation, suggesting its inhibitory...
Fig. 2. Chronic effects of pilsicainide on the expression of WT-hERG proteins and channel currents. (A) Representative Western blots for WT-hERG-FLAG proteins expressed with or without pilsicainide (left). Cells stably expressing WT-hERG were exposed to pilsicainide at 0.03–10 μM for 48 h. Cell lysates were subjected to Western blotting with anti-FLAG and anti-β-actin antibodies. Two bands for the mature form of 155 kDa and immature form of 135 kDa were observed. The band density of WT-hERG-FLAG proteins was quantified as a ratio to that of β-actin, and was then normalized to that of WT-hERG-FLAG in the absence of pilsicainide (right). Differences were tested for statistical significance by two-way ANOVA: *P < 0.05 vs. Control (n=10 each). (B) Representative traces of E4031-sensitive hERG channel currents recorded from HEK293 cells treated with or without pilsicainide at 3 μM for 48 h (left). Current-voltage relationships were determined for WT-hERG-FLAG protein-mediated peak currents during the depolarizing test pulses in the absence and presence of pilsicainide (right; n=10 each). Differences were tested for statistical significance by two-way ANOVA: *P < 0.05 vs. Control. Error bars smaller than the radius of the circles are not shown.

Fig. 3. Chronic effects of pilsicainide on the degradation of WT-hERG proteins. (A) Representative Western blots of residual WT-hERG proteins at 0–24 h after treatment with cycloheximide in the absence (Control) and presence of pilsicainide. Cells in the pilsicainide-treated group were exposed to 3 μM pilsicainide for 48 h prior to the treatment with cycloheximide. (B) Decay of WT-hERG proteins in the absence (Control) and presence of 3 μM pilsicainide. The amount of expressed hERG-FLAG was quantified as a ratio to that of β-actin, and was then normalized to that at time=0. The lines are the fits with a single exponential function. *P < 0.05, vs. Control (n=3 each).
proteins were transfected with pDsRed2-ER, pDsRed-Monomer-Golgi or pPM-mKeima-Red, and were stained with anti-FLAG and anti-marker antibodies.

therapeutic concentrations of 0.20 μM or less increased WT-hERG protein expressions. Representative Western blots are shown for WT-hERG-FLAG proteins expressed in cells with or without the membrane-impermeant pilsicainide at indicated concentrations for 48 h.

action on outward K+ currents. Wu et al. have reported that, although pilsicainide barely affected K+ and Ca2+ currents at therapeutic concentrations of 0.20–0.90 μg/mL (0.73–3.31 μM), it blocked hERG channel currents at concentrations higher than the therapeutic range [13]. In the present study, chronic treatment with pilsicainide at therapeutic concentrations significantly increased WT-hERG protein expressions via the prolongation of the half-life of WT-hERG proteins and thereby enhanced hERG channel currents, whereas acute treatment with pilsicainide at 10 μM or less influenced neither hERG protein expressions nor channel currents. Thus, while pilsicainide has been observed to cause QT prolongation occasionally, it can also shorten QT intervals by enhancing Ik1.

It is well known that Na+ channel blockers bind to the Na+ channel from inside of the cell but the charged form blocker cannot penetrate the plasma membrane [16]. In the present study, the membrane-impermeant form of pilsicainide (N-Me-PIL-MeSO4) did not increase the protein level of WT-hERG, suggesting that pilsicainide normally penetrates the plasma membrane and induces post-translational modifications of the WT-hERG protein.

Pilsicainide might increase hERG proteins via facilitating the transcription of hERG from DNA to RNA or the translation of hERG RNA into hERG protein. Qualitative RT-PCR demonstrated the absence of an increase in hERG mRNA expression levels after pilsicainide treatment [Supplemental Fig. S2], indicating that pilsicainide does not influence hERG transcription. Inhibition of the pilsicainide effect by E4031, a known chemical chaperone for hERG, suggests post-translational modifications, rather than accelerated translation, for the pilsicainide effect. Nevertheless, further experiments are necessary to confirm post-translational modifications by pilsicainide.

Chronic treatment with antiarrhythmic agents could induce post-translational modifications of ion channels and exert pro- and anti-arrhythmic actions, independent of their acute actions on ion channels. For instance, some antiarrhythmic agents could modify Na+ channel activities via phosphorylation of Akt [17]. Phosphorylated Akt is well known to increase hERG channel currents [18]. In this study, however, chronic exposure to pilsicainide significantly enhanced the expression of WT-hERG proteins without phosphorylating Akt.

It has been reported that HSF-1, hsp70 and hsp90 increase both the mature and immature forms of WT and mutant hERG proteins, and also facilitate the maturation of mutant hERG proteins [15]. Local anesthetics induced hsp70 expression [19]; thus, pilsicainide may exert its effects via modulating these regulatory proteins. In the present study, however, pilsicainide did not influence the expression of HSF-1, hsp70 or hsp90, excluding the involvement of these regulators in the pilsicainide-induced enhancement of WT-hERG protein expressions.

The most prominent finding of this study is that pretreatment with E4031 abolishes the enhancement of WT-hERG protein expression by pilsicainide. Since E4031 is well known to increase WT-hERG protein levels as a chemical chaperone, the present finding suggests that pilsicainide acts on WT-hERG proteins as a chemical chaperone, like E4031. There are three possible mechanisms for the pilsicainide-induced increase of hERG proteins via post-translational modifications: (1) phosphorylation of Akt,
(2) activation of hsp expressions, and (3) direct action as a chemical chaperone. Involvements of the Akt and hsp pathways have been denied by our experiments (Fig. 6). A chemical chaperone is defined as a chemical agent that binds to a target protein, stabilizes its structure, and delays its degradation. In our study, pilsicainide prolonged the half-life of hERG proteins, and the chemical chaperone E4031 canceled the enhancement of hERG protein expression by pilsicainide. Taken together, we conclude that pilsicainide acts as a chemical chaperone to increase hERG proteins with facilitation of their maturation.

Chronic treatment with pilsicainide at 0.03–10 μM could stabilize WT-hERG proteins. The minimum effective concentration of pilsicainide for the action as a chemical chaperone was 0.3 μM, which is far less than its therapeutic plasma concentrations of

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**Fig. 6.** Effects of pilsicainide on phosphorylation of Akt and expressions of hsp family proteins. (A) Representative Western blots for WT-hERG-FLAG, phosphorylated Akt (p-Akt), total Akt and β-actin in cells treated with or without pilsicainide in the absence and presence of the PI3-kinase inhibitor LY294002. Cells stably expressing WT-hERG were exposed to 3 μM pilsicainide for 48 h. (B) Representative Western blots for WT-hERG-FLAG, HSF-1, hsp70, hsp90 and β-actin in the cells treated with or without pilsicainide. Cells stably expressing WT-hERG were exposed to 3 μM pilsicainide for 48 h.

**Fig. 7.** Treatment with E4031 abolished the pilsicainide-induced increases in protein expression of WT-hERG. (A) Representative Western blots for WT-hERG-FLAG and β-actin in cells treated with or without pilsicainide in the presence and absence of 10 μM E4031. Cells stably expressing WT-hERG were exposed to 3 μM pilsicainide for 48 h. (B) Summary of the effects of pilsicainide on the mature (155 kDa) and immature (135 kDa) forms of WT-hERG-FLAG proteins in the presence of absence of 10 μM E4031. The amount of expressed WT-hERG-FLAG proteins was quantified as a ratio to that of β-actin, and was then normalized to that of WT-hERG-FLAG in the absence of the agents (Control). *P < 0.05 vs. Control (n = 5 each).

**Fig. 8.** Concentration-dependent effects of pilsicainide on the protein expression of the hERG mutants G601S and R752W in transfected HEK293 cells. Cells stably expressing G601S or R752W mutants were exposed to pilsicainide at 0.03–10 μM for 48 h. Cell lysates were subjected to Western blotting with the indicated antibodies.
around 3 μM [20]. The clinical relevance of the enhancing effects of pilsicainide on WT-hERG expression is clear; chronic treatment with pilsicainide could shorten the ventricular APD, because hERG channels are expressed predominantly in ventricular myocytes and significantly contribute to ventricular AP repolarization [1].

Thus, pilsicainide as a chemical chaperone will suppress early afterdepolarizations by increasing mutant hERG proteins and ï¥ in LQTS cardiomyocytes [21]. Augmentation of ï¥ results in a shortening of APDs and a reduction of Ca2+ load in ventricular muscles, and might also suppress ventricular arrhythmias [20]. However, one limitation of this study is that our experiments were performed on the heterologous system of HEK293 cells but not on cardiomyocytes; thus, it is difficult to apply our findings directly to the heart. Further experiments will be necessary to confirm whether pilsicainide can increase hERG proteins and channel currents in cardiac myocytes.

It is important to know whether pilsicainide can be used as a therapeutic agent for LQT2 patients; and if so, we should determine the types of LQT2 mutation carriers to whom pilsicainide could be administered. As shown in Fig. 8, we examined the effects of pilsicainide on two mutant hERG proteins, G601S and R752W, both of which are trafficking-defective. G601S locates in the pore region of hERG channels, while R752W in the intracellular domain. Pilsicainide at the clinical concentrations could facilitate the maturation of hERG channel proteins with the mutations in the pore region and in the intracellular domain; however, further experiments are necessary to determine which LQT2 mutation carriers will receive therapeutic benefits from pilsicainide.

5. Conclusion

Chronic treatment with 0.03–10 μM pilsicainide for 48 h enhanced the expression of WT-hERG proteins via delaying their degradation, increasing hERG channel expression and activity on the plasma membrane. Pilsicainide penetrates the plasma membrane, stabilizes WT-hERG proteins by acting as a chemical chaperone, and enhances WT-hERG channel currents without influencing phosphorylation of Akt or expressions of hsp family proteins. This mechanism may also be applicable to modulations of trafficking-defective mutant hERG proteins.

Conflict of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.joa.2016.09.003.