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Characterization of a novel mutant KCNQ1 channel subunit lacking a large part of the C-terminal domain

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ABSTRACT

A mutation of *KCNQ1* gene encoding the alpha subunit of the channel mediating the slow delayed rectifier K^+ current in cardiomyocytes may cause severe arrhythmic disorders. We identified *KCNQ1(Y461X)*, a novel mutant gene encoding KCNQ1 subunit whose C-terminal domain is truncated at tyrosine 461 from a man with a mild QT interval prolongation. We made whole-cell voltage-clamp recordings from HEK-293T cells transfected with either of wild-type *KCNQ1* [*KCNQ1(WT)*], *KCNQ1(Y461X)*, or their mixture plus *KCNE1* auxiliary subunit gene. The *KCNQ1(Y461X)*-transfected cells showed no delayed rectifying current. The cells transfected with both *KCNQ1(WT)* and *KCNQ1(Y461X)* showed the delayed rectifying current that is thought to be mediated largely by homomeric channel consisting of KCNQ1(WT) subunit because its voltage-dependence of activation, activation rate, and deactivation rate were similar to the current in the *KCNQ1(WT)*-transfected cells. The immunoblots of HEK-293T cell-derived lysates showed that KCNQ1(Y461X) subunit cannot form channel tetramers by itself or with KCNQ1(WT) subunit. Moreover, immunocytochemical analysis in HEK-293T cells showed that the surface expression level of KCNQ1(Y461X) subunit was very low with or without KCNQ1(WT) subunit. These findings suggest that the massive loss of the C-terminal domain of KCNQ1 subunit impairs the assembly, trafficking, and function of the mutant subunit-containing channels, whereas the mutant subunit does not interfere with the functional expression of the homomeric wild-type channel. Therefore, the homozygous but not heterozygous inheritance of *KCNQ1(Y461X)* might cause major arrhythmic disorders. This study provides a new insight into the structure–function relation of KCNQ1 channel and treatments of cardiac channelopathies.

1. Introduction

KCNQ1 gene encodes the alpha subunit that associates with KCNE1 beta subunit to form the KCNQ1 channel that mediates the slow delayed rectifier K^+ current in cardiomyocytes [1,2]. This current is responsible for accelerating the repolarizing phases of action potentials and prevents premature action potential regeneration [3,4]. A mutation of *KCNQ1* may cause type-1 long QT syndrome [5], which is associated with a prolongation of QT interval in the electrocardiogram and increases risk for severe arrhythmic disorders [6].

We recently found *KCNQ1(Y461X)*, a novel mutant gene from a man with a mild QT interval prolongation. Surprisingly, the subject has not shown major cardiac disorders although the KCNQ1(Y461X) subunit lacks a large part of the C-terminal domain including the sites suggested to be crucial for the functional expression of KCNQ1 channel (see Section 4). We assessed whether and how *KCNQ1(Y461X)* affects KCNQ1 channel function in heterologous expression cells using a patch-clamp technique. Moreover, we examined the molecular behavior of KCNQ1(Y461X) subunit by immunoblotting and immunocytochemistry.

Abbreviations: *KCNQ1(WT)*, wild-type *KCNQ1*; *KCNQ1(Y461X)*, mutant *KCNQ1* producing a subunit truncated at tyrosine 461; (FLAG-)WT(+E1) cell, cell transfected with (*FLAG*-fused) *KCNQ1(WT)* (and *KCNE1*); (FLAG-)YX(+E1) cell, cell transfected with (*FLAG*-fused) *KCNQ1(Y461X)* and (and *KCNE1*); (FLAG-)YX/WT(+E1) cell, cell transfected with (*FLAG*-fused) *KCNQ1(WT) and KCNQ1(461X)* (plus *KCNE1*).

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2. Materials and methods

2.1. Genetic analysis

The diagnosis of and peripheral blood sampling from the subject were performed at the Second Department of Internal Medicine, Toyama University Hospital under the approval of the university's committee on utilization of human genes (#22–9). The written informed consent was obtained from the subjects before blood sampling. Amplification and sequencing of *KCNQ1* exons 1–16 from the genomic DNA were performed as described elsewhere [7].

The amino acid sequences of KCNQ1 homologs were compared using GENETYX software (GENETYX, Tokyo, Japan).

2.2. Plasmid construction

pReceiver-M12 plasmid vector encoding 3xFLAG-fused KCNQ1(WT) subunit [FLAG-*KCNQ1(WT)*] was purchased from GeneCopoeia (Rockville, MD, USA). FLAG-*KCNQ1(Y461X)* was generated by site-directed mutagenesis on FLAG-*KCNQ1(WT)*. pCAGGS-*KCNQ1(WT)* and pCAGGS-*KCNQ1(Y461X)* encoding epitope-free KCNQ1 subunits were constructed by inserting cDNA's amplified from sites between the *EcoR* I and *Sma* I sites of FLAG-*KCNQ1(WT)* and FLAG-*KCNQ1(Y461X)* into the blunt-ended *Xho* I sites of pCAGGS plasmid vector, respectively. *KCNE1* cDNA was cloned from the genomic DNA, amplified using the primers flanked by *EcoR* I and *Sma* I sites, and inserted into pReceiver-M12 vector pre-digested at the *EcoR* I and *Sma* I sites (FLAG-*KCNE1*). HA-*KCNE1* was constructed by replacing the 3xFLAG sequence of FLAG-*KCNE1* with a 2xHA sequence.

2.3. Cell preparation

For electrophysiological analysis, HEK-293T cells were cultured in 10% fetal bovine serum-supplemented Dulbecco's modified Eagle medium (11995-065, Life Technologies) at 37 °C in 5% CO_2. Three days before the measurements, the cells were transferred to 35-mm dishes (353001, BD, Franklin Lakes, NJ, USA). Two days before the measurements, the cells were transfected with enhanced green fluorescent protein (EGFP) gene-containing pCAGGS plasmid vector (25 ng/dish), FLAG-*KCNE1* (150 ng/dish), and either of pCAGGS-*KCNQ1(WT)* (75 ng/dish), pCAGGS-*KCNQ1(Y461X)* (75 ng/dish), or a mixture of pCAGGS-*KCNQ1(WT)* and pCAGGS-*KCNQ1(Y461X)* (37.5 ng/dish each) (WT+E1, YX+E1, and YX/WT+E1 cells, respectively) using TransIT-293 reagent (Mirus Bio, Madison, WI, USA).

For immunoblot analysis, HEK-293T cells were cultured on 60-mm plastic dishes (150288, Thermo Fisher Scientific, Waltham, MA, USA). Two days before harvesting, the cells were transfected with either of FLAG-*KCNQ1(WT)* (750 ng/dish), FLAG-*KCNQ1(Y461X)* (750 ng/dish), or a mixture of pCAGGS-*KCNQ1(WT)* and FLAG-*KCNQ1(Y461X)* (375 ng/dish each) using TransIT-293 reagent.

For immunocytochemistry, HEK-293T cells were cultured on 35-mm glass-based dishes (D111300, Matsunami, Osaka, Japan). Two days before immunostaining, the cells were transfected with HA-*KCNE1* (125 ng/dish) and either of pCAGGS-*KCNQ1(WT)* (125 ng/dish), FLAG-*KCNQ1(Y461X)* (125 ng/dish), or a mixture of pCAGGS-*KCNQ1(WT)* and FLAG-*KCNQ1(Y461X)* (62.5 ng/dish each) using TransIT-293 reagent.

2.4. Electrophysiological analysis

Rupture-patch whole-cell voltage-clamp recordings were made from the EGFP-positive cells. A glass recording pipette (tip resistance, 3–5 MΩ) was filled with a solution containing (in mM) 134 potassium D-gluconic acid, 7.6 KCl, 9 KOH, 10 NaCl, 1.2 $MgCl_2$, 10 HEPES, 0.5 EGTA, and 4 adenosine triphosphate magnesium salt (pH, adjusted to 7.3 with KOH). The culture dish was perfused at a rate of 1.2 ml/min with a pre-warmed (36–38 °C) solution containing (in mM) 147 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 D-glucose (pH, adjusted to 7.4 with NaOH). The command voltages were corrected for a liquid junction potential between the pipette and bath solutions. Current signals were acquired with an EPC 8 amplifier (HEKA, Lambrecht-Pfalz, Germany) controlled by Patchmaster software (version, 2x35; HEKA). The holding potential was −80 mV. After membrane rupture, the pipette capacitance was canceled electronically and then, responses to 10 sets of bipolar voltage pulses (−75 mV, 40 ms and −85 mV, 40 ms) were recorded at a cut-off frequency of 30 kHz and a sampling rate of 50 kHz. Then, the main component of the membrane capacitance was cancelled electronically and responses to test stimuli were recorded with 60% electronic series resistance compensation and at a cut-off frequency of 300 Hz and a sampling rate of 1 kHz.

The amplitude of linear leakage and the membrane capacitance were estimated from the average of the bipolar pulse-evoked responses. The text and graphs report current densities after subtraction of the linear leakages while the traces show raw data including the linear leakages.

To quantify the voltage-dependence of activation extent of KCNQ1 channel current, a Boltzmann equation [$I_{tail} = \frac{A}{1+exp\left[\frac{V_{half} - V_{first}}{K}\right]}$, where I_{tail}, A, V_{half}, V_{first}, and K are the peak amplitude of a tail current, scale factor, voltage for half-maximal activation, first-step voltage, and slope, respectively] was fitted to the *I–V* relation of each cell using IGOR Pro software (versions, 6.22A and 6.32A; WaveMetrics, Lake Oswego, OR, USA). To quantify the activation and deactivation rates, single- and double-exponential functions were fitted to the rise (270–700 ms of a depolarizing step onset) and decay (4–3999 ms of the depolarizing step offset) of the KCNQ1 channel-mediated current, respectively using IGOR Pro software. Numerical data groups are expressed as mean ± SEM throughout the text and figures. A difference in current density between a pair of the data groups was examined using Wilcoxon rank sum test because some data groups had non-normal distributions. A difference in kinetic parameters was examined using unpaired *t*-test because most data groups had normal distributions.

2.5. Immunoblot analysis

The transfected HEK-293T cells were washed twice with chilled Dulbecco's phosphate-buffered saline (DPBS). The cells were scraped in 1.5 ml of DPBS and then spun down at 1000 rpm for 5 min. The collected cells were lysed with 300 μl of a buffer consisting of 150 mM NaCl, 20 mM Tris–HCl (pH, 7.5), 1% Triton X-100, 0.5 mM ethylenediaminetetraacetic acid, and 10 μl/ml protease inhibitor mix (80-6501-23, GE Healthcare, Little Chalfont, UK) and incubated on ice for 1 h. The lysate was centrifuged at 15,000 rpm and 4 °C for 20 min and then the supernatant was transferred to a new tube. The supernatant was incubated with a 2x sodium dodecyl sulfate (SDS) sample buffer consisting of 0.5% SDS, 100 mM Tris (pH, 6.8), 20% glycerol, and 200 mM dithiothreitol at room temperature (RT) for 30 min. Electrophoresis was performed at a constant voltage of 120 V for 1 h. The materials on the gel were transferred to a reactivated polyvinyldene fluoride membrane (Hybond-P, RPN303F, GE Healthcare) containing a buffer consisting of 0.1% SDS, 25 mM Tris–HCl (pH, 8.3), 192 mM glycine, 5% methanol. The blotted membrane was consecutively rinsed with a Tris-buffered saline (TBS) consisting of 20 mM Tris–HCl (pH7.6) and 137 mM NaCl, blocked by TBS containing 9% skim milk and 0.05% Tween20 (161-0781, Bio-Rad Laboratories, Hercules, CA,