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Original Article

Application of human stem cell-derived cardiomyocytes in safety pharmacology requires caution beyond hERG

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ABSTRACT

Human embryonic stem cell-derived cardiomyocytes (hESC-CM) have been proposed as a new model for safety pharmacology. So far, a thorough description of their basic electrophysiology and extensive testing, and mechanistic explanations, of their overall pro-arrhythmic ability is lacking. Under standardized conditions, we have evaluated the sensitivity of hESC-CM to proarrhythmic provocations by blockade of hERG and other channels. Using voltage patch clamp, some ion current densities (pA/pF) in hESC-CM were comparable to adult CM: I_{Kr} (-12.5 ± 6.9), I_{Ks} (0.65 ± 0.12), $I_{Na,peak}$ (-72 ± 21), $I_{Na,late}$ (-1.10 ± 0.36), and $I_{Ca,L}$ (-4.3 ± 0.6). I_f density was larger (-10 ± 1.1) and I_{K1} not existent or very small (-2.67 ± 0.3). The low I_{K1} density was corroborated by low KCNJ2 mRNA levels. Effects of pro-arrhythmic compounds on action potential (AP) parameters and provocation of early afterdepolarizations (EADs) revealed that Chromanol293B (100 μmol/l) and Bay K8644 (1 μmol/l) both significantly prolonged APD_{90}. ATX-II (<1 μmol/l) and $BaCl_2$ (10 μmol/l) had no effect on APD. The only compound that triggered EADs was hERG blocker Cisapride. Computer simulations and AP clamp showed that the immature AP of hESC-CM prevents proper functioning of I_{Na}-channels, and result in lower peak/maximal currents of several other channels, compared to the adult situation. Lack of functional I_{K1} channels and shifted I_{Na} channel activation cause a rather immature electrophysiological phenotype in hESC-CM, and thereby limits the potential of this model to respond accurately to pro-arrhythmic triggers other than hERG block. Maturation of the electrical phenotype is a prerequiste for future implementation of the model in arrhythmogenic safety testing.

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1. Introduction

Recently, human pluripotent stem cell-derived cardiomyocytes, including both induced pluripotent and embryonic stem cell-derived cardiomyocytes (hiPSC-CM and hESC-CM respectively) have been suggested as a *new, attractive in vitro model system* to study cardiac diseases. The hope is that they will provide a model for arrhythmogenic conditions: both inherited, such as different familial long QT syndromes, and acquired, such as drug-induced prolongation of the QT interval. Driven by the lack of sufficient and healthy human cardiac tissue for experimental research, there is a large interest in the use of stem cell derived CMs for screening of pro-arrhythmic compounds during drug development [1–8]. The ideal model for this purpose would be one that represents the adult human CM closely with respect to expression of ion channels and their modulating factors, ion current density and shape, and duration of the action potential (AP). Additionally, inclusion of an arrhythmogenic event, with a high sensitivity and specificity to respond accurately to arrhythmic triggers, is necessary for accurate evaluation of drug-induced arrhythmogenic potential.

The ICH S7B guideline was released in 2008 to offer a "strategy for assessing the potential of a test substance to delay ventricular repolarization", which is strongly associated with the life-threatening ventricular arrhythmia Torsade de Pointes (TdP). This guideline suggests to use (at least) two models: an *in vitro* hERG screen, and an *in vivo* QT assay. In line with this strategy, much focus has been on

Abbreviations: APD, action potential duration; $APD_{50/90}$, action potential duration at 50/90% of repolarization; BVR, beat-to-beat variability of repolarization; EAD, early afterdepolarization; hESC-CM, human embryonic stem cell-derived cardiomyocyte; hiPSC-CM, human induced pluripotent stem cell-derived cardiomyocyte; $I_{Ca,L}$, L-type calcium current; I_f, pacemaker (funny) current; $I_{Na,late}$, late sodium current; $I_{Na,peak}$, peak sodium current; I_{K1}, inward rectifier potassium current; I_{Kr}, rapid delayed rectifier potassium current (hERG); I_{Ks}, slow delayed rectifier potassium current; PF, purkinje fibres; TdP, Torsade de Pointes.

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the hERG current and its function in hiPSC-CM and hESC-CM [4–6]. Using both single CM and multicellular preparations, several groups have shown that established and selective blockers of the hERG current not only cause AP prolongation, but also arrhythmogenic events such as early afterdepolarizations (EADs) [1–5,7,8]. We have previously shown that the hERG blocker E-4031 prolonged the AP, and increased triangulation and beat-to-beat variability of repolarization (BVR) in hESC-CMs [5], in a similar manner to that observed in rabbit purkinje fibres (PF) — a validated (even if labour-intensive) model which is commonly used today. BVR appeared a good predictor of EAD inducibility, which occurred in 67% of hESC-CM clusters [5]. With regards to arrhythmias, the gain or loss of function of currents other than I_{Kr}, such as I_{Na}, $I_{Ca,L}$, and I_{Ks}, has been studied to a much lesser extent [1,8,9]. Moreover, several possible causes of arrhythmia related to the orchestrated interaction of ion channel activity have not been examined in hiPSC-CM or hESC-CM, and basic characterization of ion current density in combination with suitable blockers and comparison to the adult human situation is scarce and incomplete [10,11].

AP duration (APD) and shape is determined by the interaction of all cardiac ion channels, therefore modulation of any of these currents is a potential safety issue. Sodium channel block can delay cardiac conduction and trigger potentially lethal re-entrant arrhythmias [12], conversely, enhancement of the sodium current can cause triggered arrhythmias [13]. Enhancement of the calcium current, or block of potassium channels other than hERG, can also provoke arrhythmias. Additionally, two channel-specific compounds that individually seem to have limited effect on APD can, when applied in combination, cause synergistic effects that provoke EADs [14]. The same holds true for drugs targeting more than one channel, a behaviour which is frequently observed, and is highly dose-dependent [15].

For a more comprehensive picture of the predictive power that hESC-CM provide with respect to testing of proarrhythmia, we have 1) recorded all major ion currents underlying the AP and compared them to the currents reported in adult CM, 2) studied the most plausible ways of provoking arrhythmias related to ion channel function, including blocking various potassium channels and enhancement of the late Na^+-current and the L-type Ca^+-current, 3) applied computer modeling and AP clamp to predict and describe the contribution of each recorded ion current to hESC-CM vs adult AP in order to understand and explain the results obtained during arrhythmia testing.

2. Materials and methods

Detailed information on the methods is available in the online data supplement.

2.1. Stem cell preparation

Generation of multicellular CM clusters was performed as described previously, using the hESC line SA002 (Cellartis AB, Göteborg) [16]. Only clusters with a beating frequency < 50 bpm were used for AP recordings and for dissociation and use in patch clamp experiments. Additional criteria for inclusion in AP experiments were that hESC-CM clusters followed pacing at 1 Hz and that $APD_{90} > 300$ ms (at 1 Hz pacing). These properties have been previously reported as a prerequisite for hESC-CM to show a specific arrhythmogenic response that is comparable to what is seen in contemporarily used models [5].

2.2. Electrophysiological measurements from hESC-CM

Measurements of ion currents and field-stimulated APs were performed using a HEKA EPC-10 Double Plus amplifier (HEKA, Lambrecht/Pfalz, Germany) controlled by PatchMaster 2.43. APs were evoked by field stimulation (frequency 1 Hz, pulse length 2 ms) using two platinum electrodes and a Stimulator CS (Hugo Sachs Elektronik, March-Hugstetten, Germany). Except for the sodium current measurements (20 °C), all recordings were made at 37 °C.

AP recordings were made from multicellular clusters using sharp microelectrodes filled with 3 M KCl during superfusion with a modified Tyrode's solution consisting of (in mmol/l) NaCl 130, KCl 4, $NaHCO_3$ 18, $MgCl_2$ 1.2, $CaCl_2$ 1.8, HEPES 10, glucose 10, pH7.4/NaOH.

Extracellular solution for measurement of inward rectifying potassium currents (I_{K1}) was the same as for AP recordings. For measurements of hyperpolarization activated cationic currents (I_f), $BaCl_2$ 1, $CdCl_2$ 0.2, $MnCl_2$ 2, and 4-aminopyridine 0.5 were added. $NaHCO_3$ was excluded and NaCl set to 140. Pipette solution contained potassium gluconate 125, KCl 10, HEPES 5, EGTA 5, $MgCl_2$ 2, $CaCl_2$ 0.6, Na_2ATP 4, pH 7.2/KOH.

Delayed rectifier current (I_{Kr}) and I_{Kr} during AP clamp experiments was measured using the equimolar Cs^+ method which allows isolation of I_{Kr} since, in contrast to other cardiac potassium channels that have different selectivity filters in their pore regions, hERG channels are not blocked by Cs^+, but instead conduct a significant Cs^+ current [17,18]. hERG/Cs^+ currents were recorded in CsCl 135, $MgCl_2$ 1, glucose 10, HEPES 10, nifedipine 0.01, pH 7.4/CsOH. Pipette solution contained CsCl 135. $MgCl_2$ 1,EGTA 10, HEPES 10, pH 7.2/CsOH.

Slow delayed rectifier current (I_{Ks}) measurements were done in NMDG 140, $MgCl_2$ 2, glucose 11.1, HEPES 20, pH 7.35/HCl. Pipette solution contained potassium gluconate 125, KCl 20, HEPES 5, EGTA 10, $MgCl_2$ 1, Mg_2ATP 5, pH 7.40 / KOH.

Peak inward sodium currents (I_{Na}), I_{Na} during AP clamp and L-type calcium currents ($I_{Ca,L}$) measurements were performed in NMDG 140, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, glucose 6, $NaHCO_3$ 17.5, HEPES 15, pH 7.4/HCl. Pipette solution contained CsCl 120, tetraethylammonium chloride 10, $MgCl_2$ 3, $CaCl_2$ 1, Na_2ATP 2, EGTA 10, HEPES 5, pH 7.2/CsOH. I_{Na} was recorded at 20 °C. For the late component of the sodium current ($I_{Na,late}$) the extracellular solution contained NaCl 140, CsCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, glucose 5, HEPES 5 and nifedipine 0.002, pH 7.3/CsOH. The pipette solution contained NaCl 5, CsCl 133, MgATP 2, tetraethylammonium chloride 20, EGTA 10, HEPES 5, pH 7.3/CsOH.

Pipettes were pulled using a Sutter P-2000 (Sutter Instrument Company, Novato, USA) and patch pipettes had a resistance of 2–3 MΩ when fire-polished and filled with pipette solution. Sharp microelectrodes had a resistance of 60–80 MΩ when filled with 3 M KCl.

2.2.1. Pulse protocols

I_{K1} was measured as 10 μM barium-sensitive currents, elicited by 1 second voltage steps from −120 mV to +30 mV, with 10 mV increments, from a holding potential of −40 mV and an interpulse interval of 3 s (0.33 Hz). The average steady state current normalized to membrane capacitance from 900 to 990 ms was plotted versus test potential.

I_f was measured with 3 seconds test pulses with 10 mV increments ranging between −50 and −120 mV, from a holding potential of −40 mV with an interpulse interval of 5 seconds (0.2 Hz). Activating currents were fitted with mono-exponential curves and steady state current was normalized to the maximally activated current, and subsequently plotted versus test potential.

I_{Kr}/Cs^+ currents were activated by 2 second pulses between −70 and +70 mV (10 mV increments). Next, a 300 ms test pulse at −80 mV was applied that elicited a tail current. The interpulse interval was 2.5 s (0.4 Hz). Tail currents were fitted with mono-exponential curves and peak tail current was normalized to cell capacitance. Drug responses were measured using the same approach, except that the current was activated by a fixed 2 s pulse to +50 mV.

For measurement of I_{Ks}, cells were kept at a holding potential of −60 mV and currents were activated by 3 seconds pulses between −40 and +60 mV (20 mV increments). Next, a 3 second test pulse at −40 mV was applied that elicited a tail current. The interpulse

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