Development of Recombinant Cell Line Co-expressing Mutated Nav1.5, Kir2.1, and hERG for the Safety Assay of Drug Candidates

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Abstract
To provide a high-throughput screening method for human ether-a-go-go-gene–related gene (hERG) K⁺ channel inhibition, a new recombinant cell line, in which single action potential (AP)–induced cell death was produced by gene transfection. Mutated human cardiac Na⁺ channel Nav1.5 (IFM/Q3), which shows extremely slow inactivation, and wild-type inward rectifier K⁺ channel, Kir2.1, were stably co-expressed in HEK293 cells (IFM/Q3+Kir2.1). In IFM/Q3+Kir2.1, application of single electrical stimulation (ES) elicited a long AP lasting more than 30 s and led cells to die by more than 70%, whereas HEK293 co-transfected with wild-type Nav1.5 and Kir2.1 fully survived. The additional expression of hERG K⁺ channels in IFM/Q3+Kir2.1 shortened the duration of evoked AP and thereby markedly reduced the cell death. The treatment of the cells with hERG channel inhibitors such as nifekalant, E-4031, cisapride, terfenadine, and verapamil, recovered the prolonged AP and dose-dependently facilitated cell death upon ES. The EC₅₀ values to induce the cell death were 3 µM, 19 nM, 17 nM, 74 nM, and 3 µM, respectively, whereas 10 µM nifedipine did not induce cell death. Results indicate the high utility of this cell system for hERG K⁺ channel safety assay.

Keywords
cell function assays, cell-based assays, ion channels, membrane potential

Introduction
In cardiac ventricular myocytes, human ether-a-go-go-gene–related gene (hERG; KCNH2) is a critical ion channel to form early repolarization phase of action potential (AP) as a rapidly activating delayed rectifier K⁺ current (Iₖr). Loss-of-function mutation of hERG leads to potentially lethal inherited long QT syndrome.¹,² The long QT interval is also occasionally elicited by medication due to the block of the hERG K⁺ channel, and it can be a fatal side effect known as torsades de pointes.³ Although hERG channel blockers such as nifekalant are useful as antiarrhythmic drugs under some particular settings, more than 50 drugs commercially available as therapeutic agents in noncardiovascular categories have been reported to elicit long QT.⁴ Improved automated patch clamp system,⁷ ⁸ have been also developed. New methods are, however, still emerging to evaluate the potential of test compounds to block the hERG K⁺ channel as early as possible in the drug-development process with lower cost, smaller effort, and no exaggerated equipment.

The present study was undertaken to develop a novel screening system to evaluate hERG channel inhibition by a simple and popular assay of cell death, such as the MTT method. We produced a novel cell system in which mutated Na⁺ (Nav1.5) channels and wild-type inward rectifier K⁺
channel (K_r 2.1) and hERG K⁺ channels are co-expressed. When the hERG channel was suppressed by a test compound, cell death due to occurrence of a prolonged AP upon single electrical stimulation (ES) can be easily measured by MTT assay. This screening system fits well to the true HTS for hERG inhibition assay.

Materials and Methods

Cell preparation

In accordance with a previous study,⁹ we mutated the triplet IFM in the III–IV interdomain linker to QQQ in the human cardiac Nav1.5 channel (Genbank: NM_198056.2). To construct the site-directed mutants of Nav1.5, Ile¹⁴₈⁵, Phe¹⁴⁸⁶, and Met¹⁴⁸⁷ were substituted for glutamine by a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Nav1.5 and mutated Nav1.5 (IFM/Q3) were ligated into mammalian expression vectors pcDNA3.1(+)/Neo (Invitrogen, Carlsbad, CA), respectively, using the TaKaRa ligation kit version 1 (TaKaRa, Osaka, Japan). Human K_r 2.1 (Genbank: NM_000891.2) was ligated into mammalian expression vectors pcDNA3.1(+)/Zeo (Invitrogen). HEK293 cells were maintained at 37 °C in 5% CO₂ with high-glucose Dulbecco’s modified Eagle’s medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (Invitrogen), 100 U/mL penicillin (Wako), and 0.1 mg/mL streptomycin (Meiji Seika, Tokyo, Japan). Stable expression of K_r 2.1 was achieved by using calcium phosphate co-precipitation transfection techniques. After transfection, cells were cultivated for approximately 1 mo in the presence of Zeocin, and a single cell resistant to Zeocin expression of K_r 2.1 was transiently co-transfected with hERG (Genbank: NM_000238.2) and a green fluorescent protein (GFP) by the Lipofectamine 2000 (Invitrogen). Similarly, a stable cell of K_r 2.1 was transiently co-transfected with Nav1.5, G418/Zeo-resistant cells were selected as those that were IFM/Q3 and K_r 2.1 co-expressing. Double stable cells of IFM/Q3 and K_r 2.1 were transiently co-transfected with hERG by voltage-sensitive fluorescent dye, DiBAC ₃(3) (DOJINDO, Kumamoto, Japan), with excitation wavelength at approximately 488 nm and emission at 512 nm, respectively.¹⁰ Data were collected and analyzed using the ARGUS/HiSCA imaging system (Hamamatsu Photonics, Hamamatsu, Japan). ES was performed through paired platinum wire electrodes placed in the recording chamber by applying square pulses of 200 ms in duration and 200 mA in amplitude, every 1 s three times.

Membrane Potential Measurements by Voltage-Sensitive Fluorescent Dye

In some experiments, membrane potentials were measured by voltage-sensitive fluorescent dye, DiBAC ₃(3) (DOJINDO, Kumamoto, Japan), with excitation wavelength at approximately 488 nm and emission at 512 nm, respectively.¹⁰ Data were collected and analyzed using the ARGUS/HiSCA imaging system (Hamamatsu Photonics, Hamamatsu, Japan). ES was performed through paired platinum wire electrodes placed in the recording chamber by applying square pulses of 200 ms in duration and 200 mA in amplitude, every 1 s three times.

MTT Assay

Cell viability was monitored by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO) assay as described previously.¹¹,¹² MTT was dissolved in phosphate-buffered saline at 5 mg/mL and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. Cells were seeded onto 96-well plates (approximately 5000 cells per well) and cultured for 24 to 48 h after transfection.

Solutions

The standard HEPES-buffered solution for electrophysiological recording and image analyses had an ionic composition of 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 14 mM glucose, and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. The pipette solution for whole-cell recordings contained 140 mM KCl, 4 mM MgCl₂, 10 mM HEPES, 2 mM Na₂ATP, and 0.05 mM EGTA. The pH of the pipette solution was adjusted to 7.2 with KOH. The 140 mM K⁺ HEPES-buffered solution for DiBAC ₄(3) fluorescence imaging had an ionic composition of 2.9 mM NaCl, 140 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 14 mM glucose, and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. Phosphate-buffered saline had an ionic composition of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, and 1.5 mM KH₂PO₄.

Electrophysiological Experiments

The whole-cell patch clamps were applied to single cells using the CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan), EPC-7 amplifier (List Electronics, Darmstadt, Germany), and data acquisition/analysis system (Digidata 1440A and pClamp software version 10; Molecular Devices, Sunnyvale, CA). A small tip of cover glass, on which cells were cultured, was placed at the bottom of a recording chamber of 500 µL in volume on the stage of the inverted microscope (TE300; Nikon, Tokyo, Japan). Cells were continuously perfused with an extracellular solution. Whole-cell recordings were carried out at room temperature.
dimethyl formamide and demineralized water) was added to all wells, and the plates were incubated at 37 °C for 6 to 8 h. These plates were then analyzed following absorption on a Multiscan JX (Ver1.1 system; Thermo Labsystems), using a test wavelength of 595 nm and a reference wavelength of 650 nm. Unless specified in the text, no mixing was performed, and no medium was removed prior to the addition of any ingredient.

Trypan Blue Exclusion

Cell viability was determined also by the measurement of the capacity to exclude the vital dye trypan blue. ES was performed in the same manner as membrane potential measurements with DiBAC₄(3). After the ES, cells were incubated for 5 min, 15 min, 30 min, and 1 h at room temperature and then stained with phosphate-buffered saline containing 0.15% trypan blue (Sigma-Aldrich) for 5 min. Blue-stained and unstained cells were counted in the viewing field of the microscope to calculate the percentage of nonviable cells.

Chemicals

Lidocaine, nifedipine, cisapride, terfenadine, and verapamil were obtained from Sigma-Aldrich. Nifekalant and barium chloride were obtained from Wako Pure Chemicals. Quinidine was obtained from Tokyo Chemical Industry (Tokyo, Japan). E-4031 was a gift from Eisai (Tokyo, Japan). The test compounds were dissolved with dimethyl sulfoxide or distilled water.

Statistics

Data are expressed as means ± SEM. The statistical significance between the two groups and among multiple groups was evaluated using Student’s t-test and Scheffe’s test after F-test or one-way analysis of variance, respectively. The Z’ factor was calculated from each dose response set as follows:

\[ Z' \text{ factor} = 1 - 3(\text{SD of positive controls} + \text{SD of negative controls})/(\text{mean of positive controls} - \text{mean of negative controls}) \]

Results

Production of a Cell Line Expressing Mutated Nav1.5 Channel

First, we aimed to produce a recombinant cell line that elicits an extremely long AP by transfecting HEK293 cells with a mutated voltage-dependent Na⁺ channel, Nav1.5. A prolonged AP due to slow inactivation of mutated Nav1.5 may lead the cell to death. The replacement of the three amino acids (Ile¹⁴₈₅, Phe¹⁴₈₆, and Met¹⁴₈₇) with glutamine (IFM/Q3) completely removes fast inactivation. This mutation, however, killed cells presumably because of sustained Na⁺ influx through the noninactivating Na⁺ channel. It has been also known that co-expression with IFM/Q3 of the inward-rectifier K⁺ channel K⁺2.1, which provides more negative resting membrane potential, is effective to prevent the cell death. Based on these lines of information, a cell line stably expressing both K⁺2.1 and IFM/Q3 was generated.

In HEK293 cells stably co-expressing K⁺2.1 and wild-type or mutant Nav1.5 channels (WT+Kir and IFM/Q3+Kir), membrane currents were measured using whole-cell patch-clamp recordings (Fig. 1A,B; see also I-V relationships in Supplemental Fig. 1). It is apparent that the Na⁺ current in IFM/Q3+Kir had a sustained component even at the end of the 50 ms pulse. The current at the pulse end in WT+Kir was slightly outward (n = 9), and that in IFM/Q3+Kir was markedly inward (n = 13, p < 0.01; Fig. 1C). The functional expression of K⁺2.1 in IFM/Q3+Kir resulted in a resting membrane potential of approximately –80 mV. Addition of 100 µM Ba²⁺ changed the resting membrane potential in IFM/Q3+Kir from −77 mV to about −30 mV (Fig. 1D). The K⁺2.1 currents were also blocked by 100 µM Ba²⁺ (see also Supplemental Fig. 1). Similar depolarization by Ba²⁺ was observed also in WT+Kir (not shown). The summarized data indicate significantly more negative resting membrane potential in IFM/Q3+Kir than that in native HEK (n = 7, p < 0.01; Fig. 1E).

Occurrence of Prolonged AP in IFM/Q3+Kir and Effects of Lidocaine

In the next step, APs elicited by current injection of depolarizing square pulses of 5 ms in duration and 400 pA in amplitude were measured from WT+Kir and IFM/Q3+Kir (Fig. 2A, B). APs in WT+Kir showed the peak within 5 ms and progressively turned out within 100 ms (Fig. 2A). In contrast, those in IFM/Q3+Kir possessed a long-lasting plateau phase, which gradually declined from +10 to −20 mV and lasted for more than 30 s (Fig. 2B). The summarized data of AP duration (APD) indicate significantly prolonged APD in IFM/Q3+Kir (n = 5) compared with that in WT+Kir (n = 5, p < 0.01; Fig. 2C).

Effects of lidocaine, a Na⁺ channel blocker, on APs were examined in WT+Kir and IFM/Q3+Kir (Fig. 3A, B). In the presence of 1 mM lidocaine, APs were not elicited by current injection in WT+Kir (Fig. 3A, B). Addition of 1 mM lidocaine during a long AP in IFM/Q3+Kir repolarized the cell (Fig. 3B,a). Even in the presence of 1 mM lidocaine, electrical responses with shorter duration (<5 s) and smaller amplitude were observed in all IFM/Q3+Kir examined (n = 3; Fig. 3B,b). Therefore, there appeared a tendency that APs in IFM/Q3+Kir were more resistant to 1 mM lidocaine than those in WT+Kir. To examine the possibility more
exactly, the sensitivity of Na⁺ currents to 1 mM lidocaine was compared between WT+Kir and IFM/Q3+Kir (Fig. 3C). Accordingly, the block by lidocaine of Na⁺ current was significantly smaller in IFM/Q3+Kir (n = 6, p < 0.01, respectively).

To measure the prolonged AP in a larger number of cells, membrane potentials were recorded using a slow-response voltage-sensitive fluorescent dye, DiBAC4(3). In IFM/Q3+Kir, a gradual increase in relative fluorescent intensity (F/F<sub>140K</sub>) was observed following the application of a set of three electrical square pulses (arrow, Fig. 4B) but not in WT+Kir (Fig. 4A). The increase in F/F<sub>140K</sub> upon ES indicates the occurrence of large depolarization, presumably prolonged AP. The ES-induced increase was not observed in the presence of 1 mM lidocaine, whereas that by 140 mM K⁺ was detected (not shown). The summarized changes in F/F<sub>140K</sub> from a larger number of cells indicate that the increase occurred only in IFM/Q3+Kir but not in WT+Kir (Fig. 4C, D).
Detection of Cell Death Induced by a Long AP with MTT Assay

The cell death following application of electrical pulses was examined using 96-well plates and MTT assay. The methods for ES and the process of assay are shown in Figure 5A. Cells cultured in each well were activated by ES, applied three times with an interval of 1 to 3 min, and were again cultured for 2 to 6 h prior to MTT assay. The viability in WT+Kir and IFM/Q3+Kir was approximately 1.0 and 0.15, respectively, when the viability of nonstimulated WT+Kir and IFM/Q3+Kir was taken as 1.0 (Fig. 5B,C). The low viability in IFM/Q3+Kir was recovered by the presence of 1 mM lidocaine (Fig. 5C). The relative viability of IFM/Q3+Kir in the absence of lidocaine was not apparently affected when the intensity of pulse was ranged from 50 to 200 mA.

Co-expression of the hERG Channel for hERG Channel Inhibition Assay

To apply this cell line to a potential drug-safety assay for hERG channel inhibition, we generated novel cells by additionally transfecting IFM/Q3+Kir with hERG (IFM/Q3+Kir+hERG). In these experiments, hERG together with GFP were transiently expressed in IFM/Q3+Kir. If the hERG channels are activated enough to overcome the sustained Na⁺ current during the plateau phase of AP in IFM/Q3+Kir+hERG, the AP would be turned out, and the cell death would be prevented.

In IFM/Q3+Kir+hERG, application of 10 µM nifekalant markedly reduced the outward current during prepulse at 0 mV and the tail current at –50 mV to a similar extent (see Supplemental Fig. 2). The Na⁺ current and Kr2.1 current were not affected by nifekalant. APs were elicited by current injection in most IFM/Q3+Kir+hERG and characteristic of apparently shorter duration than those in IFM/Q3+Kir. It is notable that the application of 10 µM nifekalant markedly prolonged the AP. The APD was 2.43 ± 0.82 s (n = 5) in the absence of nifekalant and 9.75 ± 2.93 s in the presence of 10 µM nifekalant (n = 6, **p < 0.01).

To measure the prolongation of AP by nifekalant in a larger number of cells, membrane potentials were recorded using DiBAC₄(3). Neither the first ES to IFM/Q3+Kir+hERG nor the addition of 10 µM nifekalant per se changed the F/F₀ (left arrow and bar in Fig. 6A). However, the second
Figure 3. Effects of lidocaine on action potentials (APs) and Na⁺ current amplitude. (A) APs evoked by injection of square currents of 5 ms in duration and 300 pA in amplitude in the absence (a) and presence (b) of 1 mM lidocaine in WT+Kir. (B) APs evoked by injection of square currents of 5 ms in duration and 300 pA in amplitude in IFM/Q3+Kir. Addition of 1 mM lidocaine turned off the prolonged AP (a). Only depolarization response to ES with shorter duration and smaller amplitude than AP was elicited in the presence of 1 mM lidocaine (b). (C) The comparison of Na⁺ current inhibition by 1 mM lidocaine in WT+Kir (filled column) and IFM/Q3+Kir (open column). Cells were depolarized from –80 to –30 mV for 50 ms. The Na⁺ inward current amplitude at the peak in the absence of lidocaine was taken as 1.0, respectively. The relative inhibition of peak current amplitude by 1 mM lidocaine was 0.96 ± 0.01 in WT+Kir (n = 6) and 0.37 ± 0.08 in IFM/Q3+Kir (n = 6, **p < 0.01).
stimulation in the presence of nifekalant induced a gradual but large increase in \( F/F_{140k} \). The \( F/F_{140k} \) at 5 min after stimulation was significantly increased by 10 µM nifekalant, suggesting that the block of hERG channel by nifekalant prolonged the AP in IFM/Q3+Kir+hERG (Fig. 6B, C).

In IFM/Q3+Kir+hERG, the cell death induced by ES and effects of 10 µM nifekalant on it were examined. The efficacy of the transient expression of hERG in IFM/Q3+Kir+hERG was high (more than 80%) based on the patch-clamp experiments (not shown). The ES did not significantly affect the viability of IFM/Q3+Kir+WT+Kir at 5 min after the ES. Ratios of WT+Kir and IFM/Q3+Kir were 0.22 ± 0.01 and 0.67 ± 0.04, respectively. **\( p < 0.01 \).

Effects of four compounds known to block the hERG channel—E-4031, cisapride, terfenadine, and verapamil—were also examined using the system. The EC \( 50 \) and Hill coefficient of nifekalant determined by this methods were 2.70 ± 0.77 µM and 2.31 ± 0.11 (n = 4; Fig. 7B). The mean Z’ factor value was calculated from the data shown in Figure 7B as 0.54.

Figure 4. Effects of electrical stimulation (ES) on membrane potential in IFM/Q3+Kir and WT+Kir. Membrane potential changes by ES were monitored as images using DiBAC \(_4\) (3) and a fluorescent microscope system. (A, B) Time courses of changes in ratio in WT+Kir and IFM/Q3+Kir, respectively. ES was applied to cells at the times indicated by arrows. A set of three square pulses of 200 ms in duration, 200 mA in amplitude, and 1 s intervals was applied for stimulation at one time through paired platinum wire electrodes placed in the chamber. At the end of each experiment, 140 mM K+ HEPES-buffered solution was applied, and the fluorescence intensity was taken as \( F_{140k} \) (1.0) to evaluate changes in intensity (F) as the ratio (F/ \( F_{140k} \)). The increase in the ratio indicates membrane depolarization. (C) Summarized data of the time courses after ES in WT+Kir (closed squares) and IFM/Q3+Kir (open squares). (D) Summarized data of IFM/Q3+Kir and WT+Kir at 5 min after the ES. Ratios of WT+Kir and IFM/Q3+Kir were 0.22 ± 0.01 and 0.67 ± 0.04, respectively. **\( p < 0.01 \).
for cisapride (Fig. 7D), 74.0 ± 2.9 nM and 1.61 ± 0.15 (n = 6) for terfenadine (Fig. 7E), and 275 ± 36 nM and 1.81 ± 0.51 (n = 6) for verapamil (Fig. 7F), respectively. 

The Cell Death by ES Occurred Quickly in IFM/Q3+Kir

To examine the time course of cell death, the capacity to exclude the vital dye trypan blue was measured in IFM/Q3+Kir following ES. The percentages of cells stained by trypan blue were measured by eye in the viewing field of a microscope at 5, 15, 30, and 60 min after the application of ES. The results indicate that the cell death in IFM/Q3+Kir mostly occurred within 5 min from ES (Supplemental Fig. 4). A similar quick cell death was also observed in IFM/Q3+Kir+hERG in the presence of nifekalant.

Discussion

The present results showed that the novel cell line, which was produced by co-expression of K,2,1 and mutated Nav1.5 (IFM/Q3) in HEK293 cells (IFM/Q3+Kir), caused autoclasis by a prolonged AP occurrence in response to ES.
The key issue is that the additional expression of hERG channel to IFM/Q3+Kir markedly shortened APD and significantly prevented cell death induced by ES.

Moreover, this new assay system allows us to quantify the potential of test compounds to inhibit the hERG K+ channel because the hERG block again prolonged evoked APs, resulting in cell death in IFM/Q3+Kir+hERG. The potency of nifekalant, cisapride, E-4031, terfenadine, and verapamil, which have been known as hERG channel blockers, to induce cell death was evaluated in IFM/Q3+Kir+hERG. The EC50s of these drugs were 2.7 µM, 19 nM, 42 nM, 74 nM, and 275 nM, respectively, and these values are close to their EC50s for hERG K+ current in heterologous expression system; 7.8 µM, 28 nM, 26 nM, 45 nM, and 143 nM, respectively. In contrast, 10 µM nifedipine did not affect the cell death (Supplemental Fig. 4). Z or Z' value is a useful parameter to evaluate and validate screening methods, and a method with a Z' value between 0.5 and 1.0 is considered to be an excellent assay.13 Z' values greater than 0.5 were obtained in this study.

Co-expression of K2.1 with IFM/Q3 is essential for this assay system to keep the resting membrane potential negative to −75 mV, because the noninactivating Na+ current through IFM/Q3 is large enough to kill HEK293 cells at less negative resting membrane potentials.14,15 Actually, 100 µM Ba2+, a blocker of Kir2.1, induced marked membrane depolarization in IFM/Q3+Kir (Fig. 1D) and killed the cells without ES (EC50 of 14.5 ± 2.3 µM, n = 6; Supplemental Fig. 4). A test compound, which blocks Kir2.1 but not hERG, induces cell death of IFM/Q3+Kir+hERG and might be evaluated as a false-positive hERG blocker. Cytotoxic compounds might be also evaluated as false-positive.

Moreover, a test compound, which blocks both Nav1.5 and hERG, will prevent cell death of IFM/Q3+Kir+hERG upon ES and could be evaluated as false-negative. Quinidine
Figure 7. Validating the potency of HERG inhibitors using IFM/Q3+Kir+hERG. (A) Effects of nifekalant on IFM/Q3+Kir+hERG cell death upon electrical stimulation (ES) were measured with an MTT assay. Experimental procedures were the same as shown in Figure 5. The cell was stimulated by ES with three square pulses of 200 ms in duration, 150 mA in amplitude, and 60 s in interval. The relative viability was calculated taking the averaged viability of the control without ES as 1.0 (1.00 ± 0.06, n = 7). The relative viability of ES (150 mA) was 1.03 ± 0.14 (n = 6, p > 0.05 versus control), and that of ES (150 mA) +10 µM nifekalant was 0.39 ± 0.06 (n = 6, **p < 0.01 versus control and ES [150 mA]). (B–F) Concentration-dependent effects of nifekalant (B), E-4031 (C), cisapride (D), terfenadine (E), and verapamil (F) on cell death were examined in IFM/Q3+Kir+hERG. Each data set of a dose-response curve for four or six doses of drugs was obtained from triplicate (average from three wells as N = 1) and was fitted by the following equation, taking the cell death with the highest concentration of each drug as 1.0: relative cell death = 1/[1 + (ED50/X)^h], where X and h are a test dose and Hill coefficient, respectively.
blocks both hERG and Nav1.5 in cardiac myocytes, but the effect on hERG appears at lower concentrations. Accordingly, cell death of IFM/Q3+Kir+hERG upon ES was observed in the presence of quinidine at low concentrations (<3 μM) and the inhibition of cell death at higher concentrations (Supplemental Fig. 4). The blocking effect of a test compound on K_\text{r}2.1 or Na^+ channel (IFM/Q3) can be resolved by comparing its effect on cell death in IFM/Q3+Kir and IFM/Q3+Kir+hERG. In other words, the assay comparing cell death in IFM/Q3+Kir and IFM/Q3+Kir+hERG with or without ES allows us to assess whether the test compound has blocking effects on Nav1.5 or K_\text{r}2.1 in addition to that on hERG. This may therefore be a cost-effective method that provides basic information about the potential of test compounds on these three important channels.

The cause of cell death in IFM/Q3+Kir upon ES is not clear in this study but is presumably the rapid increase in intracellular Na^+ concentration ([Na^+]_i), because the block of sustained Na^+ influx by lidocaine prevented cell death. In previous studies, the persistent opening of a sodium channel by treatment with veratridine causes the elevation of [Na^+]_i, which perturbs ion homeostasis and triggers cell death in the HEK cells expressing Na^+ channel and the neuronal cells. The staining of IFM/Q3+Kir by trypan blue revealed that more than 90% of cell death occurred within 30 min after the application of ES (Supplemental Fig. 3). This finding strongly suggests that the major part of cell death was not apoptotic but necrotic.

Long QT syndrome upon medication occasionally leads to a delay in cardiac AP repolarization and predisposes patients to potentially fatal arrhythmias. Consequently, evaluating the effect of new drugs on hERG function is now a mandatory step in the development of novel pharmaceuticals. The assessment of compound activities on hERG channels is conducted with use of an automated, electrophysiological, ion flux method and so on. The advantages of the hERG channel safety assay by use of IFM/Q3+Kir+hERG can be listed as follows: (1) The procedure and results are so simple that the assay including analyses can be performed without electrophysiological techniques. (2) The initial cost for equipment and running cost are low. In this study, cell death was evaluated by the most popular MTT methods. (3) This assay fits well to HTS because of the low cost and ease of automation. The whole procedure of the assay will be easily automated. The method of field ES in a 96-well has been reported for HTS.

There are several options for the use of IFM/Q3+Kir and IFM/Q3+Kir+hERG in assays. Instead of ES, application of 25 to 45 mM K^- solution induced cell death in IFM/Q3+Kir (not shown). In HEK293 cells co-expressing K_\text{r}2.1 and T-type Ca^{2+} channel, Ca^{2+} channel was activated by high K^- 25 The hERG inhibition assay by use of DiBAC(3) as HTS has been applied using CHO cells expressing hERG and high K^- solution for hERG channel activation. 6 The advantage of this new method is as follows. (1) Test compounds are evaluated by their effects on AP occurrence triggered by ES in IFM/Q3+Kir+hERG, suggesting that the state- and/or voltage-dependent effects can be detected. (2) The viability of IFM/Q3+Kir+hERG upon ES is determined by AP occurrence in an all-or-none manner within 30 min after ES. Therefore, this system easily fits to HTS by developing a 96-/384-well plate, in which ES can be applied at one time.

In addition to the application of IFM/Q3+Kir to the hERG K^- channel inhibition assay, it may be available for evaluation of new compounds acting on other voltage-dependent K^- channels, such as Kv1.3, Kv1.5, or KCNQ1+KCNE2. It may even be available for the screening of compounds acting on ligand-gated ion channels. The advantage of this system against other simple cell systems such as yeast may be that the validation using AP allows us to evaluate state-dependent effects on voltage-sensitive ion channels.

In conclusion, the new assay system for hERG channel inhibition using IFM/Q3+Kir+hERG is a novel tool because of its simplicity, accuracy, and high cost performance. Further application of methods for the screening of drug-development targeting on ion channels will be also expected. The technology developed in this study has been applied to patent filings (PCT/JP2011/064967), and the laid-open disclosure number is WO2012/002460.

Declaration of Conflicting Interests
The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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