Full Paper

Electrophysiological Characterization of Cardiomyocytes Derived From Human Induced Pluripotent Stem Cells

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Abstract. Cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CMs) hold great promise for development of in vitro research tools to assess cardiotoxicity, including QT prolongation. In the present study, we aimed to clarify the electrophysiological/pharmacological characteristics of hiPS-CMs using the patch-clamp technique. The hiPS cells were differentiated into beating cardiomyocytes by the embryoid body method. The expression of genes related to cardiac ion channels and differentiation markers in cardiomyocytes were detected by RT-PCR. Whole-cell patch-clamp recordings were performed using single hiPS-CMs dispersed from beating colonies. We confirmed voltage-dependence of major cardiac ion currents (I_{Na} , I_{Ca} , I_{Kr} , and I_{Ks}) and pharmacological responses to ion-channel blockers. Action potential duration (APD) was prolonged by both I_{Kr} /hERG and I_{Ks} blockers, whereas it was shortened by an I_{Ca} blocker, indicating that these ion current components contribute to action potential generation in hiPS-CMs. As for multiple ion channel blockers, terfenadine prolonged APD, but verapamil did not, results which were identical to clinically relevant pharmacological responses. These data suggest that patch-clamp assay using hiPS-CMs could be an accurate method of predicting the human cardiac responses to drug candidates. This study would be helpful in establishing an electrophysiological assay to assess the risk of drug-induced arrhythmia using hiPS-CMs.

Keywords: human induced pluripotent stem cell, cardiomyocyte, patch-clamp, action potential duration, safety pharmacology

Introduction

Human induced pluripotent stem (hiPS) cells were established by transfecting human fibroblasts with the transcription factors Oct3/4, Sox2, Klf4, and c-Myc (1). Since these cells exhibit the ability to differentiate into various cells including cardiomyocytes, they hold great promise as an in vitro safety assessment tool to detect cardiotoxicity, such as QT-interval prolongation (2, 3). In nonclinical drug development, we examine the activity of drug candidates on the key cardiac ion channels and action potential (AP) waveforms using animal-derived primary cardiomyocytes/tissues to predict the QT risk in the clinic (4, 5); however, the drug responses from animal hearts may be different from human results because cardiomyocytes have distinct properties of ion channels

among species (6, 7). Therefore, using primary cardiomyocytes from animals would be a less relevant choice for predicting the arrhythmic risk in humans in the above-mentioned studies. Since cardiomyocytes derived from hiPS cells (hiPS-CMs) would express human cardiac ion channels in a native environment, the use of them for cardiac safety assessment is expected to generate results that more accurately predict the relevant in vivo human response.

A series of studies using hiPS-CMs has demonstrated the temporal expression pattern of genes associated with the process of cardiomyogenesis in hiPS cells (8, 9). Moreover, their pharmacological responses were shown by measuring the surface electrogenic activities of cell clusters as field potential waveforms (9, 10). Thus, hiPS cells have a capacity to differentiate functional cardiomyocytes, but to our knowledge, there are no reports on the electrophysiological/pharmacological properties of major cardiac ion currents in hiPS-CMs. Because cardiac ion currents essentially determine the electrical activity

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of the heart (11, 12), we should clarify the characteristics of major ion currents in hiPS-CMs for their use as a drug discovery tool. The balance of functional ion currents in cardiomyocytes is also important for evaluating the effects of drugs on cardiac APs because AP waveforms are the result of the balanced activity of several ion channels (11, 12). Hence, the investigation of pharmacological responsiveness of AP waveforms is necessary to elucidate the ion current components that contribute to the generation of AP in hiPS-CMs.

The purpose of this study was to clarify the electrophysiological/pharmacological characteristics of hiPS-CMs in detail so as to apply these cells to drug discovery. We showed the gene expressions of the key ion channels in hiPS-CMs and examined the responses to several drugs in each ion current and on AP parameters by conventional patch-clamp recordings.

Materials and Methods

The present study complied with the “Ethical Guidelines for Research that Uses Human-derived Test Material” promulgated in Chugai Pharmaceutical Co., Ltd. and was approved by the company’s Research Ethics Committee.

Cell culture

We used hiPS cell line 201B7, which was generated by retroviral transduction of human fibroblasts with Oct3/4, Sox2, Klf4, and c-Myc (1). These cells were provided by Dr. Shinya Yamanaka (Kyoto University, Kyoto). The undifferentiated hiPS cell colonies were cultured with a mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder layer (ReproCELL, Inc., Kanagawa) in DMEM/F-12 medium (Invitrogen, Inc., Carlsbad, CA, USA) that was supplemented with 20% knock-out serum replacement (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acid (NEAA, Invitrogen), 0.11 mM β -mercaptoethanol (Sigma-Aldrich, Inc., St. Louis, MO, USA), 50 U/mL penicillin / 50 μ g/mL streptomycin (Invitrogen), and 4 ng/mL basic fibroblast growth factor (Wako Pure Chemical Industries, Ltd., Osaka).

Embryoid body formation and cardiac differentiation

The hiPS colonies were dispersed into small clumps using CTK dissociation solution (Primate ES Cell Dissociation Solution, ReproCELL), which allows for gentle detachment of cells, providing high levels of viability in subsequent passages (13). The cells were then transferred to suspension culture in plastic Petri dishes for 4 days, where they aggregated to form embryoid bodies (EBs). The EBs were then plated on 0.1% gelatin-

coated culture dishes. Differentiation medium, consisting of DMEM/F-12 supplemented with 20% fetal bovine serum (Japan Bioserum, Inc., Hiroshima), 2 mM L-glutamine, 0.1 mM NEAA, 0.11 mM β -mercaptoethanol, and 50 U/mL penicillin / 50 μ g/mL streptomycin, was used to initiate cardiac differentiation. During differentiation, the medium was replaced every 2 to 4 days. Contracting colonies appeared 7 to 11 days after the differentiation was initiated.

Patch-clamp

The whole-cell configuration of the patch-clamp technique was used. Beating colonies (28 – 44 days after differentiation was initiated) were dissociated using 0.25% trypsin with EDTA 4Na (Invitrogen). Cardiomyocytes were plated on 0.1% gelatin-coated glass coverslips and cultured for 4 – 12 days. According to their typical morphology (single adherent cells which were round; see Fig. 1B) and spontaneous beating activity, individual hiPS-CMs were selected for patch-clamp experiments. We used ventricular-like cells that were classified according to AP morphology (showing a clear plateau phase) and APD₉₀ (AP duration at 90% repolarization) value of 150 ms in this study, as they were in previous reports (8, 14 – 16). Mean cell capacitance was 45.0 ± 2.6 pF (N = 42). All recordings were performed at room temperature. Cells were superfused with the respective external solutions at a rate of 4 mL/min. Data acquisition was performed using an Axopatch 1D amplifier and pClamp 8 software (Molecular Devices, Inc., Sunnyvale, CA, USA). Analog signals were low-pass filtered at 5 kHz and sampled at 20 kHz (Digidata 1322A, Molecular Devices).

In the voltage-clamp mode, we recorded four ion currents (I_{Na} , I_{Ca} , I_{Kr} , and I_{Ks}) and the pharmacological response to each ion-channel blocker because these currents are essential for AP generation (11, 12). For recordings of Na^+ and Ca^{2+} currents, the pipette solution consisted of 120 mM CsCl, 3 mM $MgCl_2$, 10 mM EGTA, 5 mM HEPES, 5 mM Mg-ATP, pH adjusted to 7.3 with CsOH, while the bath recording solution consisted of 120 mM NaCl, 5 mM KCl, 3.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 20 mM tetraethylammonium (TEA) chloride, pH adjusted to 7.4 with TEA-OH. To record I_{Kr} and I_{Ks} , the pipette solution contained 140 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 5 mM Mg-ATP, pH adjusted to 7.2 with KOH, while the bath recording solution contained 150 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH. For the recording of I_{Kr} , the bath solution contained initially 150 mM NaCl and 4 mM KCl, which was changed to a solution containing 15 mM NaCl and 140 mM KCl to increase the driving force

for potassium influx. The internal pipette solution was prepared in batches, stored frozen and freshly thawed on each day of use. Peak (I_{Na} , I_{Ca} , and I_{Kr}) or steady state current (I_{Ks}) during voltage steps was used to plot the current–voltage relationship. Current amplitudes were normalized for membrane capacitance to give current density values and to compensate for differences in cell size. We could record Ca^{2+} currents without contamination from Na^{+} inward currents, as voltage-dependent Na^{+} channel was inactivated with the holding potential at -40 mV.

In the current-clamp mode, we recorded typical APs of the hiPS-CMs. The patch pipette solution consisted of 140 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 5 mM Mg-ATP, pH adjusted to 7.2 with KOH. The bath recording solution consisted of 150 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH adjusted to 7.4 with NaOH. After achieving a conventional whole-cell configuration, the amplifier was switched to current-clamp mode. Spontaneous beating activity of the single hiPS-CMs stopped then, so we could not record AP waveform of spontaneous activity. A constant holding current was added to adjust the membrane potential to levels ranging from -65 to -75 mV. A brief current pulse (4-ms duration) at the smallest values needed to evoke excitation (100–300 pA) was used to measure APs at a cycle length of 10 s (0.1 Hz). Some recorded hiPS-CMs showed spontaneous activity after application of the current pulses, but most of the activity stopped during the stabilization period of AP waveforms. hiPS-CMs that could not be paced stably were not included in the analysis. The concentrations of each tested drug were sequentially applied to hiPS-CMs from lowest to highest concentration. Following stabilization of AP waveforms, the average responses from five recorded APs were analyzed for each test concentration. Data analysis was performed using Microsoft Excel 2007. Membrane potential at 30%, 50%, or 90% repolarization of AP was calculated from resting membrane potential [RMP (mV): average of membrane potential before the stimulation of current pulse] and action potential amplitude [APA (mV): difference between peak membrane potential and RMP]. Then, AP duration at the corresponding membrane potential was measured. The following AP parameters were quantitated: APD_{30} , APD_{50} , and APD_{90} (AP duration at 30%, 50%, and 90% repolarization, respectively; ms) and the APD_{90}/APD_{50} ratio. APD_{30-90} was also calculated as the difference in APD between 90% and 30% repolarization. APD parameters are presented as percent change ($\Delta\%$) from baseline at each concentration of the drugs.

Reverse transcription PCR (RT-PCR)

Total RNA was isolated from undifferentiated hiPS cells and contracting areas of differentiated iPS cells (38 days after differentiation was initiated) using the RNeasy Mini Kit (QIAGEN, Inc., Valencia, CA, USA) and treatment with RNase-free DNase I (QIAGEN). Total RNA obtained from human adult left ventricle (BioChain Institute, Inc., Hayward, CA, USA) was also used as a positive control. The cDNA was synthesized using ReverTra Ace[®] qPCR RT Kit (TOYOBO Co., Ltd., Osaka). PCR was carried out using PrimeSTAR[®] HS DNA Polymerase (Takara Bio Inc., Shiga). PCR conditions included denaturation at $98^{\circ}C$ for 10 s, annealing at $60^{\circ}C$ for 5 s, and extension at $72^{\circ}C$ for 30 s for 30 or 40 cycles, with a final extension at $72^{\circ}C$ for 5 min. Primers used are listed in Table 1.

Drugs

Chromanol 293B, diltiazem, E-4031, lidocaine, terfenadine, and verapamil were purchased from Sigma-Aldrich. The drugs were dissolved in 100% dimethylsulfoxide (DMSO) and the solutions were diluted to 0.1% DMSO in the respective external solutions.

Statistics

Data are presented as mean $\pm$ S.E.M., and N equals the number of data. Statistical significance was determined by 1-way ANOVA with post hoc testing using Dunnett's method. Statistical procedures were performed by SAS system (Version 8.2; SAS Institute Japan Ltd., Tokyo).

Results

Cardiomyocyte differentiation from hiPS cells

To induce cardiomyocyte differentiation, the hiPS cells were removed from the MEF feeder layer and cultivated in suspension, where they formed EBs. Fractions of the EBs generated from the tested hiPS cells formed contracting colonies of cells, suggesting cardiac differentiation. The morphology of differentiated colonies and the dispersed single cardiomyocytes (7 days after dispersion) are shown in Fig. 1. Rhythmically contracting areas appeared as early as 7 days into EB formation. The efficiency of forming contracting EBs after 38 days in culture was around 20% (data not shown).

Expressions of cardiac markers and ion channel genes in hiPS-CMs

Although some seminal studies demonstrated the expression of several genes in hiPS-CMs (8, 9), the changes in gene expressions of major cardiac ion channels were not shown comprehensively. Therefore, the expression levels of various cardiac markers and ion channel-related

Table 1. Oligonucleotide primers used for RT-PCR

Target genes	Gene description	Primer sequence	PCR cycle
SCN5A	Nav1.5	Forward 5'-TCATCGTAGACGTCTCTCTGGT-3' Reverse 5'-GGCTCTTGTTGTTACGATGGT-3'	40
CACNA1C	Cav1.2	Forward 5'-AAGGCTACCTGGATTGGATCAC-3' Reverse 5'-GCCACGTTTTTCGGTGTGAC-3'	40
CACNA1D	Cav1.3	Forward 5'-GGGCAATGGGACCTCATAAATAA-3' Reverse 5'-TTACCTGGTTGCGAGTGCATTA-3'	40
KCNH2	Kv11.1 (hERG/I _{Kr})	Forward 5'-CATTGGCTCCCTCATGTATGCT-3' Reverse 5'-GCGTGCTGGAAGTACTCCTCG-3'	40
KCND3	Kv4.3 (I _{to})	Forward 5'-AGAGAGCTGATAAACGCAGGG-3' Reverse 5'-CAGGCAGTGCAGCAGGTGAT-3'	40
KCNQ1	Kv7.1 (KvLQT1, I _{Ks})	Forward 5'-GCGCGGAAGCCTTACGATGT-3' Reverse 5'-CAGCTGCGTCACCTTGTCCTC-3'	40
KCNJ2	Kir2.1 (I _{K1})	Forward 5'-TGTTGGGTTTGACAGTGGAA-3' Reverse 5'-CCCACAGGATTTTCATTGCT-3'	40
KCNJ12	Kir2.2 (I _{K1})	Forward 5'-GCCAGCTAGGCTCTGTTTGTG-3' Reverse 5'-CTGAGACACATCTCTAAGGTAC-3'	40
HCN4	Hyperpolarization-activated cyclic nucleotide-gated channel (I _h)	Forward 5'-CAGCCTCTTACGCCTGTTAC-3' Reverse 5'-CCAGGAGTTGTTACCATGTTG-3'	40
HCN2	Hyperpolarization-activated cyclic nucleotide-gated channel (I _h)	Forward 5'-CGCCTGATCCGCTACATCCAT-3' Reverse 5'-AGTGCGAAGGAGTACAGTTCAC-3'	40
OCT3/4	Octamer-binding transcription factor 3	Forward 5'-CAGTGCCCGAAACCCACAC-3' Reverse 5'-GGAGACCCAGCAGCCTCAAA-3'	40
MYH6	Myosin heavy chain 6	Forward 5'-TCTCCGACAACGCCTATCAGTAC-3' Reverse 5'-GTCACCTATGGCTGCAATGCT-3'	30
MYL2	Myosin light chain 2	Forward 5'-CCTTGGGCGAGTGAACGTGA-3' Reverse 5'-CAGCATTTCGCCAACGTAATCA-3'	30
TNNT2	Troponin T type 2	Forward 5'-CCAAAGCCCAGGTCGTTTCATGC-3' Reverse 5'-CTGCAACTCATTAGGTCCTTC-3'	30
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward 5'-CCTGTTTCGACAGTCAGCCG-3' Reverse 5'-CGACCAAATCCGTTGACTCC-3'	30

Fig. 1. Typical morphology of hiPS-CMs. Spontaneous beating colony (A) and single beating cells dispersed from the beating colony (7 days after dispersion: B).

genes were analyzed by RT-PCR (Fig. 2). Undifferentiated hiPS cells strongly expressed Oct3/4 (undifferenti-

ated marker), but the contracting areas of differentiated hiPS cells and human adult left ventricle did not express

Fig. 2. Gene expression profiles of various ion channels (A) and cardiac markers or sarcomeric proteins (B). RT-PCR was performed on undifferentiated hiPS cells (Undiff hiPS) and a hiPS beating colony. Human adult left ventricle (Human heart LV) was used as a positive control.

it. In contrast, genes of several cardiac myofilament proteins including myosin heavy chain 6 (MYH6), myosin light chain 2 (MYL2), and troponin T type 2 (TNNT2) were identified in the differentiated hiPS cells and the human heart, whereas they were not detected in the undifferentiated cells. Moreover, RT-PCR showed that hiPS-CMs expressed the sodium channel ($\text{Na}_v1.5$; SCN5A), α -1C subunit of the L-type calcium channel ($\text{Ca}_v1.2$; CACNA1C), α -1D subunit of the L-type calcium channel ($\text{Ca}_v1.3$; CACNA1D), the rapidly activating delayed rectifier potassium channel (hERG; KCNH2), the transient outward potassium channel ($\text{K}_v4.3$; KCND3), the slowly activating delayed rectifier potassium channel ($\text{K}_v\text{LQT1}$; KCNQ1), the inward rectifier potassium ion channel ($\text{K}_{ir2.1}$; KCNJ2), the ATP-sensitive inward rectifier potassium channel ($\text{K}_{ir2.2}$; KCNJ12), and the hyperpolarization-activated cyclic nucleotide-gated channels (HCN2, HCN4). Thus, gene expressions of major cardiac ion channels were identified in hiPS-CMs and human adult left ventricle.

Voltage dependence of ion currents and pharmacology

The character of voltage dependence in the major cardiac ion currents was confirmed using voltage-step protocols. Inward sodium current elicited by stepwise test pulse from -80 to $+40$ mV was inhibited by lidocaine (I_{Na} blocker) in a concentration-dependent manner (Figs. 3A and 4A). Ca^{2+} current elicited by step pulses ranging from -40 to $+60$ mV was suppressed by diltiazem (I_{Ca} blocker), where I_{Na} was inactivated and should not open theoretically (Figs. 3B and 4B). Inward I_{Kr} current could not be detected in the general assay condition, but was identified using a bath solution with a high concentration of potassium (Figs. 3C and 4C). The I_{Kr} current was inhibited by E-4031 (I_{Kr} blocker), concentration-dependently. The slow component of I_K (I_{Ks}) in hiPS-CMs was observed by I_{Ks} blocker. Current amplitudes measured at the end of depolarization (steady state current) were plotted against membrane potentials. E-4031 and chromanol 293B (I_{Ks} blocker) reduced the I_K current, and chromanol 293B-sensitive currents are shown in Figs. 3D and 4D.

Fig. 3. Voltage dependence of ion currents in hiPS-CMs. A) Inward I_{Na} measured at depolarizing test pulses, -80 to $+40$ mV (500 ms) in 10-mV increments from holding potential (V_h) -80 mV. B) Inward I_{Ca} measured at depolarizing test pulses, -40 to $+60$ mV (500 ms) in 10-mV increments from V_h -40 mV. C) Inward I_{Kr} measured at -110 mV after depolarization ranging from -40 to $+50$ mV (1 s) in 10-mV increments from V_h -80 mV. I_{Kr} recorded in normal bath solution (upper trace) and high potassium solution (lower trace). D) Typical outward I_K (Pre) measured at steady state currents of test pulses, -40 to $+60$ mV (4 s) in 10-mV increments from V_h -80 mV. I_{Ks} (Chromanol 293B-sensitive currents) were obtained by digital subtraction.

Table 2. AP parameters in hiPS-CMs

APD ₉₀ (ms)	APD ₃₀ (ms)	APD ₃₀₋₉₀ (ms)	APD ₅₀ (ms)	APD ₉₀ /APD ₅₀	RMP (mV)	RMP _{Hold} (mV)	APA (mV)
429 ± 34	287 ± 27	141 ± 14	357 ± 29	1.21 ± 0.01	-60.1 ± 1.1	-69.5 ± 0.5	101.5 ± 2.5

APD₉₀: action potential duration at 90% repolarization, APD₃₀: action potential duration at 30% repolarization, APD₃₀₋₉₀: difference between action potential duration at 30% and 90% repolarization, APD₅₀: action potential duration at 50% repolarization, RMP: resting membrane potential without a constant holding current, RMP_{Hold}: resting membrane potential with a constant holding current, APA: action potential amplitude. Mean ± S.E.M., N = 26.

Characterization of AP waveform and pharmacology

To characterize the cells' recorded APs, the AP-related parameters were quantified (Table 2). Figure 5A shows the effect of vehicle (0.1% DMSO) on AP waveforms. The parameters did not change after application of vehicle for 20 min ($\Delta\%$ APD₉₀: 0.1 ± 1.3 , $\Delta\%$ APD₃₀: -2.5 ± 2.6 , $\Delta\%$ APD₃₀₋₉₀: 3.6 ± 2.1 ; N = 6). Diltiazem, a blocker of I_{Ca} , shortened APD₃₀ and APD₉₀, whereas it did not affect APD₃₀₋₉₀ (Fig. 5B and Table 3). E-4031, a blocker of hERG/ I_{Kr} , prolonged APD₃₀, APD₉₀, and APD₃₀₋₉₀ in a concentration-dependent manner (Fig. 5C

and Table 3). In addition, early afterdepolarizations were induced by E-4031 (1 μ M) in two cells out of four (Fig. 6). As shown in Fig. 5D and Table 3, concentration-dependent AP prolongation was observed with chromanol 293B, a blocker of I_{Ks} , although the magnitude of AP prolongation was lower than was observed in the presence of hERG/ I_{Kr} blockers (17). As for the multiple ion channel blockers, terfenadine prolonged APD₃₀, APD₉₀, and APD₃₀₋₉₀ (Fig. 5E and Table 3). Significant shortenings of APD₃₀ and APD₉₀ were observed, but APD₃₀₋₉₀ was prolonged by verapamil, a blocker of both hERG/ I_{Kr}

Fig. 4. Current-voltage relationship of an ion current (A: I_{Na} , B: I_{Ca} , C: I_{Kr} , and D: I_{Ks}) and its pharmacological responses in hiPS-CMs. Peak inward I_{Na} (A) and I_{Ca} (B) measured at depolarizing pulses or peak inward I_{Kr} (C) measured at -110 mV after depolarization pulses were presented. Outward currents measured at the end of depolarization (steady state current) were plotted against membrane potentials (D). Current is expressed as current density. Data are represented as the mean $\pm$ S.E.M. ($N = 4$).

Fig. 5. Effects of I_{Ca} , I_{Kr} , or I_{Ks} inhibitors on AP waveform in hiPS-CMs. Representative traces showing the effect of vehicle (A), diltiazem (B), E-4031 (C), chromanol 293B (D), terfenadine (E), and verapamil (F).

Table 3. Changes of AP parameters induced by I_{Ca} , I_{Kr} , or I_{Ks} inhibitors in hiPS-CMs

Drug	Conc.	N	APD ₉₀ [#]	APD ₃₀ [#]	APD ₃₀₋₉₀ [#]	RMP (mV)	APA (mV)
Diltiazem (I_{Ca} blocker)	Baseline		564 ± 115	430 ± 93	134 ± 26	-68.8 ± 0.2	111.5 ± 2.3
	0.1 μ M	4	-12.7 ± 3.0	-17.6 ± 4.3	5.2 ± 5.3	-68.5 ± 1.4	109.6 ± 2.1
	1 μ M		-33.4 ± 7.3**	-42.2 ± 9.6**	-1.7 ± 10.2	-69.0 ± 1.5	106.2 ± 1.7
E-4031 (I_{Kr} blocker)	Baseline		346 ± 53	184 ± 28	163 ± 36	-70.0 ± 1.9	112.3 ± 0.7
	0.01 μ M	4	8.3 ± 2.4	5.2 ± 1.6	10.4 ± 5.9	-69.7 ± 2.2	111.7 ± 1.6
	0.1 μ M		18.7 ± 3.2**	13.7 ± 2.3**	24.9 ± 6.2*	-71.3 ± 2.1	112.0 ± 2.3
	1 μ M		38.1 ± 7.1**	31.1 ± 4.8**	46.8 ± 9.1**	-75.9 ± 2.3	117.0 ± 2.2
Chromanil 293B (I_{Ks} blocker)	Baseline		463 ± 132	275 ± 60	189 ± 73	-71.2 ± 1.6	98.2 ± 5.9
	1 μ M	4	5.1 ± 1.6	4.1 ± 1.8	6.5 ± 2.6	-70.8 ± 1.8	97.5 ± 6.7
	10 μ M		12.1 ± 3.3*	5.4 ± 0.6*	22.4 ± 9.1	-72.8 ± 2.4	100.9 ± 4.0
	100 μ M		19.2 ± 4.2**	10.6 ± 1.0**	32.2 ± 9.2**	-73.3 ± 2.3	102.8 ± 4.2
Terfenadine (Multi channel blocker)	Baseline		367 ± 72	234 ± 62	133 ± 25	-68.5 ± 1.5	91.1 ± 5.9
	0.01 μ M	4	7.3 ± 3.7	9.7 ± 4.2	2.1 ± 3.6	-69.2 ± 1.8	92.5 ± 6.2
	0.1 μ M		16.1 ± 5.5	5.8 ± 2.6	41.3 ± 18.7	-70.7 ± 2.4	92.7 ± 7.4
	1 μ M		29.3 ± 7.8**	11.7 ± 4.6*	75.7 ± 40.9*	-70.0 ± 2.4	92.1 ± 6.4
Verapamil (Multi channel blocker)	Baseline		386 ± 71	270 ± 54	116 ± 18	-70.1 ± 1.5	97.5 ± 9.9
	0.1 μ M	4	-5.1 ± 1.2	-8.1 ± 1.1	1.7 ± 3.3	-71.1 ± 1.8	96.9 ± 9.7
	1 μ M		-8.1 ± 2.1	15.5 ± 2.6*	8.1 ± 3.0	-73.0 ± 1.3	95.5 ± 10.4
	10 μ M		-20.0 ± 4.5**	-33.0 ± 5.6**	11.6 ± 1.1*	-73.6 ± 2.2	91.5 ± 11.5

[#]Baseline values are in ms. All other values are percent change from baseline. Mean ± S.E.M.; average responses from five recorded APs were analyzed for each concentration. * $P < 0.05$, ** $P < 0.01$ vs. vehicle-treatment.

Fig. 6. Typical waveform of early afterdepolarization (EAD) induced by 1 μ M E-4031.

and L-type calcium channels (Fig. 5F and Table 3).

Discussion

The purpose of this study was to clarify the electrophysiological/pharmacological characteristics of hiPS-CMs using the patch-clamp technique. We differentiated the hiPS cells into beating cardiomyocytes and showed the gene expressions of cardiac ion channels, biophysiological properties of ion currents, and pharmacological

responses of AP waveforms. Functional cardiac ion channels were identified in hiPS-CMs and changes of AP waveforms were identical to clinically relevant pharmacological responses. Thus, hiPS-CMs would be a useful tool to predict the QT risk in the clinic.

To confirm the differentiation of hiPS cells into cardiomyocytes, we demonstrated the expression of heart-specific structural genes, including sarcomeric-related proteins and ion channels in contracting cells. Myosin, troponin, and various cardiac ion channel-related genes were observed in beating colonies, while a pluripotent marker (Oct 3/4) was not detected. It is well known that cardiomyocytes generated from human ES/iPS cells and human cardiomyocytes express these cardiac myofibrillar proteins and ion channels (9, 18, 19). These findings suggest the cells used in this study have differentiated into cardiomyocytes from hiPS cells in terms of their gene expression profiles.

From the physiological point of view, identifying the functional activities of major ion channels is necessary because the biophysical action of each ion channel plays an important role in the electrical activity of the heart. It is well known that the AP waveforms of cardiomyocytes consist of four major ion currents (I_{Na} , I_{Ca} , I_{Kr} , and I_{Ks}).

Voltage-gated sodium and calcium channels in ventricular cells induce inward currents, which cause the initial rapid depolarization and plateau phases of AP, respectively (20, 21). Both rapidly (I_{Kr}) and slowly (I_{Ks}) activating components of the delayed rectifier potassium current contribute to the repolarization phase (22, 23). We recorded each ion current from hiPS-CMs using different internal/external buffer compositions to isolate it in this study. Activation of I_{Na} was observed at voltage positive to -60 mV, and the maximum amplitude was obtained at pulse to -40 mV. I_{Na} was inhibited by lidocaine in a concentration-dependent manner. Peak current–voltage relation of I_{Ca} revealed a threshold at about -30 mV and the maximum amplitude occurred near 0 mV, which was suppressed by diltiazem. Although the composition of the external solution was different from physiological conditions, inward I_{Kr} current showed clear voltage dependence and E-4031, the hERG/ I_{Kr} blocker, inhibited it concentration-dependently in hiPS-CMs. Depolarizing pulse elicited the delayed rectifier outward current and a chromanol 293B-sensitive component, known as I_{Ks} current (22), was observed in this study. Thus, hiPS-CMs express functional cardiac ion channels as well as human cardiomyocytes (11, 12, 20–23) and would be suitable for evaluating cardiotoxicity of a drug that affects these ion channels.

Cardiac AP is formed via a balanced activity of each ion channel and is necessary for the electrical conduction system of the heart. Because the duration of AP reflects a delicate balance between outward and inward currents related to various ion channels (11, 12), investigating the effects of pharmacological modulators on APD is essential to clarify the characteristics of AP waveforms in hiPS-CMs. Application of $1\text{ }\mu\text{M}$ diltiazem induced significant shortening of APD_{30} and APD_{90} , whereas $0.1\text{ }\mu\text{M}$ E-4031 and $10\text{ }\mu\text{M}$ chromanol 293B induced significant prolongation of APD_{30} , APD_{90} , and APD_{30-90} in this study. It has been reported that $10\text{ }\mu\text{M}$ diltiazem shortened APD_{40} (24) and $1\text{ }\mu\text{M}$ E-4031 and $1\text{ }\mu\text{M}$ chromanol 293B prolonged APD_{90} (22, 25) in human isolated cardiomyocytes. These data indicate that three ion current components (I_{Ca} , I_{Kr} , and I_{Ks}) regulate APD in ventricular type hiPS-CMs as well as in human isolated cells.

Pharmacological responses to multiple ion channel blockers in hiPS-CMs correlated to clinically relevant pharmacological responses, indicating that an APD assay using hiPS-CMs would be useful for a prediction of drug-induced QT prolongation. Terfenadine induces QT prolongation and torsade de pointes ventricular tachycardia in the clinical setting (26). However, it simultaneously inhibits the inward Na^+ and Ca^{2+} currents in addition to outward hERG/ I_{Kr} current in cardiomyocytes (27), which may cause the lack of prolongation of APD in

canine Purkinje fibers or guinea-pig papillary muscles (28, 29). In hiPS-CMs, terfenadine produced a concentration-dependent prolongation of APD_{30} , APD_{90} , and APD_{30-90} . Verapamil also inhibits hERG/ I_{Kr} current, but does not induce the QT prolongation in the clinic because of multiple interactions with cardiac ion channels (26). Consequently, it is well known as a “false positive” in a conventional hERG assay. This study showed that verapamil shortened APD_{30} and APD_{90} , but it prolonged APD_{30-90} . Inhibition of L-type calcium channel by verapamil would cause the shortening of APD_{30} and APD_{90} in hiPS-CMs. In contrast, the inhibitory effect of hERG/ I_{Kr} current would reflect the prolongation of APD_{30-90} in this study. Thus, electrophysiological assay using hiPS-CMs would be an accurate method to predict the human cardiac responses to drug candidates.

Previous studies using cardiomyocytes derived from human embryonic stem cells (hES-CMs) or hiPS-CMs showed that their AP waveforms could be classified into three types (nodal-, atrial-, and ventricular-like cells) by the shape of AP or by APD ($\text{APD}_{90} > 150$ ms) (8, 14–16). We also identified ventricular-like cells according to AP morphology (showing a clear plateau phase) and an APD_{90} value of 150 ms and clarified their characteristics in this study. The presence of the clear plateau phase in ventricular-like cells was confirmed by the $\text{APD}_{90}/\text{APD}_{50}$ ratio. In this study, the range of the $\text{APD}_{90}/\text{APD}_{50}$ ratio (1.07 to 1.31) was similar to that in a previous study (15) using hiPS-CMs (1.10 to 1.30).

Because hiPS cells can differentiate into various types of cells, it is important to set up a standard of single hiPS-CMs for use in patch-clamp assays. We selected single adherent cells that were round (see Fig. 1B) and approximately 45 pF in membrane capacitance. AP waveforms of these cells were classified as ventricular-like and reproducible pharmacological responses were observed in this study. Therefore, the morphological characteristics of single hiPS-CMs could help us identify ventricular-like hiPS-CMs and obtain reliable responses to drugs in a patch-clamp assay. However, the number of cells that had these characteristics was variable in every beating colony of hiPS-CMs.

Although chromanol 293B prolonged APD of hiPS-CMs in this study as well as in previous studies in human isolated ventricular myocytes (22) and hES-CMs (16), it has also been reported that I_{Ks} blocker in the absence of sympathetic stimulation has little influence on normal human ventricular APD (17). The actual reason underlying the greater sensitivity of APD to I_{Ks} blocker in hiPS-CMs is unclear; however, a difference in balance between I_{Kr} and I_{Ks} components in the AP repolarization phase may be important. Jost et al. showed that an I_{Ks} current elicited by an action potential-like test pulse was rela-

tively small compared with I_{K_r} in human ventricular cardiomyocytes and I_{K_s} blockade did not lengthen APD under those conditions (17). In contrast, we could not detect an I_{K_r} tail current of hiPS-CMs in the general assay condition, indicating that I_{K_r} plays a lesser role and I_{K_s} is more responsible for the repolarization phase of AP in hiPS-CMs than in human ventricular cardiomyocytes. While the I_K of hiPS-CMs may be slightly different from that of human ventricle, APD changes to I_{K_r} and multiple ion channel blockers in hiPS-CMs correlated to clinically relevant pharmacological responses. These data suggest that hiPS-CMs provide a useful tool to predict the QT risk in the clinic.

One of the important properties of cardiomyocytes is an ability to respond appropriately to neurohormonal regulation such as those by noradrenaline and acetylcholine (30, 31). Stimulation of β_1 -adrenergic receptor mediates a positive chronotropic and inotropic effect on the heart (30). On the other hand, acetylcholine reduces heart rate and contractile forces in heart through activation of muscarinic M_2 receptor (31). Recent studies revealed that the positive and negative chronotropic responses were induced by isoproterenol and carbachol in hiPS-CMs, respectively (8, 9). Tanaka et al. (10) and Yokoo et al. (32) also reported that adrenergic stimulation by adrenaline or isoproterenol increased beating frequency and contractility in hiPS-CMs derived from the same cell line (201B7) used in this study. These data support the view that the cells used in this study would express functional β_1 and M_2 receptors as they did in previous reports.

The hiPS-CMs also have several limitations with regard to their potential use as an in vitro electrophysiological model. First, although it is reported that several cardiac ion channels showed temperature-dependent changes of activation/inactivation kinetics (33–35), we clarified the fundamental characteristics of hiPS-CMs by patch-clamp recordings at room temperature in this study. When we investigate the physiological similarities in detail, electrophysiological experiments should be performed at physiological temperature. Second, we could not evaluate the effect of test compounds on the maximum upstroke velocity (V_{max}) of the AP. Since membrane depolarization of AP waveform in cardiomyocytes is elicited by the inward sodium current, V_{max} of the AP can be used as a qualitative index of sodium channel availability (29, 36). However, transient change of membrane potential evoked by current injection in the current-clamp mode could affect V_{max} because that response overlaps with the depolarization phase of APs in hiPS-CMs. Hence, V_{max} was excluded from items evaluated in this study. Third, while hiPS-CMs showed the expected pharmacological responses to each ion channel

blocker, the electrophysiological properties of hiPS-CMs may be relatively immature, as found in previous reports (8, 9). The RMP in hiPS-CMs was more depolarized than that in human adult ventricular myocytes (around -85 mV) (37). Because I_{K1} , an inward rectifier potassium channel, regulates the RMP in human cardiomyocytes (38), the expression of functional I_{K1} channel in hiPS-CMs may be lower than in adult cardiomyocytes. Finally, the reason for the lack of inward I_{K_r} current under general solution composition remains unclear. The balance of I_K components which contributes to AP waveforms of hiPS-CMs may be slightly different from that of human ventricle, although the gene expression of KCNH2 and the APD prolongation induced by two hERG/ I_{K_r} blockers were shown in this study. Further studies are needed to clarify the biophysical character of I_K in hiPS-CMs.

In conclusion, we demonstrated the gene expressions of cardiac ion channels and electrophysiological characteristics in hiPS-CMs. These cells would be a powerful new research tool that can potentially be used to establish an electrophysiological assay to predict human cardiac risk accurately. Our results provide the first description of the electrophysiological properties of major cardiac ion currents and the pharmacological responsiveness of AP waveforms in hiPS-CMs. These data would assist in establishing an electrophysiological assay to assess the potential risk of drug-induced arrhythmia using hiPS-CMs.

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