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Original article

Novel intracellular transport-refractory mutations in *KCNH2* identified in patients with symptomatic long QT syndrome

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ABSTRACT

Background: Missense mutations in *KCNH2*, a gene encoding the Kv11.1 channel, cause long QT syndrome (LQTS) type 2 primarily by disrupting the intracellular transport of Kv11.1 to the plasma membrane. The present study aimed to clarify the functional changes by two novel *KCNH2* missense mutations.
Methods: We performed genetic screening of three unrelated symptomatic LQTS probands with family histories of cardiac symptoms. Chinese hamster ovary cells were transfected with wild-type (WT) and/or mutant *KCNH2* plasmid and examined by patch-clamp technique. Immunostaining and confocal microscopy were performed to evaluate the intracellular localization of WT and homozygous mutant Kv11.1 in human embryonic kidney cells. For the study of trafficking rescue, we used low-temperature incubation (30 °C). We also examined pharmacological rescue of homozygous mutant Kv11.1 current in cells treated with E-4031 or dofetilide.
Results: We identified two novel *KCNH2* missense mutations, G785D and T826I. Electrophysiological study showed that both mutant channels were nonfunctional in homozygous condition and reduced current densities by half in heterozygous condition compared with WT Kv11.1. Heterozygous Kv11.1-G785D produced a significant positive shift in activation and a significant negative shift in inactivation, whereas heterozygous Kv11.1-T826I caused no kinetic changes. Immunostaining revealed that both were transport-refractory mutations. Incubation at 30 °C rescued plasma membrane expression of Kv11.1-T826I but not G785D. We confirmed low-temperature-induced restoration of homozygous Kv11.1-T826I transport by functional current measurements. In contrast, incubation with E-4031 or dofetilide failed to produce measurable currents in both homozygous mutant channels.
Conclusions: Two novel *KCNH2* mutations disrupted the intracellular transport of Kv11.1. Low-temperature incubation rescued plasma membrane expression of Kv11.1-T826I but not G785D. Both mutations exerted loss-of-function effects on Kv11.1 and explained the phenotypes of the mutation carriers.

Introduction

Congenital long QT syndrome (LQTS) is an inherited disease characterized by prolonged corrected QT (QTc) interval on an electrocardiogram (ECG) that can lead to syncope and sudden cardiac death in patients because of ventricular arrhythmias [1,2]. To date, 16 genes have been identified to be responsible for LQTS [3]. *KCNH2* (human ether-à-go-go-related gene) encodes the

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α-subunit of the voltage-gated Kv11.1 channel which produces a rapid component of the cardiac delayed rectifier potassium current (I_{Kr}), and loss-of-function mutations in *KCNH2* have been shown to cause reduction in the outward current during repolarization and LQTS type 2 (LQT2), the second most common form of LQTS [4,5]. A lot of *KCNH2* missense mutations primarily disrupt the intracellular transport of Kv11.1 [5–8]. Most of the transport-refractory *KCNH2* mutations are located in the Per-Arnt-Sim domain at the amino (N)-terminus, pore domain, or cyclic nucleotide-binding domain at the carboxyl (C)-terminus [6]. Some of these transport-refractory mutations are sensitive to pharmacological or temperature-dependent restoration [5,6].

In the present study, we performed genetic screening of three unrelated symptomatic LQTS probands with family histories of cardiac symptoms and identified two novel C-terminal *KCNH2* missense mutations. We performed functional analyses of the mutant Kv11.1 channels, including trafficking rescue, to clarify the mechanism underlying LQT2 caused by these mutations. Furthermore, we performed immunostaining to evaluate the intracellular localization of wild-type (WT) and mutant Kv11.1 proteins.

Methods

Probands and clinical investigation

Three LQTS probands from unrelated families were referred to our hospital for genetic screening. Their QT intervals were manually measured on lead V_5 of the 12-lead ECG and corrected for heart rate (QTc) using Bazett's formula. All subjects submitted written informed consent in accordance with the guidelines approved by our institutional review board.

Genetic analysis

DNA was isolated from peripheral blood leukocytes of the three probands. Genetic screening for LQTS-related genes, including *KCNQ1*, *KCNH2*, and *SCN5A*, was performed using denaturing high-performance liquid chromatography (WAVE system Model 3500; Transgenomic, Omaha, NE, USA) [9] followed by direct sequencing as previously described [10]. Direct sequencing was performed with an automated capillary sequencer (ABI3130; Applied Biosystems, Foster City, CA, USA).

Mutagenesis and transfection

The human WT *KCNH2* cDNA was subcloned into the pRc/CMV vector. Mutant constructs for *KCNH2* missense mutations were generated using the Quick Change-II-XL kit (Stratagene, La Jolla, CA, USA) and verified by DNA sequencing. *KCNH2* plasmids (1 μg of WT, 1 μg of mutant, or 0.5 μg each of WT and mutant) and 0.25 μg of green fluorescent protein cDNA were transfected into Chinese hamster ovary (CHO) cells using lipofectamine 3000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After transfection, the cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12; Nacalai, Kyoto, Japan) supplemented with 10% fetal bovine serum and antibiotics (100 IU ml^{-1} of penicillin and 100 mg ml^{-1} of streptomycin) and incubated in a humidified atmosphere with 5% CO_2. CHO cells were incubated at 37 °C except where noted. For low-temperature-induced trafficking rescue, CHO cells transfected with WT or mutant *KCNH2* plasmids were incubated at 30 °C.

Electrophysiological study

After 48–72 h of transfection, CHO cells attached to glass coverslips were transferred to a recording chamber perfused with extracellular solution and maintained at 36–37 °C. The extracellular solution contained (in mmol/L) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, and HEPES 5.0 (pH was adjusted to 7.4 with NaOH). The pipette solution contained (in mmol/L) potassium aspartate 70, KCl 40, KH_2PO_4 10, EGTA 5, $MgSO_4$ 1, Na_2-ATP 3, Li_2-GTP 0.1, and HEPES 5.0 (pH was adjusted to 7.4 with NaOH). Only green fluorescent protein-positive cells were used for patch-clamp experiments. In all experiments, giga-ohm seal resistances were attained. The I_{Kr} was measured using a whole-cell patch-clamp technique with an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany) and normalized to cell membrane capacitance [11]. Voltage-dependencies of I_{Kr} activation and inactivation were evaluated by fitting the current–voltage (*I–V*) relations of the tail currents to the Boltzmann equation,

$$f(V) = I_{max}/(1 + \exp[(V_h - v)/k]),$$

where I_{max} is the maximum current density of activation or inactivation, V_h is the half-maximum activation or inactivation voltage, v is the test voltage, and k is the slope factor. The deactivation kinetics of I_{Kr} after depolarization were determined using a double exponential equation,

$$I_{K,tail} = A_{fast}\exp(-t/\tau_{fast}) + A_{slow}\exp(-t/\tau_{slow}),$$

where $I_{K,tail}$ is I_{Kr} current of deactivation, A_{fast} and A_{slow} are decay phases of the currents, and τ_{fast} and τ_{slow} are the time constants of deactivation.

Immunofluorescence

Immunostaining was performed to evaluate the intracellular localization of WT and mutant Kv11.1 proteins. Human embryonic kidney (HEK) cells were colabeled with anti-Kv11.1 and endoplasmic reticulum (ER) marker pDsRed2-ER and examined under a confocal microscope. Briefly, 1 μg of WT or 1 μg of mutant *KCNH2* plasmid was transfected into HEK cells together with 1 μg of pDsRed2-ER vector. After transfection, the cells were incubated at 37 °C or 30 °C (where noted) for 48 h, then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 min. Fixed cells were permeabilized using 0.5% Triton X in PBS for 2 min and incubated with anti-Kv11.1 goat polyclonal IgG (1:200) overnight at 4 °C. Subsequently, the cells were washed with PBS and incubated at 37 °C for 2 h with Alexa 488-conjugated donkey anti-goat IgG (1:400). Labeled cells were rinsed with PBS and counterstained with diluted DAPI (1:2000) before observation under a confocal laser scanning microscope (Nikon C1 si, Tokyo, Japan).

Pharmacological rescue

We also examined pharmacological rescue of homozygous mutant Kv11.1 current in cells treated with E-4031 (Eisai; Tokyo, Japan) or dofetilide (SIGMA-ALDRICH, St. Louis, MO, USA) [5,12]. Stock solutions (200 μM) were prepared by dissolving E-4031 in distilled water and dofetilide in distilled water acidified to pH 3.0 by adding HCl. The final concentration of E-4031 and dofetilide was obtained by adding the stock solution to the incubation medium. For patch-clamp experiments, CHO cells were incubated with 1 μM of E-4031 or 1 μM of dofetilide for 48 h and then incubated in drug-free medium for >1 h.

Statistical analysis

All averaged data are expressed as mean ± standard error. Statistical comparisons between two groups were evaluated using

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