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Original article

Mechanism of automaticity in cardiomyocytes derived from human induced pluripotent stem cells

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ABSTRACT

Background and objectives. The creation of cardiomyocytes derived from human induced pluripotent stem cells (hiPS-CMs) has spawned broad excitement borne out of the prospects to diagnose and treat cardiovascular diseases based on personalized medicine. A common feature of hiPS-CMs is their spontaneous contractions but the mechanism(s) remain uncertain. Methods. Intrinsic activity was investigated by the voltage-clamp technique, optical mapping of action potentials (APs) and intracellular Ca^{2+} (Ca_i) transients (Ca_iT) at subcellular-resolution and pharmacological interventions. Results. The frequency of spontaneous Ca_iT (sCa_iT) in monolayers of hiPS-CMs was not altered by ivabradine, an inhibitor of the pacemaker current, I_f despite high levels of HCN transcripts (1–4). HiPS-CMs had negligible I_f and I_{K1} (inwardly-rectifying K^+-current) and a minimum diastolic potential of -59.1 ± 3.3 mV ($n = 18$). APs upstrokes were preceded by a depolarizing-foot coincident with a rise of Ca_i. Subcellular Ca_i wavelets varied in amplitude, propagated and died-off; larger Ca_i-waves triggered cellular sCaTs and APs. SCa_iTs increased in frequency with $[Ca^{2+}]_{out}$ (0.05-to-1.8 mM), isoproterenol (1 μM) or caffeine (100 μM) ($n \geq 5$, $p < 0.05$). HiPS-CMs became quiescent with ryanodine receptor stabilizers (K201 = 2 μM); tetracaine; Na–Ca exchange (NCX) inhibition (SEA0400 = 2 μM); higher $[K^+]_{out}$ (5 → 8 mM), and thiol-reducing agents but could still be electrically stimulated to elicit Ca_iTs. Cell–cell coupling of hiPS-CM in monolayers was evident from connexin-43 expression and Ca_iT propagation. SCa_iTs from an ensemble of dispersed hiPS-CMs were out-of-phase but became synchronous through the outgrowth of inter-connecting microtubules. Conclusions. Automaticity in hiPS-CMs originates from a Ca^{2+}-clock mechanism involving Ca^{2+} cycling across the sarcoplasmic reticulum linked to NCX to trigger APs.

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1. Introduction

A milestone of stem cell biology has been the generation of embryonic stem (ES) cells from human blastocysts [1,2] and their differentiation into cells of all three germ layers including cardiomyocytes (CMs) [3,4]. Embryoid bodies (EBs) consisting of myocytes derived from human ES cells (hES-CMs) have the ultra-structural, electrical and Ca^{2+} cycling properties of early-stage or developing myocytes [3,5]. In contrast to mature ventricular myocytes, hES-CMs (20–35 days post-differentiation) exhibit spontaneous excitability and contractions which were attributed to hyperpolarization-activated cyclic nucleotide-gated (HCN) channels or the pacemaker current, I_f that triggered voltage-gated Na^+ channels and current (I_{Na}) to elicit action potentials [6]. Evidence of their immature phenotype includes a notable absence of Kir2.1 (the channel protein that encodes for the inward rectifying K^+ current, I_{K1}) and a lack of functional sarcoplasmic reticulum (SR) network [5,6]. Enhanced expression of Kir2.1in cultured hES-CMs ablated the pro-arrhythmic action potential traits [7]. Based on action potential recordings from EBs, hES-CMs are heterogeneous in their cell

Abbreviations: iPSC, inducible pluripotent stem cells; CM, cardiomyocytes; hiPS-CMs, human cardiomyocytes derived from iPS cells; ESCs, embryonic stem cells; hESCs, human ESCs; Ca_i, intracellular Ca^{2+}; SR, sarcoplasmic reticulum; APs, action potentials; Ca_iT, intracellular Ca^{2+} transients; RyR2, cardiac ryanodine receptor; K201, (1,4-benzothiazepine); NCX, sodium–calcium exchanger; DTT, dithiothreitol; SAN, sinoatrial node; CX43, connexin 43; I_f, funny current; I_{K1}, inwardly rectifying K^+-current; HCN, hyperpolarization-activated cycling nucleotide-gated channel.

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type and consist of a mixture of nodal, atrial and ventricular-like myocytes [8].

The reprogramming of human fibroblasts into induced pluripotent stem cells (hiPSCs) [9,10] has made it possible to generate patient specific iPSCs from a readily accessible source of cells. hiPSCs possess similar proliferative properties to hESCs and the potential to differentiate into CMs. CMs derived from human iPSCs (hiPS-CMs) are genetically identical to the native donor CMs, avoid possible immune response in clinical applications and ethical concerns faced by the procurement of hESCs. HiPS-CMs are comparable to hES-CMs with similar differentiation pattern, action potential properties, distribution of cell types (nodal, atrial and ventricular) and responses to α- and β-adrenergic stimulation [11–14]. HiPS-CMs generated from patients with LEOPARD [15] or long QT syndrome [16,17] were shown to retain the expected characteristics of the patients' cardiac phenotypes, demonstrating great promise for patient specific diagnostics and treatments [14,17,18].

Studies of Ca^{2+} handling in hES-CMs and hiPS-CMs have yielded contradictory results. Studies reported a lack of contractile response to ryanodine and thapsigargin and a relatively immature SR function in hES-CMs [5,19], or functional SR but with an immature Ca^{2+} cycling phenotype in 38% of hES-CMs [20] or operative Ca^{2+}-induced Ca^{2+} release despite a leaky SR in hES-CMs enriched for ventricular-like cells [21]. A study of pacemaker mechanisms in hES-CMs (11–21 days post-EB formation) found marked heterogeneities of action potential shape and pacemaker properties yet all cells had substantial I_f levels [22]. Spontaneous activity was reduced by I_f inhibitors in some but not all cells while other cells responded to Na–Ca exchange (NCX) inhibitors and still others responded to both types of inhibitors, suggesting that voltage and Ca^{2+} clock mechanisms can co-exist. Blockade of both I_f and I_{NCX} did not eliminate pacemaker activity and the residual pacemaker current was attributed to the turn-off of Ca^{2+} activated K^+ channels (I_{KCa}/SK4). Overexpression of SK4 channels in embryonic stem cells leads to an increase in pacemaker cell differentiation emphasizing its importance in the early development of the conduction system [23]. A comparison of Ca^{2+} handling properties of hES-CMs and hiPS-CMs reported that iPS-CMs have smaller amplitude and slower Ca^{2+} kinetics than hES-CMs suggesting that 20-day old hiPS-CMs are more immature than hES-CMs [24]. Others found that hiPS-CMs have Ca^{2+} cycling properties of adult ventricular myocytes [25] and hiPS-CMs generated from a CPVT (catecholaminergic polymorphic ventricular tachycardia) patient carrying a mutation in RyR2 (cardiac ryanodine receptor) recapitulated the Ca^{2+} signaling phenotype of the CPVT syndrome [25,26]. Despite these inconsistencies about Ca^{2+} cycling properties, spontaneous contractions of CMs are commonly used as evidence of successful differentiation to cardiomyocytes from pluripotent stem cells. Yet, the mechanisms underlying automaticity have not been systematically investigated making it difficult for hiPS-CMs to reach their full potential as a therapeutic tool.

Here, we studied the mechanisms underlying spontaneous electrical and mechanical activity (through changes in Ca^{2+} transients: CaT) of cultured hiPS-CMs using optical mapping of action potentials (APs) and CaT and using the whole-cell voltage-clamp technique. CaT were measured using low (Rhod-2/AM, $K_D = 500$ nM) [27] and high (GCaMP2; $K_D = 146$ nM) [28] affinity Ca^{2+} sensors to insure the detection of low amplitude intracellular Ca^{2+} (Ca_i) signals in monolayers of hiPS-CMs and single cells. Ionic and pharmacological interventions were applied to show that a Ca^{2+}-clock mechanism involving Ca^{2+} cycling across the sarcoplasmic reticulum (SR) linked to membrane potential changes via the sodium–calcium exchanger (NCX) is responsible for their spontaneous activity.

2. Methods

2.1. Maintenance and cardiomyocyte differentiation of human iPS cells

A human Y1 iPS cell line was generated from human fibroblast line HDF-α and was fully characterized as previously described [29]. The following conditions were used for cardiomyocyte differentiation [30] from Y1 iPSCs by forming EBs using the basal StemPro®-34 medium (Invitrogen) as described in our previous studies [29,30]: days 0–1, BMP4 (5 ng/ml); days 1–4, BMP4 (10 ng/ml), bFGF (5 ng/ml) and Activin (1.5 ng/ml); days 4–20, DKK1 (150 ng/ml) and VEGF (10 ng/ml). Cultures were maintained in a 5% CO_2/5% O_2/90% N_2 environment for the first 20 days and were then transferred into a 5% CO_2/air environment. All cytokines were purchased from R&D Systems. To study dissociated hiPS-CMs, spontaneously beating EBs were dissociated by incubation with 1 mg/ml collagenase B for 30 min at 37 °C, followed with 0.25% trypsin for 5 min at 37 °C, and then plated on laminin-coated cover slips or dishes. The purity of our hiPS-CMs was previously shown to be >80% [31].

2.2. Optical mapping of human iPS-CMs

Cells were loaded with Rhod-2/AM [32] or infected with AAV-GCaMP2 to measure intracellular Ca^{2+} (Ca_i) [33] and/or stained with di-4-ANEPPS or Pittsburgh1 (PGHI) to measure voltage [34]. Cells were allowed to anchor on laminin-coated plasticware and were placed on the heated stage of an Olympus BX1f microscope. Optical mapping was performed at high spatiotemporal resolution (100 × 100 pixels, 150 μm × 150 μm; 100 Hz) at 37 °C using two SciMedia CMOS cameras (Ultima One) with 100 × 100 pixels, as previously described [35]. Excitation light intensity was carefully adjusted not to cause cell damage due to phototoxicity. Membrane potential (V_m) and intracellular Ca^{2+} (Ca_i) were simultaneously measured in spontaneously beating hiPS-CM clusters or single cells followed by various ionic or pharmacological interventions to manipulate automaticity. K201 (3-(4-Benzylcyclohexyl)-1-(7-methoxy-2, 3-dihydrobenzo[*f*] [1,4] thiazepin-4(5*H*)-yl)propan-1-one) was synthesized and generously provided by Dr. Robert Strongin and Dr. Jonathan Abramson (Portland State University). SEA0400 was the generous gift of Dr. András Varró and Dr. Norbert L. Iost (University of Szeged). Field electrical shocks were applied to pace iPS-CMs.

2.3. Calcium mapping of hiPS-CMs with genetically encoded probes

Cells were genetically encoded with the Ca^{2+} sensor GCaMP2 using an adeno-associated viral vector AAV-GCaMP2 infection at MOI of 10^5 for 48 h, as previously described [28].

2.4. Immunolabeling and fluorescence microscopy

HiPS-CMs were fixed in 2% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100, washed in PBS, and blocked with 2% BSA in PBS. Fixed cells were incubated with primary then secondary antibodies with extensive washings to remove unbound antibodies. Cells were imaged with an inverted Nikon Eclipse TS100 microscope using a CCD camera (Quantitative Imaging Corporation) and software from iVision (Biovision Technologies). The following antibodies were used for fluorescence microscopy: anti-human Troponin T from Lab Vision; anti-human α-Actinin from Sigma Aldrich and anti-Connexin 43/GJA1 from Abcam (1:200 dilution). Alexa 488 and Cy3-conjugated secondary antibodies were purchased from Invitrogen; slides were also stained with DAPI (Invitrogen, 1:1000 dilution) to visualize nuclei.

2.5. Electrophysiology

HiPS-CMs were seeded at a density of 20,000–40,000 viable cells in DMEM (Dulbecco's Modified Eagle Medium)/FBS (fetal bovine serum) media on twelve 15-mm cover-slips coated with 0.1% (w/v) gelatin (Sigma) solution placed in 12 well plates, and incubated for at least 2 days at 37 °C, 5% CO_2, before recording. Action potentials (APs) and ionic currents were recorded from single hiPS-CMs using the whole-cell patch clamp technique at room temperature using Axopatch1D,

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