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Original Article

Molecular mechanisms underlying the pilsicainide-induced stabilization of hERG proteins in transfected mammalian cells

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ABSTRACT

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.

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 ($C_{17}H_{24}N_2O$) is a widely used antiarrhythmic agent, especially for treating atrial fibrillation [9]. It belongs to the class Ic antiarrhythmic 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 and found that chronic treatment with pilsicainide as a chemical chaperon could stabilize hERG proteins in transfected mammalian cells.

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 (D-MEM; 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% CO_2 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 aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstain, 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:

$$D = (D_{max} - 100) \times [C]/([C] + EC_{50}) + 100, \quad (1)$$

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-ER (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 permealized 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, $CaCl_2$ 1.8, $MgCl_2$ 0.53, NaH_2PO_4 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, $CaCl_2$ 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 $\tau = 98 \pm 8$ μs and $C_m = 46 \pm 2$ 4 pF ($n = 5$). After 50–60% compensation of R_s, voltage errors arising from the R_s 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 determined by digital subtraction of the currents recorded in the presence 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) *in vivo* (Supplemental Fig. S1, *top*), incorporated into the target cell through the cell membrane, and then performs various actions. We envisioned that a membrane-impermeant pilsicainide analogue might be useful to determine the origin of its physiological actions, and thus turned our attention to the methylsulfate of N-methylpilsicainide (N-Me-PIL-$MeSO_4$) which has the same structural 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-$MeSO_4$ 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 membrane. Thus, we decided to prepare N-Me-PIL-$MeSO_4$ as a model compound 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) to eliminate genomic DNA, and cDNA was synthesized using SuperScriptTM II reverse transcriptase (Thermo Fisher Scientific, Yokohama, Japan). Primers used were as follows: hERG forward primer, GGGCTCCATCGAGATCCT; hERG reverse primer, AGGCCTTGCATACAGGTTCA; GAPDH forward primer, TGAACGGGAAGCTCACTGG; GAPDH reverse primer, TCCACCACCCTGTTGCTGTA.

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 EC_{50} 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 pilsicainide at 3 μM on the E4031-sensitive WT-hERG current (Fig. 2B). Chronic treatment with 3 μM pilsicainide for 48 h significantly 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 pilsicainide, 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. Immunofluorescence demonstrated that the signals of WT-hERG-FLAG colocalized 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), respectively. 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-$MeSO_4$) on the expression of WT-hERG.

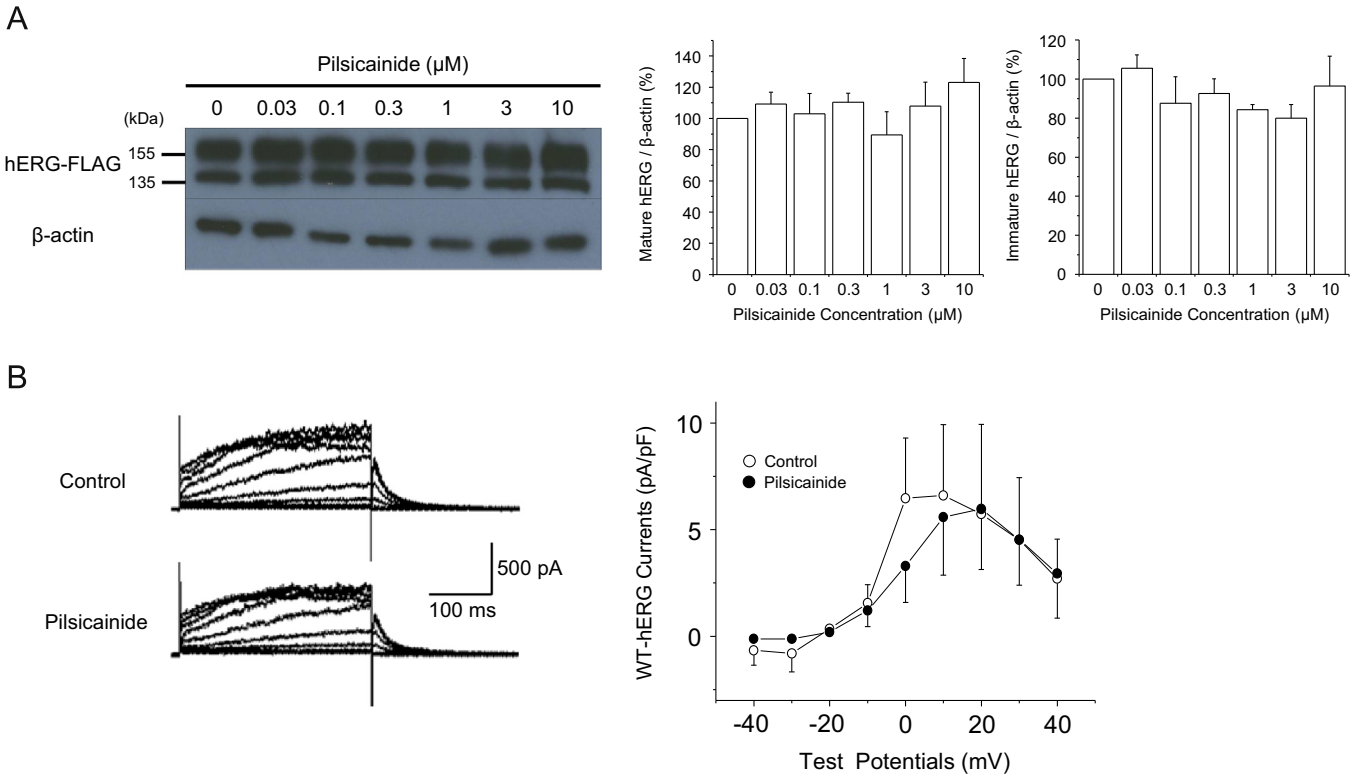

Fig. 1. Acute effects of pilsicainide on the expression of WT-hERG proteins and channel currents. (A) Effects of 10 min application of pilsicainide at 0.03–10 μM on WT-hERG protein expressions. Representative Western blots show bands of hERG proteins at 135 kDa (immature form) and 155 kDa (mature form) with those of β-actin being used for normalizing the band density of hERG proteins (*left*). The density of the immature and mature forms of hERG proteins determined at each concentration of the agent was normalized first to that of β-actin and then to that of WT-hERG-FLAG in the absence of pilsicainide (*right*; $n=4$ each). (B) Effects of 10 min application of pilsicainide on hERG channel currents. Shown are representative traces of E4031-sensitive hERG channel currents recorded in HEK293 cells expressing WT-hERG-FLAG in the absence (Control) and presence of pilsicainide at 3 μM (*left*). Current–voltage relationships were determined for WT-hERG-FLAG protein-mediated peak currents during the depolarizing test pulses (*right*; $n=10$ each). Error bars smaller than the radius of the circles are not shown.

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 WT-hERG proteins [15]; therefore, we tested whether pilsicainide would increase the expressions of these regulatory proteins. Fig. 6B shows the effects of pilsicainide on the expressions of HSF-1, hsp70 and hsp90 as well as WT-hERG-FLAG proteins. Pilsicainide at 3 μM increased the expression levels of WT-hERG without enhancing the expression of HSF-1, hsp70 or hsp90.

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_{Kr}. 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 Ic antiarrhythmic agent that blocks Na^+ channels and reduces the maximum rate of AP upstroke. Pilsicainide occasionally caused QT prolongation, suggesting its inhibitory

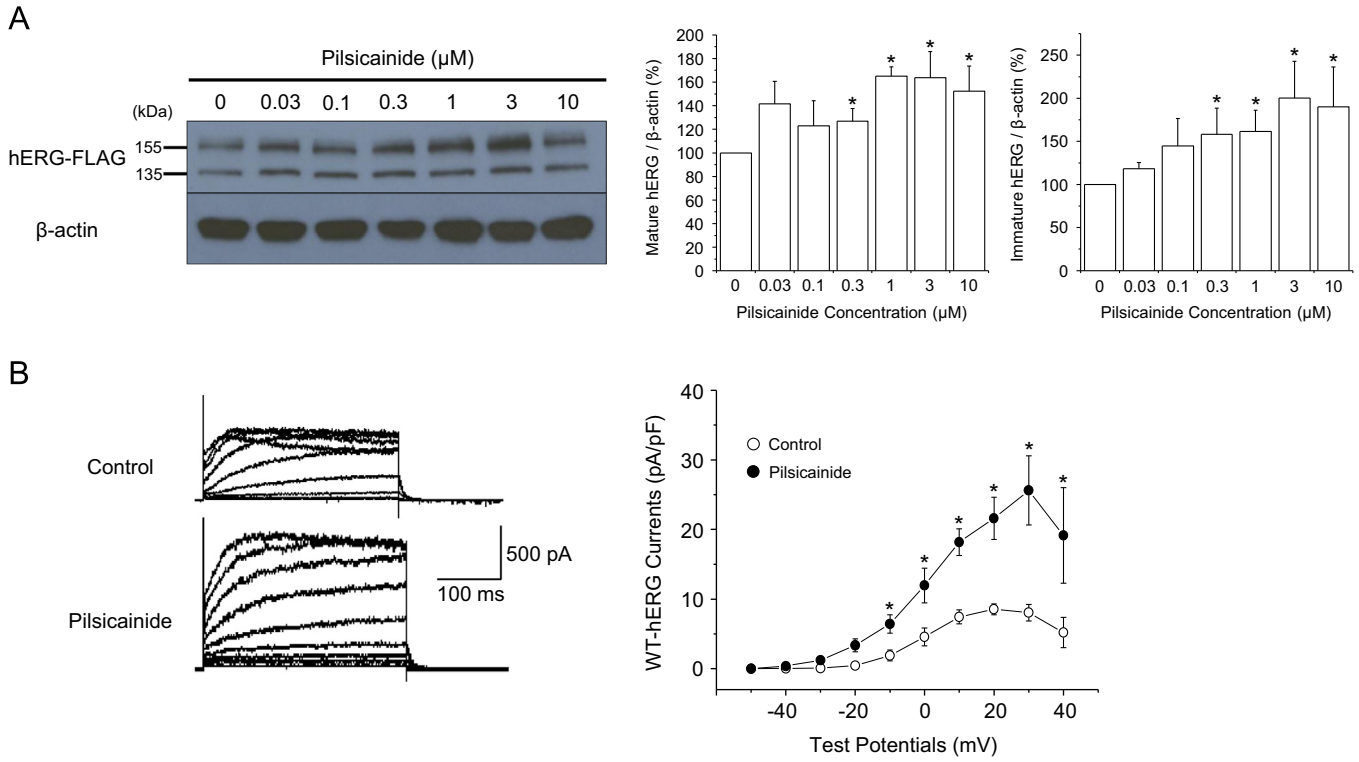

Fig. 2. Chronic effects of pilsicainide on the expression of WT-hERG proteins and channel currents. (A) Representative Western blots for WT-hERG-FLAG proteins expressed with or without pilsicainide (*left*). Cells stably expressing WT-hERG were exposed to pilsicainide at 0.03–10 μM for 48 h. Cell lysates 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 (0) ($n = 10$ each). (B) Representative traces of E4031-sensesitive 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.

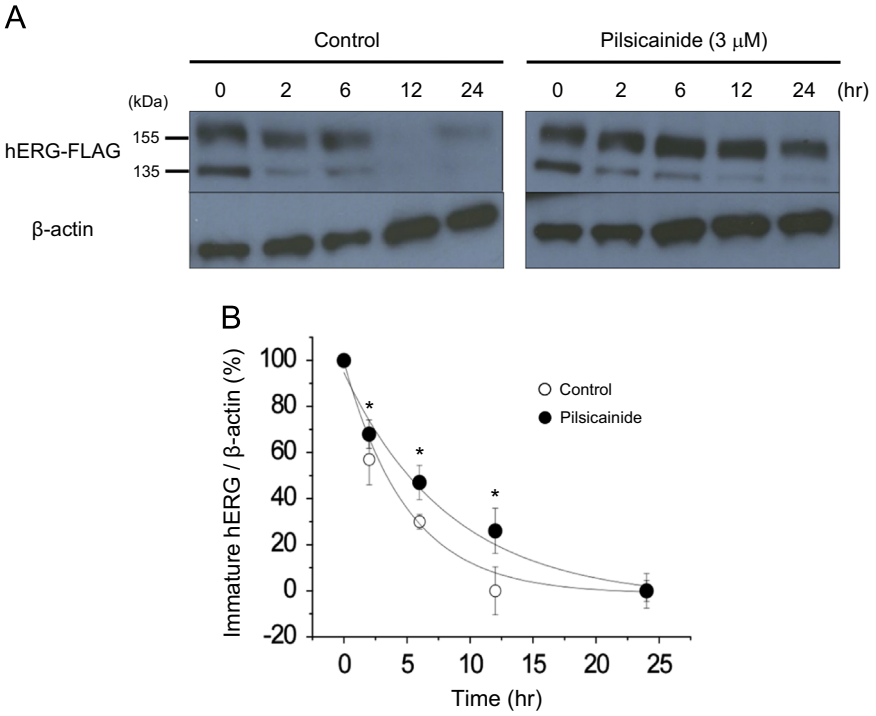

Fig. 3. Chronic effects of pilsicainide on the degradation of WT-hERG proteins. (A) Representative Western blots of residual WT-hERG proteins at 0–24 h after treatment with cycloheximide in the absence (Control) and presence of pilsicainide. Cells in the pilsicainide-treated group were exposed to 3 μM 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).

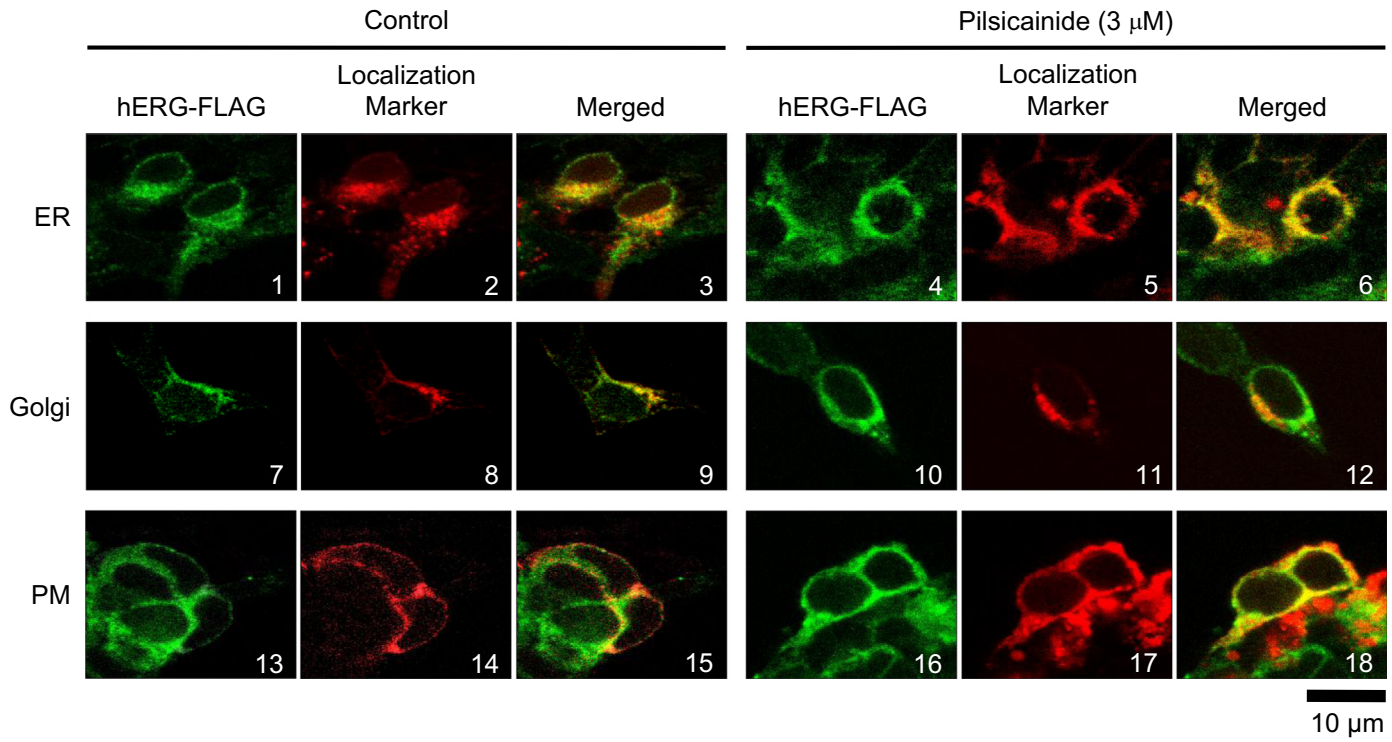

Fig. 4. Effects of pilsicainide on the intracellular localization of WT-hHERG-FLAG proteins. Representative immunofluorescence images obtained by a confocal microscope are shown for the WT-hERG-FLAG protein and DsRed2-ER (*top*), DsRed-Monomer-Golgi (*middle*) or PM-mKeima-Red (*bottom*). Cells stably expressing WT-hERG-FLAG proteins were transfected with pDsRed2-ER, pDsRed-Monomer-Golgi or pPM-mKeima-Red, and were stained with anti-FLAG and anti-marker antibodies.

action on outward K^+ currents. Wu et al. have reported that, although pilsicainide barely affected K^+ and Ca^{2+} 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 I_{Kr}.

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-$MeSO_4$) 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 chaperon 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

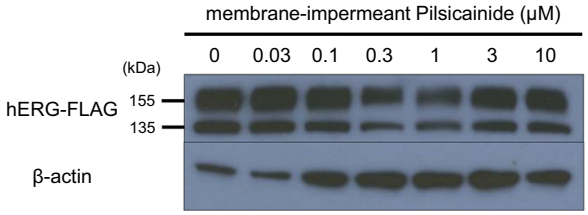

Fig. 5. Effect of membrane-impermeant pilsicainide (N-Me-PIL-$MeSO_4$) on protein expression of hERG. Representative Western blots are shown for WT-hERG-FLAG proteins expressed in the cells with or without the membrane-impermeant pilsicainide at 0.03–10 μM. Cells stably expressing WT-hERG were exposed to pilsicainide at indicated concentrations for 48 h.

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,

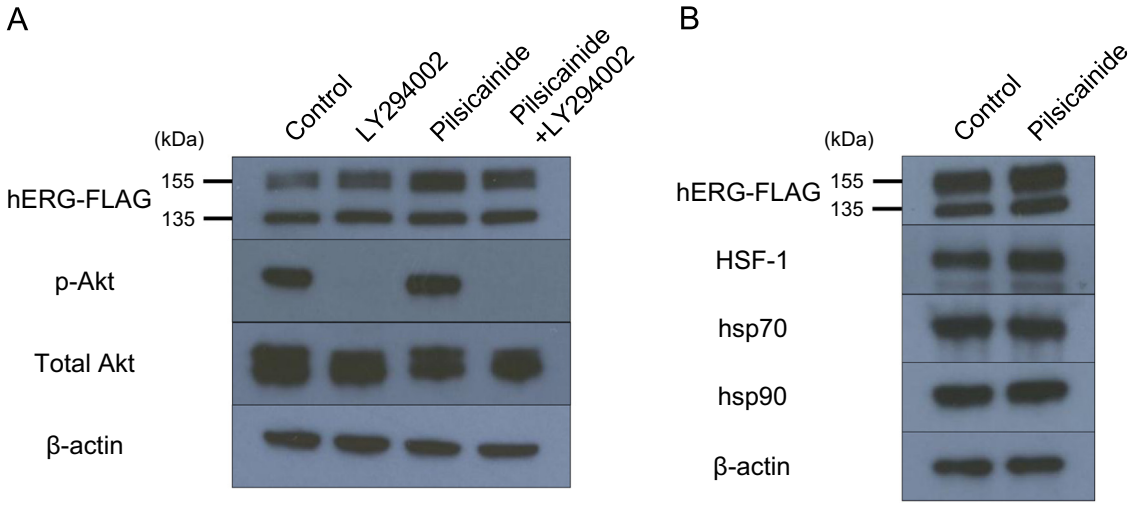

Fig. 6. Effects of pilsicainide on phosphorylation of Akt and expressions of hsp family proteins. (A) Representative Western blots for WT-hERG-FLAG, phosphorylated Akt (p-Akt), total Akt and β-actin in cells treated with or without pilsicainide in the absence and presence of the PI3-kinase inhibitor 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.

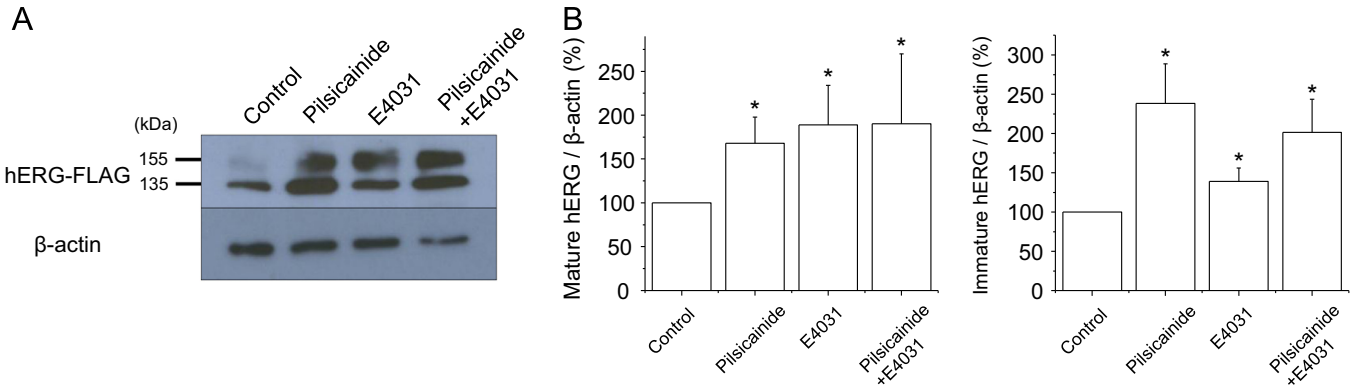

Fig. 7. Treatment with E4031 abolished the pilsicainide-induced increases in protein expression of WT-hERG. (A) Representative Western blots for WT-hERG-FLAG and β-actin in cells treated with or without pilsicainide in the presence and absence of 10 μM E4031. Cells stably expressing WT-hERG were exposed 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).

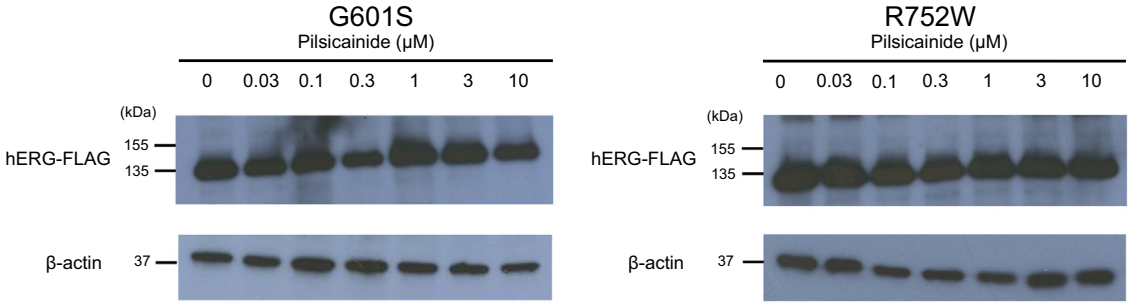

Fig. 8. Concentration-dependent effects of pilsicainide on the protein expression of the hERG mutants G601S and R752W in transfected HEK293 cells. Cells stably expressing G601S or R752W mutants were exposed to pilsicainide at 0.03–10 μM for 48 h. Cell lysates were subjected to Western blotting with the indicated antibodies.

(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

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 I_{Kr} in LQTS cardiomyocytes [21]. Augmentation of I_{Kr} results in a shortening of APDs and a reduction of Ca^{2+} 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.

References

[1] Sanguinetti MC, Jiang C, Curran ME, Keating MT. A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the I_{Kr} potassium channel. Cell 1995;81:299–307.
[2] Surawicz B. Electrophysiologic substrate of torsade de pointes: dispersion of repolarization or early afterdepolarizations? J Am Coll Cardiol 1989;14:172–84.
[3] Li P, Ninomiya H, Kurata Y, et al. Reciprocal control of hERG stability by hsp70 and hsc70 with implication for restoration of LQT2 mutant stability. Circ Res 2011;108:458–68.
[4] Gordo-Gilart R, Andueza S, Hierro L, Jara P, Alvarez L. Functional rescue of trafficking-impaired ABCB4 mutants by chemical chaperones. PLoS One 2016;11:e0150098.
[5] Zhang KP, Yang BF, Li BX. Translational toxicology and rescue strategies of the hERG channel dysfunction: biochemical and molecular mechanistic aspects. Acta Pharmacol Sin 2014;35:1473–84.
[6] Guo J, Zhang X, Hu Z, et al. A422T mutation in HERG potassium channel retained in ER is rescurable by pharmacologic or molecular chaperons. Biochem Biophys Res Commun 2012;422:305–10.
[7] Zhou Z, Gong Q, January CT. Correction of defective protein trafficking of a mutant HERG potassium channel in human long QT syndrome: pharmacological and temperature effects. J Biol Chem 1999;274:31123–6.
[8] Ficker E, Dennis AT, Wang L, Brown AM. Role of the cytosolic chaperones Hsp70 and Hsp90 in maturation of the cardiac potassium channel HERG. Circ Res 2003;92:e87–100.
[9] Komatsu T, Tachibana H, Sato Y, et al. A randomized study on the efficacy of intravenous cibenzoline and pilsicainide administered prior to electrical cardioversion in patients with lone paroxysmal and persistent atrial fibrillation. J Cardiol 2009;53:35–42.
[10] Williams EM. The development of new antiarrhythmic drugs. Schweiz Med Wochenschr 1973;103:262–71.
[11] Hattori Y, Inomata N, Aisaka K, Ishihara T. Electrophysiological actions of N-(2,6-dimethylphenyl)−8-pyrrolizidine-acetamide hydrochloride hemihydrate (SUN 1165), a new antiarrhythmic agent. J Cardiovasc Pharmacol 1986;8:998–1002.
[12] Yatani A, Akaike N. Effects of a new antiarrhythmic compound SUN 1165[N-(2,6-dimethylphenyl)−8-pyrrolizidineacetamide hydrochloride] on the sodium currents in isolated single rat ventricular cells. Naunyn Schmiedebergs Arch Pharmacol 1984;326:163–8.
[13] Wu LM, Orikabe M, Hirano Y, Kawano S, Hiraoka M. Effects of Na^+ channel blocker, pilsicainide, on HERG current expressed in HEK-293 cells. J Cardiovasc Pharmacol 2003;42:410–8.
[14] Fukuda K, Watanabe J, Yagi T, et al. A sodium channel blocker, pilsicainide, produces atrial post-repolarization refractoriness through the reduction of sodium channel availability. Tohoku J Exp Med 2011;225:35–42.
[15] Kondo T, Hisatome I, Yoshimura S, et al. Characterization of the novel mutant A78T-HERG from a long QT syndrome type 2 patient: instability of the mutant protein and stabilization by heat shock factor 1. J Arrhythmia 2016, http://dx.doi.org/10.1016/j.joa.2015.10.005, in press.
[16] Sato R, Hisatome I, Tanaka Y, et al. Aprindine blocks the sodium current in guinea-pig ventricular myocytes. Naunyn Schmiedebergs Arch Pharmacol 1991;344:331–6.
[17] Tao Y, Young WC, Dina MS, et al. Screening for acute I_{Kr} block is insufficient to detect Torsades de Pointes liability: Role of late sodium current. Circulation 2014;130:224–34.
[18] Zhang Y, Wang H, Wang J, Han H, Nattel S, Wang Z. Normal function of HERG K^+ channels expressed in HEK293 cells requires basal protein kinase B activity. FEBS Lett 2003;534:125–32.
[19] Blake MJ, Buckley AR, Buckley DJ, LaVoi KP, Bartlett T. Neural and endocrine mechanisms of cocaine-induced 70-kDa heat shock protein expression in aorta and adrenal gland. J Pharmacol Exp Ther 1994;268:522–9.
[20] Qiu Q, Liu W, Li J, et al. Pharmacokinetics of pilsicainide hydrochloride for injection in healthy Chinese volunteers: A randomized, parallel-group, open-label, single dose study. Clin Ther 2014;36:255–63.
[21] Janse MJ, Wilde AA. Molecular mechanisms of arrhythmias. Rev Port Cardiol 1998;17:41–6.