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Ca^{2+} signaling in human induced pluripotent stem cell-derived cardiomyocytes (iPS-CM) from normal and catecholaminergic polymorphic ventricular tachycardia (CPVT)-afflicted subjects

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ABSTRACT

Derivation of cardiomyocytes from induced pluripotent stem cells (iPS-CMs) allowed us to probe the Ca^{2+}-signaling parameters of human iPS-CMs from healthy- and catecholaminergic polymorphic ventricular tachycardia (CPVT1)-afflicted individuals carrying a novel point mutation p.F2483I in ryanodine receptors (RyR2). iPS-CMs were dissociated on day 30–40 of differentiation and patch-clamped within 3–6 days. Calcium currents (I_{Ca}) averaged ~8 pA/pF in control and mutant iPS-CMs. I_{Ca}-induced Ca^{2+}-transients in control and mutant cells had bell-shaped voltage-dependence similar to that of I_{Ca}, consistent with Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism. The ratio of I_{Ca}-activated to caffeine-triggered Ca^{2+}-transients was ~0.3 in both cell types. Caffeine-induced Ca^{2+}-transients generated significantly smaller Na^+–Ca^{2+} exchanger current (I_{NCX}) in mutant cells, reflecting their smaller Ca^{2+}-stores. The gain of CICR was voltage-dependent as in adult cardiomyocytes. Adrenergic agonists enhanced I_{Ca}, but differentially altered the CICR gain, diastolic Ca^{2+}, and Ca^{2+}-sparks in mutant cells. The mutant cells, when Ca^{2+}-overloaded, showed longer and wandering Ca^{2+}-sparks that activated adjoining release sites, had larger CICR gain at −30 mV yet smaller Ca^{2+}-stores. We conclude that control and mutant iPS-CMs express the adult cardiomyocyte Ca^{2+}-signaling phenotype. RyR2 F2483I mutant myocytes have aberrant unitary Ca^{2+}-signaling, smaller Ca^{2+}-stores, higher CICR gains, and sensitized adrenergic regulation, consistent with functionally altered Ca^{2+}-release profile of CPVT syndrome.

1. Introduction

Recent breakthroughs in stem cell biology have made it possible to develop pluripotent stem cells from adult fibroblasts by transfecting them with a set of 4 "stemness" genes (inducible pluripotent stem cells, iPSC, [1]). This reprogramming allows experimental approaches that drive such cells to acquire cardiac molecular and electrophysiological phenotypes [2–4], thus creating opportunities for therapy of a host of cardiac pathologies using patient-derived cells. This approach has made it also possible to examine patient-specific mutations in ion channels and Ca^{2+} signaling proteins that might lead to arrhythmia and heart failure in iPS-CM in a laboratory setting, thus devising pharmacological patient-specific paradigms for therapy [5–11]. In light of such potentials, it is imperative that the electrophysiological and Ca^{2+} signaling properties of human iPS-CM as well as their pharmacology are fully identified and quantified.

Ca^{2+}-signaling in mammalian hearts is characterized by: (1) I_{Ca}-gated Ca^{2+}-release (CICR), providing for the characteristic bell-shaped voltage-dependence of Ca^{2+} transients that closely reflect the voltage-dependence of I_{Ca}; (2) the gain of CICR is voltage-dependent, not predicted from a strictly Ca^{2+}-dependent process [12–14]; (3) β-adrenergic agonists enhance I_{Ca}, Ca^{2+} content of the sarcoplasmic reticulum (SR), Ca_i-transients and accelerate their decay kinetics, consistent with PKA-mediated phosphorylation of DHPRs, phospholamban/SERCA2a complex, and the RyR2; (4) Caffeine-triggered Ca^{2+}-release activates an inward current (I_{NCX}) with time-course and kinetics similar to rise and fall of cytosolic Ca^{2+}, reflecting the efflux of Ca^{2+} on the electrogenic Na^+–Ca^{2+} exchanger (NCX). Although there are already a number of reports on the electrophysiology of iPS-CM [15–17] there are few detailed reports on their Ca^{2+} signaling pathways and their regulation beyond measurements of Ca^{2+} transients in intact non-voltage clamped cells [9,18] and in embryonic stem cell-derived cardiomyocytes [19].

In this report, we describe the Ca^{2+} signaling properties of human iPS-CM by quantifying the activities of Ca^{2+}-signaling

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proteins that include the density, kinetics, and regulation of Ca^{2+} channels and NCX transporter, the size of SR Ca^{2+}-stores, its regulation by β-adrenergic agonists, the voltage-dependence of I_{Ca} and Ca^{2+}-transients, the gain of CICR, the efficiency of Ca^{2+}-release mechanism, and the properties of the individual dyadic calcium release (sparks). In addition, we have attempted to quantify possible abnormalities in these parameters in cells derived from a patient afflicted with catecholaminergic polymorphic ventricular tachycardia (CPVT), carrying a recently identified ryanodine receptor mutation (p.F2483I) [9]. Our data suggests that Ca^{2+}-signaling properties of adult cardiac myocytes are closely replicated in human iPS-CM. That is, I_{Ca}-gated SR Ca^{2+}-release is the primary mechanism for the release of Ca^{2+} on depolarization of the cell by the action potential. Relaxation, in a manner similar to mammalian myocardium, is mediated by reuptake of Ca^{2+} into the SR and extrusion of Ca^{2+} by the Na^{+}–Ca^{2+} exchanger, producing currents often in excess of 2–3 pA/pF. While adrenergic agonists strongly enhanced I_{Ca}, and accelerated the rate of decay of the Ca^{2+}-transients, they had insignificant effects on NCX currents, consistent with findings in adult mammalian hearts [20,21]. These findings led us to conclude that human iPS-CM represent a reliable Ca^{2+}-signaling model of mammalian cardiomyocytes.

Numbers of recent reports have implicated RyR2-mutations and the resultant abnormal Ca^{2+} signaling in development of arrhythmia and sudden death associated with intense adrenergic stimulation in patients with CPVT. It has been proposed that such mutation renders the RyRs "leaky" on exposure to β-adrenergic agonists (hyper-phosphorylation & dissociation of calstabin from RyR2, [22,23] producing localized increases in Ca^{2+} that is extruded on NCX generating local depolarization (EADs & DADs), triggering at times fatal arrhythmias. Alternatively, overloading of SR Ca^{2+}-stores by adrenergic agonists has been proposed [24,25] to lead to increased probability of RyR2 channel openings, resulting in abnormal release of Ca^{2+} and the resultant membrane-depolarization and arrhythmias. These ideas have been tested in a number of knock-in mice and *in vitro* models [26–29], but their validity in the human disease remain still somewhat clouded by both the variability of the RyR2 point mutations producing CPVT, the locus of phosphorylation on RyR2, lack of universal confirmatory results, and absence of clear-cut pharmacology [25].

Our data here suggests that despite significant quantitative intercellular differences in Ca^{2+}-signaling parameters of control and RyR2 mutant cells, they both had equivalent and elevated densities of Ca^{2+} currents and NCX activity, similar bell-shaped voltage-dependence of I_{Ca}-gated Ca^{2+}-release, and voltage-dependent CICR gain. Mutant cells, however, were consistently found to have smaller caffeine-triggered Ca^{2+}-stores higher CICR gain, especially at −30 mV, consistent with longer, recurrent and often wandering Ca^{2+}-sparks, compared to sporadic and brief sparks of control iPS-CM. Though adrenergic agonists produced equivalent and large enhancements of I_{Ca} in both mutant and control cells, they differentially altered the CICR gain, diastolic Ca^{2+}, and Ca^{2+}-sparks in mutant cells consistent with aberrant Ca^{2+}-release profiles of Ca^{2+}-overloaded CPVT-mutant myocytes, and the higher proclivity for generation of DADs and EADs in mutant hearts.

2. Methods

2.1. Cultivation of human iPS cells and preparation of cardiomyocytes

2.1.1. Culture of undifferentiated human iPS cells

The human iPS cell lines were derived from dermal fibroblasts of a CPVT-afflicted patient carrying a *de novo* heterozygous autosomal dominant p.F2483I mutation in RYR2 and a healthy subject. The generation, cardiac differentiation and characterization of these cell lines were reported recently [9]. The iPS cells were maintained on mitomycin C treated murine embryonic fibroblasts (MEF) prepared in our laboratory in DMEM/F12 medium supplemented with Glutamax, 20% knockout serum replacer, 1% nonessential amino acids (NAA), 0.1 mM β-mercaptoethanol (βME, Invitrogen, Darmstadt, Germany), 50 ng/ml FGF-2 (PeproTech, Hamburg, Germany). Cells were passaged by manual dissection of cell clusters every 5–6 days.

2.1.2. Cardiac differentiation

Cardiac differentiation of human iPS cells was carried out on the murine visceral endoderm-like cell line (END_2), which was provided by C. Mummery (Leiden University Medical Center, The Netherlands). END_2 cells were mitotically inactivated for 3 h with 10 μg/ml mitomycin C (Sigma–Aldrich Chemie GmbH, Munich, Germany) and 1.2×10^6 cells were plated on 6 cm dishes coated with 0.1% gelatin one day before initiation of iPS cell differentiation. To initiate co-cultures, iPS cell colonies were dissociated into clumps by using collagenase IV (Sigma-Aldrich, 1 mg/ml in DMEM/F-12 at 37 °C for 5–10 min). The differentiation was carried out in 1% knockout-DMEM containing 1 mM L-glutamine, 1% NAA, 0.1 mM βME and 1% penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). The co-culture was left undisturbed at 37 °C/5% CO_2 for 5 days. First medium change was performed on day 5 and later on days 9, 12 and 15 of differentiation. Spontaneously contracting clusters were dissociated into single cardiomyocytes for experiments.

2.1.3. Preparation of iPS-CM for patch-clamp experiments

Beating areas were micro-dissected mechanically at day 30–40 of differentiation, dissociated with collagenase B, and single iPS-CM then plated on fibronectin (2.5 μg/ml)-coated glass coverslips in 6 well plates. Cells were incubated for 36–72 h before their use in electrophysiological experiments.

2.2. Measurements of cellular currents and global Ca^{2+}

iPS-CM were voltage-clamped in the whole-cell configuration. L-type Ca^{2+} current (I_{Ca}) and I_{NCX} were activated by depolarizing pulses or exposure to caffeine. The voltage-clamped cells were dialyzed with a Cs^{+}-based, moderately Ca^{2+}-buffered pipette solution containing (in mM): 110 Cs^{+}-Aspartate, 15 or 5 NaCl, 20 TEACl, 5 Mg-ATP, 0.2 EGTA and 0.1 Fluo-4 pentapotassium, 0.1 $CaCl_2$ ($[Ca^{2+}]_i \sim 100$ mM), 10 glucose and 10 HEPES (titrated to pH 7.2 with CsOH; measured osmolarity: 295 mOsm) allowing simultaneous measurements of intracellular Ca^{2+} transients. L-type Ca^{2+} current (I_{Ca}) was measured by depolarization to 0 mV from a holding potential of −50 or −40 mV using a Dagan amplifier and pClamp (Clampex 10.2) software. Borosilicate patch pipettes were prepared using a horizontal pipette puller (Model P-87, Sutter Instruments, CA). The pipettes had a resistance of 3–5 MΩ. The extracellular solution in the experimental chamber contained (in mM): 137 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 HEPES (titrated to pH 7.4 with NaOH). To facilitate recordings of I_{Ca} and I_{NCX}, we blocked K^{+} currents, not only by including TEA in the dialyzing solution, but also by using an electromagnetically controlled puffing system that applied K^{+}-free solutions in the immediate vicinity of the voltage-clamped cell. I_{Ca} was modulated by adding isoproterenol or Bay-K 8644 to the K^{+}-free puffing solutions while rapid application of 3 mM caffeine was used to probe the magnitude of SR Ca^{2+} stores and activate I_{NCX}. I_{Ca} was normalized relative to the membrane capacitance and plotted in units of pA/pF. All experiments were carried out at room temperature (22–24 °C).

Cellular Ca^{2+} transients in voltage-clamped cells were measured fluorometrically by including 0.1 mM Fluo-4 pentapotassium salt in

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