Published in final edited form as:

J Mol Cell Cardiol. 2013 July ; 60: 36–46. doi:10.1016/j.yjmcc.2013.03.014.

Identification and characterization of a transient outward K^+ current in human induced pluripotent stem cell-derived cardiomyocytes

Jonathan M. Cordeiro^a, Vladislav V. Nesterenko^a, Serge Sicouri^a, Robert J. Goodrow Jr.^a, Jacqueline A. Treat^b, Mayurika Desai^b, Yuesheng Wu^b, Michael Xavier Doss^b, Charles Antzelevitch^{a,*}, and José M. Di Diego^{a,*}

^aDepartment of Experimental Cardiology, Masonic Medical Research Laboratory, Utica, NY 13501, USA

^bStem Cell Center, Masonic Medical Research Laboratory, Utica, NY 13501, USA

Abstract

Background—The ability to recapitulate mature adult phenotypes is critical to the development of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) as models of disease. The present study examines the characteristics of the transient outward current (I_{to}) and its contribution to the hiPSC-CM action potential (AP).

Method—Embryoid bodies were made from a hiPS cell line reprogrammed with Oct4, Nanog, Lin28 and Sox2. Sharp microelectrodes were used to record APs from beating-clusters (BC) and patch-clamp techniques were used to record I_{to} in single hiPSC-CM. mRNA levels of Kv1.4, KChIP2 and Kv4.3 were quantified from BCs.

Results—BCs exhibited spontaneous beating (60.5 ± 2.6 bpm) and maximum-diastolic-potential (MDP) of 67.8 ± 0.8 mV ($n = 155$). A small 4-aminopyridine-sensitive phase-1-repolarization was observed in only 6/155 BCs. A robust I_{to} was recorded in the majority of cells (13.7 ± 1.9 pA/pF at +40 mV; $n = 14$). Recovery of I_{to} from inactivation (at -80 mV) showed slow kinetics ($\tau_1 = 200 \pm 110$ ms (12%) and $\tau_2 = 2380 \pm 240$ ms (80%)) accounting for its minimal contribution to the AP. Transcript data revealed relatively high expression of Kv1.4 and low expression of KChIP2 compared to human native ventricular tissues. Mathematical modeling predicted that restoration of I_{K1} to normal levels would result in a more negative MDP and a prominent phase-1-repolarization.

Conclusion—The slow recovery kinetics of I_{to} coupled with a depolarized MDP account for the lack of an AP notch in the majority of hiPSC-CM. These characteristics reveal a deficiency for the development of *in vitro* models of inherited cardiac arrhythmia syndromes in which I_{to} -induced AP notch is central to the disease phenotype.

Keywords

Repolarization; Electrophysiology; Mathematical modeling; Ion channel currents

© 2013 Elsevier Ltd. All rights reserved.

*Corresponding authors at: Department of Experimental Cardiology, Masonic Medical Research Laboratory, 2150 Bleecker Street, Utica, NY 13501, USA. Tel.: +1 315 735 2217; fax: +1 315 735 5648. ca@mmrl.edu (C. Antzelevitch), didiego@mmrl.edu (J.M. Di Diego).

Conflict of interest disclosures

The authors have nothing to disclose.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yjmcc.2013.03.014>.

1. Introduction

Human induced pluripotent stem cells (hiPSCs) derived by reprogramming somatic cells can be differentiated into a variety of cell types, including cardiomyocytes [1,2]. The use of hiPSC-derived cardiomyocytes (hiPSC-CM) holds promise for a variety of applications including: 1) drug development and safety pharmacology; 2) creation of *in vitro* human models of cardiac genetic diseases to better understand the pathophysiological mechanisms underlying electrocardiographic and arrhythmic manifestations, thus enabling the development of patient-specific treatments [2].

Repolarization of the cardiac action potential is initiated and controlled by activation of a number of time- and voltage-dependent K^+ channel currents [3]. In native ventricular myocytes, four K^+ currents play important roles in regulating the cardiac action potential (AP) duration: (i) the rapidly and slowly activating delayed rectifier K^+ channel currents (I_{Kr} and I_{Ks} , respectively), (ii) an inwardly rectifying K^+ current (I_{K1}) and (iii) a Ca^{2+} -independent transient outward K^+ current (I_{to}).

In the adult human heart, a prominent I_{to} is recorded in atrial [4] as well as ventricular epicardial (Epi) cells [5–7]. Molecular analysis of I_{to} in human ventricle has demonstrated that $K_v4.3$ channels comprise the majority of I_{to} channels with lower levels of $K_v1.4$. [7,8]. Evidence from several studies also suggests that several α -subunits including KChIP2 associate with $K_v4.3$ and serve to alter I_{to} density and kinetics [8,9]. In contrast to adult human ventricle, there are few data regarding I_{to} in young or neonatal ventricular myocardium. However, because electrophysiological analysis has established that I_{to} is small [10] or nearly absent [11] in the neonatal ventricle of other mammalian species, it is likely that newborn humans also lack I_{to} .

During embryonic development, the mesodermal layer differentiates into a number of cell types including vascular smooth muscle and cardiac muscle. The mechanism by which mesodermal cells integrate the various signals they receive and how they resolve this information to regulate their morphogenetic behavior is largely unknown [12]. Nonetheless, many investigators have successfully created hiPSC-CM from patients afflicted with arrhythmic syndromes and showed that they closely recapitulate the disease phenotype [13–21].

We recently demonstrated that maximum diastolic potential (MDP) of hiPSC-CM is critically dependent on I_{Kr} due to a minimal contribution of the I_{K1} [22]. In the present study, we examine the characteristics of I_{to} in single hiPSC-CM and its contribution to phase 1 of the AP in beating clusters (BCs). Preliminary results have been presented in abstract form [23].

2. Methods

2.1. Human iPSC culture and in vitro cardiac differentiation

The human iPS cell line IMR-90-C4 (WiCell, Madison, WI, USA), reprogrammed with Oct4, Sox2, Lin28 and Nanog as described previously [24,25] was maintained in serum-free, feeder-free conditions with mTeSR1 media (Stem Cell Technologies, Vancouver, Canada) on BD Matrigel™ (BD Biosciences, San Jose, CA) coated dishes for routine expansion. We used directed differentiation protocols to derive cardiomyocytes using serum-free, chemically-defined media supplemented with BMP4, Activin A, bFGF, VEGF and DKK-1 in stage specific manner as previously described. Our protocol yielded contractile clusters from up to 90% of the total embryoid bodies (EB) by days 8–10 post-differentiation. BCs

were micro-dissected from EBs ranging between 11–119 days of maturity and plated on gelatin coated dishes with EB10 media (DMEM + GlutaMAX™-I) supplemented with 10% fetal calf serum pretested for cardiac differentiation (Cat# 100–625, lot# A00C00Z, Gemini Bio-Products, West Sacramento, CA, USA), 100 μ M MEM non-essential amino acids and 100 μ M β -mercaptoethanol (all except otherwise stated from Gibco, Life Technologies, Grand Island, NY, USA). Single cells dissociated with collagenase II (Worthington Biochemical Corp, Lakewood, NJ, USA) from the contractile clusters were plated on fibronectin (Invitrogen, Life Technologies, Grand Island, NY, USA) coated dishes for at least 72 h prior to single cell electrophysiological recordings. Spontaneously contracting single cells were assumed to be of cardiac cells and were used for study.

2.2. Action potential recordings

Sharp microelectrodes (40–60 M Ω) filled with 2.7 M KCl were used to record APs from spontaneously beating clusters superfused with HEPES-Tyrodé's solution of the following composition (in mM): NaCl 140, KCl 4, MgCl₂ 1, HEPES 10, D-Glucose 10 and CaCl₂ 2; pH was adjusted to 7.4 with NaOH. The microelectrodes were connected to an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA, USA) operating in bridge mode. All signals were digitized (sampling rate = 40 kHz), stored on magnetic media and analyzed using Spike 2 for Windows (Cambridge Electronic Design [CED], Cambridge, UK). Following the control recordings, the preparations were exposed to 1 mM 4-aminopyridine (4-AP).

We assessed the spontaneous rate and the cycle length in each of the BCs studied and measured the following action potential (AP) parameters: AP amplitude, MDP (maximum diastolic potential), V_{max} (dV/dt_{max}), Notch magnitude (phase 1 repolarization), APD₃₀, APD₄₀, APD₅₀, APD₇₀, APD₈₀ and APD₉₀ (action potential duration at 30, 40, 50, 70, 80 and 90% of repolarization, respectively), Bazett's APD₉₀ (Bazett's correction: APD₉₀/CL); Hodge's APD₉₀ (Hodge's correction: ADP₉₀ + 1.75 $\times$ (heart rate – 60)); Fredericia's APD₉₀ (Fredericia's correction: ADP₉₀/(CL)^{1/3}), APD₅₀/APD₉₀ ratio (RO) and APD₃₀–40/APD₇₀–80 ratio (RO). Although the latter parameter was introduced by Ma et al. in an effort to distinguish between atrial-like (RO $\leq$ 1.5) and ventricular-like (RO > 1.5), [26] in the present study we did not separately analyze the different phenotypes. Nonetheless, we included the ROs “overall” for each of the groups evaluated (i.e. APs with and without a notch; Table 1) and specified the percentage of atrial-like and ventricular-like that comprised each group.

Right ventricular (RV), endocardial (Endo), and Epi tissues were obtained from hearts isolated from anesthetized (sodium pentobarbital, 35 mg/kg) mongrel dogs of either sex. The preparations consisted of dermatome shavings (Davol Simon Dermatome Power Handle 3293 with cutting head 3295) obtained from the anterobasal regions of the RV free wall. The tissues were superfused with oxygenated (95% O₂/5% CO₂) Tyrodé's solution maintained at 36.5 °C to 37 °C. The composition of the Tyrodé's solution was (in mmol/L) NaCl 129, KCl 4, NaH₂PO₄ 0.9, NaHCO₃ 20, CaCl₂ 1.8, MgSO₄ 0.5, and D-glucose 5.5; pH 7.4. The tissues were stimulated at basic cycle lengths (BCLs) of 1000 ms through thin silver bipolar electrodes. Transmembrane action potentials were recorded using glass microelectrodes filled with 2.7 mol/L KCl connected to a high-input-impedance amplification system. Signals were stored on magnetic media, digitized at 40 kHz and analyzed with Spike 2 (Cambridge Electronic Designs, Cambridge, UK). Following the control recordings, the preparations were exposed to 300 μ M BaCl₂.

2.3. Solutions

Single hiPSC-CM were superfused with HEPES buffer (mM): NaCl 126, KCl 5.4, MgCl₂ 1.0, CaCl₂ 2.0, HEPES 10, glucose 11, pH = 7.4 with NaOH. In addition, 300 μ M CdCl₂ was present in the extracellular solution to block I_{Ca}. The pipette solution consisted of (mM): K-aspartate 125, KCl 10, MgCl₂ 1, EGTA 5, MgATP 5, HEPES 10, NaCl 10, pH = 7.2 with KOH.

2.4. Electrophysiology

I_{to} recordings were made as previously described [27,28] and all experiments were performed at 36 °C. Voltage-clamp and conventional recordings were made using a MultiClamp 700A amplifier and MultiClamp Commander (Axon Instruments, Molecular Devices, LLC, Sunnyvale, CA, USA). Patch pipettes were fabricated from borosilicate glass capillaries (1.5 mm O.D., Fisher Scientific, Pittsburg, PA, USA). Pipettes were pulled using a gravity puller (Narishige Corp., East Meadow, NY, USA) and the resistance ranged from 1 to 4 M Ω when filled with the internal solution. Cell capacitance was measured by applying -5 mV voltage steps. Electronic compensation of series resistance to 60–70% was applied. All analog signals were acquired at 10–25 kHz, filtered at 4–6 kHz, digitized with a Digidata 1322 converter (Axon Instruments, Molecular Devices, LLC, Sunnyvale, CA, USA) and stored using pClamp9 software.

2.5. Quantitative Real Time-PCR

qPCR analysis was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Total RNA was extracted with RNeasy Micro Kit (Qiagen, CA). 50 ng total RNA from each of the pooled clusters ranging from 27 to 68 days post-differentiation (n = 8). Beating clusters were reverse transcribed with SuperScript™ First Strand Synthesis System for RT-PCR (Invitrogen, CA). Real-time PCR was performed in triplicates using the primers listed in Supplementary Table 1 using FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics, IN). Averaged C_t values of each qPCR reaction from the target gene were normalized with the average C_t values of the housekeeping gene *GAPDH*.

2.6. Statistics

Pooled data are presented as Mean $\pm$ SEM. Statistical analysis was performed using an ANOVA followed by a Student-Newman-Keuls test using SigmaStat software. A value of p < 0.05 was considered statistically significant.

2.7. Action potential simulations

Automatic activity of hiPSC-CMs was reproduced using the Luo-Rudy II (LRII) cellular action potential model [29,30] by decreasing the maximal conductance (G_{K1}) of the I_{K1} below 11% of normal value. The LRII model does not include hyperpolarization-activated inward current (I_f) and does not exhibit automatic activity under normal conditions.

Two models for I_{to} were employed in this study: (1) the previously published “normal” I_{to} model with fast inactivation (10 ms above 0 mV) and fast reactivation (68 ms at -80 mV) [31] and (2) the modification of this model to reproduce I_{to} behavior in hiPSC-CM with slower inactivation (16 ms above 0 mV) and very slow recovery from inactivation (2000 ms at -80 mV). Equations describing both normal and modified I_{to} models can be found in the on-line supplement together with the plots of the voltage dependence of kinetic parameters (Fig. S1).

In order to reproduce the shape of the epicardial AP reported in human ventricular cardiomyocytes (e.g. notch at 0 mV) using LRII cellular model, the density of I_{to} in the epicardial cell model G_{to} was set at 0.5 mS/ μ F.

Propagating APs were simulated in a short 4 mm cable comprised of 41 epicardial cells (LRII plus modified I_{to} model). The maximal conductance of I_{K1} in the cable was linearly distributed in the range between 0% (cell 1) and 30% (cell 41) to mimic heterogeneous expression of I_{K1} in BCs. Cells between 1 and 15 exhibit intrinsic automaticity due to low I_{K1} density. Cells in the rest of the cable are not automatically active and are excited by the propagating AP. Automatic activity was observed in this cable only when average resistivity was set at or above 3200 Ω cm, i.e. 8 times above normal myocardial resistivity.

3. Results

We studied 155 BCs and performed a detailed analysis of their electrophysiological characteristics. The vast majority of cells (149 out of 155) exhibited a slow upstroke velocity and no phase 1 repolarization or spike and dome morphology (notch). However, a small phase 1 repolarization could be observed in APs recorded from 6 BCs (Fig. 1A). The spontaneous rate (bpm; beats per min) and AP duration (APD) at 90% repolarization (APD₉₀) from all BCs were measured. The average rate of spontaneous APs was 60.4 ± 2.6 bpm for clusters without a phase 1 ($n = 149$), and 62.7 ± 16.9 bpm in clusters with a phase 1 ($n = 6$) ($p = n.s.$). In these 6 clusters, a more negative MDP (-87.8 ± 1.4 vs. -67.0 ± 0.8 mV; $p < 0.001$) and a higher V_{max} (141.8 ± 18.5 vs. 29.3 ± 1.7 V/s; $p < 0.001$) were observed (Table 1).

The small notch in these clusters suggested the presence of I_{to} , hypothesis that was supported by its abolition with the I_{to} inhibitor 4-aminopyridine (1 mM, 4-AP, Fig. 1B). Table 2 summarizes the effects of 4-AP (1 mM) on AP characteristics recorded from 13 BCs, 4 of which displayed an AP notch under control conditions.

The absence of phase 1 in BCs suggested a lack of I_{to} . In neonatal cardiac myocytes from most mammalian species, I_{to} is small [10] or absent [11] but increases with age. Given that I_{to} is absent in neonates, we anticipated that I_{to} would also be absent in hiPSC-CM in that they are developmentally immature. To test this hypothesis, we used voltage clamp techniques to record I_{to} from isolated hiPSC-CM. Spontaneously active single dissociated cells were studied. After a brief step to -50 mV to inactivate sodium channels, voltage steps from -40 to $+50$ mV elicited a rapidly activating and inactivating I_{to} (Fig. 2A). Surprisingly, the vast majority of these cells exhibited a relatively large I_{to} (peak current 13.7 ± 1.91 pA/pF at $+40$ mV, Fig. 2B), an amplitude comparable to that of canine left ventricular Epi and midmyocardial (Mid) cells. The time constant of decay (τ) of I_{to} was best fit with a single exponential yielding a value of 15.3 ± 1.5 ms at $+40$ mV and 22.4 ± 2.7 ms at 0 mV (Fig. 2C). These values are slower when compared to canine Epi cells (range from 7.95 to 12.0 ms at $+40$ mV), but comparable to Endo cells (16.9 ms at $+40$ mV) [28,32] under identical recording conditions.

The basis for the apparent disconnect between the absence of phase 1 repolarization in the majority of BCs and the presence of a robust I_{to} in single hiPSC cells suggests that alterations in the gating parameters of I_{to} in these cells may be responsible for the absence of phase 1 repolarization. We first measured recovery from inactivation of I_{to} and found it was very slow in hiPSC-CM (Fig. 3A). Recovery of I_{to} (at -80 mV) showed a fast and slow phase with $\tau_1 = 200 \pm 110$ ms ($12\% \pm 3\%$) and $\tau_2 = 2380 \pm 240$ ms ($80\% \pm 2\%$) (Fig. 3C, $n = 8$ cells). Recovery of I_{to} in hiPS-CM proved to be markedly slower than previously reported in human adult Epi ventricular myocytes [5,7], but comparable to values reported

for human adult Endo myocytes [5] recorded at room temperature. Interestingly, the recovery values recorded in hiPSC cells are comparable to those recorded in human Purkinje cells at 37 °C [33].

We next measured the steady-state inactivation of I_{to} using a prepulse-test pulse voltage-clamp protocol (top of Fig. 3B). Peak current following a 2 s prepulse was normalized to the maximum current and plotted as a function of the prepulse voltage to determine channel availability (Fig. 3D). A Boltzmann function was fit to the data which yielded a $V_{1/2} = -41.1 \pm 0.2$, $k = 6.68 \pm 0.19$. The steady state inactivation is shifted in the positive direction by 5 to 8 mV and shows a more shallow slope than had been typically reported in the literature (3.5 to 5 mV). [5,7,28] These characteristics of hiPSC-CM I_{to} are similar to what had been recorded in endocardial cells at 36 °C [5,7,28].

The very slow recovery of I_{to} suggests that at a physiologically relevant rate (60–75 bpm), very little I_{to} would be observed. To further test this hypothesis, we measured the magnitude of I_{to} at different stimulation frequencies. In the next set of experiments, changes in I_{to} were recorded from hiPSC-CM in response to pacing at progressively faster frequencies (Fig. 4A). Cells were held at -80 mV and I_{to} was activated at various pacing rates. The red trace in each panel shows the first activation pulse following a prolonged rest period. An increase in the pacing rate results in a progressive reduction in the amplitude of I_{to} . At a pacing rate of once per second, only a small fraction of I_{to} remains. The mean data showing the reduction in I_{to} as a function of pacing rate is illustrated in Fig. 4B. Our results show that I_{to} is present in hiPSC-CM cells but the slow recovery kinetics (at -80 mV) suggests that its contribution to the action potential is minimal. The depolarized MDP, secondary to a deficiency in I_{K1} [22], further reduced I_{to} via steady-state inactivation and slower recovery.

To address the question of which of the two factors (i.e. depolarized MDP due to low expression of I_{K1} current or slow inactivation and recovery of hiPSC-CM I_{to} current) is predominantly responsible for the lack of the spike-and-dome morphology in hiPSC-CM APs, we performed another set of experiments in which native Epi and Endo ventricular preparations with normal I_{to} kinetics were depolarized by exposure to 300 μ M BaCl₂, a concentration known to block nearly 100% of I_{K1} (Fig. 5A and B) [34,35]. Note the presence of phase 1 repolarization (Endo) and a spike-and-dome morphology (Epi) under control conditions (Panel A) and the lack thereof following 20 min of superfusion with barium (Panel B). Application of barium resulted in depolarization of both tissue types followed by spontaneous beating, which made them indistinguishable from each other. Thus, in the absence of I_{K1} , I_{to} -mediated phase 1 repolarization in adult Endo and Epi tissues resembled the immature AP morphologies of the hiPSC-CM.

These results suggest that at least part of the hiPSC-CM AP phenotype is due to a deficiency of I_{K1} . To test this hypothesis, we used the (LRII) model to simulate APs in the presence of normal (adult CM) and reduced (hiPSC-CM) levels of I_{K1} while incorporating normal adult I_{to} (Fig. 5C and D). Adult-CM were not automatically active and were stimulated at a CL of 500 ms. Left panel on Fig. 5C shows APs in the absence of I_{to} (Endo). Central panel illustrates APs in the presence of I_{to} with normal kinetics, which mediates spike-and-dome morphology (Epi). Reduction of I_{K1} to 10% of normal resulted in significant depolarization and development of automatic activity with a cycle length of 510 ms. The presence of I_{to} in the depolarized hiPSC-CM cellular model did not produce a spike and dome morphology (compare left and central panels on Fig. 5D) but decreased the APD30-APD40/APD70-APD80 ratio from 1.53 to 0.90 thus converting ventricular-like AP into atrial-like AP. The ionic currents contributing to the early phase of APs simulated with normal and decreased I_{K1} are shown in the right panels on Fig. 5C and D. Note the absence of fast I_{Na} in the right panel on Fig. 5D due to the failure to recover from the inactivation at depolarized MDPs,

which accounts for the absence of a “spike” in the Epi AP morphology. Also, because the AP upstroke is generated by slow inward current (I_{CaL}) only the “dome” portion of AP remains. The slower recovery of I_{to} from inactivation was not programmed in these simulations, demonstrating that the absence of the notch in AP recorded from hiPSC-CM can be primarily due to the depolarized MDP because no notch can be observed even with fast recovering normal adult I_{to} . Additional simulation results including effects of modified I_{to} model on simulated AP shape are provided in the Online Supplement.

The simulations described above are in apparent disagreement with the experimental data showing a small spike and dome in AP recordings from 6 BCs (out of 155). To explore this phenomenon, the modified I_{to} model, which reproduces slow kinetic of the current in hiPSC-CM (see Supplemental material for the model description), was incorporated into LRII to closely reproduce the hiPSC-CM behavior. Propagated APs were simulated in a 4 mm cable comprised of 41 modified Epi cells with linear distribution of I_{K1} to mimic heterogeneous expression of this current in BCs. The maximal conductance of I_{K1} in the cable was linearly distributed in the range between 0% (cell 1) and 30% (cell 41). Cells between 1 and 15 exhibited intrinsic automaticity due to low I_{K1} density. Cells in the rest of the cable were not automatically active and were excited by the propagating AP. A notch was observed in cells between 31 and 41. As shown on Fig. 6, the shape of the spike and dome in cell 35 is similar to the experimental recordings shown in Fig. 1. These simulations indicate that even a moderate increase in I_{K1} can hyperpolarize the MDP to values negative enough to allow I_{Na} to partially recover from inactivation which, in turn, allows a “spike” and “dome” (I_{to} -mediated notch) to manifest.

Next we attempted to address the question of what shape of AP could be expected if we succeed to fully express I_{K1} in hiPSC-CM. Fig. 7 (two upper panels) shows the effects of two different pacing rates on the shape of Epi APs simulated using the normal I_{to} model (upper traces) and the modified (slow) I_{to} model (bottom traces) under conditions of full I_{K1} (100%). The model predicts that slowly inactivating I_{to} observed in hiPSC-CM would nullify the contribution of I_{CaL} current (which has a similar inactivation rate) thus preventing the development of the dome at any pacing rate. On the other hand, the shape of simulated AP with normal I_{to} kinetics shows prominent spike and dome morphology at both pacing rates.

In the final series of simulations we evaluated the individual and combined contributions of slow inactivation rate and slowed recovery from inactivation of I_{to} in a single cell model (Fig. 7, lower panels; BCL = 1000 ms). Slow recovery of I_{to} from inactivation reduced the availability of the current leading to a smaller AP notch (panel B). Under these conditions, the notch is shown to remain even when the inactivation rate is reduced (panel D). However, when I_{to} is adjusted to recover normally, the slow inactivation prevents the development of the dome (panel C).

We have previously shown that the I_{to} agonist NS5806 works through KChIP2 to produce a pharmacological enhancement of I_{to} carried through $K_v4.3$ channels [28,36], although application of the agonist to $K_v1.4$ channels resulted in blockade of the current. In the next series of experiments, we attempted to enhance I_{to} in hiPSC-CM by application of 10 μ M NS5806 (Fig. 8). The I_{to} agonist failed to enhance I_{to} and produced a small reduction of the current in iPSC-CM (12.9 ± 1.0 pA/pF control vs. 10.8 ± 1.0 pA/pF following NS5806 ($n = 4$, $p = n.s.$).

Fig. 9 (left panel) displays mRNA levels of KCNA4 ($K_v1.4$), KCNIP2 (KChIP2) and KCND3 ($K_v4.3$) measured from BCs of 27 to 68 days post-differentiation ($n = 8$) and normalized to the mRNA levels of GAPDH. In agreement with the electrophysiological data

suggesting relatively low expression of KChIP2 and/or high expression of Kv1.4, our transcript data demonstrate that Kv1.4 is indeed highly expressed ($Kv1.4/Kv4.3 = 0.80$), whereas KChIP2 expression is disproportionally low ($KChIP2/Kv4.3 = 0.12$) when compared to human ventricular tissues as shown in the right panel of Fig. 9 (from Gaborit et al. [8]). The values for genes expression in human RV were re-normalized to GAPDH using published expression ratio of GAPDH to HPRT ≈ 61 [37,38].

4. Discussion

4.1. Summary of main findings

Our results demonstrate that AP recordings from hiPSC BCs 11 to 119 days post-differentiation exhibit phase 4 depolarization, spontaneous activity and a generally low upstroke velocity. A small population of BCs (6 out of 155; 3.87%) was found to exhibit a 4-AP sensitive phase 1, a more negative MDP, and a higher V_{max} . Patch clamp analysis of single iPSC-CM revealed that the majority of cells display a prominent I_{to} exhibiting slow inactivation and recovery kinetics. This apparent disconnect was further investigated in adult ventricular tissue exposed to $BaCl_2$ and with computer simulation. These results suggest that the depolarized MDP coupled with slow inactivation and recovery kinetics of I_{to} results in the absence of a phase 1-mediated AP notch in hiPSC-CM.

4.2. Biophysical and molecular analysis of I_{to} in hiPSC-CM

Our data indicate that hiPSC-CM exhibit robust I_{to} , but because reactivation kinetics are very slow (on the order of several seconds) and MDPs are depolarized (because of a deficiency in I_{K1}) an AP notch is rarely observed. The molecular mechanisms underlying slow reactivation of I_{to} remains to be elucidated. We have previously demonstrated that the I_{to} agonist NS5806 works through KChIP2 to produce a pharmacological enhancement of I_{to} carried through $Kv4.3$ channels, while application of the agonist to $Kv1.4$ channels resulted in blockade of the current [28,36]. In that the I_{to} agonist failed to enhance I_{to} and produced a small reduction of the current in hiPSC-CM, the data suggest that KChIP2 levels may be deficient and/or that $Kv1.4$ may be a major component of I_{to} in hiPSC-CM.

The molecular identity of I_{to} in canine and human adult ventricle is thought to be due to $Kv4.3$ and KChIP2, although $Kv1.4$ shows considerable expression in endocardial cells and in Purkinje fibers [8]. Several studies suggest that $Kv4.3$ levels are uniform throughout the canine and human adult ventricle and that a transmural gradient in I_{to} expression is due largely to a gradient in KChIP2 [15,16]. KChIP2 interacts with $Kv4.3$ channels resulting in increased peak $Kv4.3$ current density as well as accelerated recovery from inactivation.

The slow recovery of I_{to} in hiPSC-CM cells in the present study (Fig. 3C and Fig. 4) as compared with much faster I_{to} recovery in human ventricular Epi cells [7] suggests that the molecular composition of this current differs from that of human adult Epi ventricular myocytes. Nabauer et al. [39] showed that human endocardial and epicardial I_{to} recovery is a double exponential function. However, in the endocardial cells the slow recovery dominates (96%) and in the epicardial cells the fast recovery dominates (89%). Our cells show some characteristics of endocardial I_{to} , recovering with the time constant similar to the slow time constant obtained in the human endocardial cells, while the fast component of recovery is rather small and its amplitude is statistically not significantly different from zero. For that reason we chose to describe and simulate I_{to} found in hiPSC-CM as a slow inactivating and slow recovering current ignoring the small fast component. A greater expression of $Kv1.4$ and low expression of KChIP2 underlies the slow recovery of I_{to} in endocardial cells and our finding of relatively high expression of $Kv1.4$ vs. $Kv4.3$ is consistent with these electrophysiologic characteristics of I_{to} in our iPSC-CM. The precise

stoichiometry of the alpha and beta subunits responsible for the I_{to} in hiPSC-CM remains to be elucidated.

4.3. Physiological role of I_{to} in stem cells

Our study demonstrates that I_{to} is present in hiPSC-CM but that its functional role at physiological rates is small due to depolarized MDP and slow upstroke velocity. The slow recovery of I_{to} from inactivation in hiPSC-CM further reduces the available levels of this current. Our experimental studies clearly demonstrate that inhibition of I_{K1} in adult ventricular myocardium results in AP morphologies resembling hiPSC-CM. Our findings suggest that future studies should be directed at enhancing I_{K1} and I_{to} in an effort to generate hiPSC-CM that display more mature ventricular electrophysiological phenotypes. Once this goal is achieved, the potential of hiPSC-CM for safety pharmacology or for cell replacement therapy and heart regeneration may be expanded. The ability to recapitulate a mature adult phenotype is also critical to development of hiPSC-CM as human models of inherited cardiac arrhythmias syndromes, particularly Brugada and early repolarization syndromes in which I_{to} is believed to be pivotal in generating the disease phenotype.

4.4. Limitation of the study

Previous studies from our group have shown that the biophysical characteristics of I_{to} vary among adult Purkinje, atrial and ventricular cells [27,28,32,40]. Since hiPSC-CM have been characterized as exhibiting nodal, atrial and ventricular-like phenotypes based on their AP morphology, they are expected to exhibit different I_{to} characteristics. A detailed correlation of cardiac phenotype based on AP waveforms and I_{to} was not performed in the current study. Studies are underway to investigate whether different cell-types can be identified, including ventricular-Endo-like and Epi-like, which are known to display contrasting I_{to} amplitudes, inactivation and recovery kinetics as a consequence of differences in the relative contribution of ancillary subunits to channel function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Judy Hefferon.

Funding

This study was supported by a Grant-in-Aid (10GRNT4210016) from the American Heart Association (Dr. Di Diego), a New York Stem Cell Science (NYSTEM) grant C026424 (Dr. Antzelevitch), a grant HL47678 from NHLBI (Dr. Antzelevitch), an American Heart Association (AHA) grant 11SDG5410034 (Dr. Doss), and by New York State, Florida, Massachusetts and Connecticut Masons.

References

1. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007; 131:861–872. [PubMed: 18035408]
2. Gunaseeli I, Doss MX, Antzelevitch C, Hescheler J, Sachinidis A. Induced pluripotent stem cells as a model for accelerated patient- and disease-specific drug discovery. *Curr Med Chem*. 2010; 17:759–766. [PubMed: 20088756]
3. Nerbonne JM, Kass RS. Molecular physiology of cardiac repolarization. *Physiol Rev*. 2005; 85:1205–1253. [PubMed: 16183911]

4. Amos GJ, Wettwer E, Metzger F, Li Q, Himmel HM, Ravens U. Differences between outward currents of human atrial and subepicardial ventricular myocytes. *J Physiol.* 1996; 491(Pt 1):31–50. [PubMed: 9011620]
5. Wettwer E, Amos GJ, Posival H, Ravens U. Transient outward current in human ventricular myocytes of subepicardial and subendocardial origin. *Circ Res.* 1994; 75:473–482. [PubMed: 8062421]
6. Wettwer E, Amos GJ, Posival H, Ravens U. Regional differences in transient outward current I_{to} in ventricular myocytes from subendocardium and subepicardium in human hearts. *Proceeding of IUPS (Glasgow).* 1993:13.
7. Akar FG, Wu RC, Deschenes I, Armoundas AA, Piacentino III V, Houser SR, et al. Phenotypic differences in transient outward K^+ current of human and canine ventricular myocytes: insights into molecular composition of ventricular I_{to} . *Am J Physiol Heart Circ Physiol.* 2004; 286:H602–H609. [PubMed: 14527940]
8. Gaborit N, Le BS, Szuts V, Varro A, Escande D, Nattel S, et al. Regional and tissue specific transcript signatures of ion channel genes in the non-diseased human heart. *J Physiol.* 2007; 582:675–693. [PubMed: 17478540]
9. Deschenes I, Di Silvestre D, Juang GJ, Wu RC, An WF, Tomaselli GF. Regulation of $K_v4.3$ current by KCHIP2 splice variants: a component of native cardiac I_{to} ? *Circulation.* 2002; 106:423–429. [PubMed: 12135940]
10. Paciorek LM, Gilmour RF Jr. Developmental changes in the transient outward potassium current in canine epicardium. *Am J Physiol.* 1995; 268:H2513–H2521. [PubMed: 7611502]
11. Jeck CD, Boyden PA. Age-related appearance of outward currents may contribute to developmental differences in ventricular repolarization. *Circ Res.* 1992; 71:1390–1403. [PubMed: 1423935]
12. Gaspar JA, Doss MX, Winkler J, Wagh V, Hescheler J, Kolde R, et al. Gene expression signatures defining fundamental biological processes in pluripotent, early, and late differentiated embryonic stem cells. *Stem Cells Dev.* 2012; 21:2471–2484. [PubMed: 22420508]
13. Moretti A, Bellin M, Welling A, Jung CB, Lam JT, Bott-Flugel L, et al. Patient-specific induced pluripotent stem-cell models for long-QT syndrome. *N Engl J Med.* 2010; 363:1397–1409. [PubMed: 20660394]
14. Itzhaki I, Maizels L, Huber I, Zwi-Dantsis L, Caspi O, Winterstern A, et al. Modelling the long QT syndrome with induced pluripotent stem cells. *Nature.* 2011; 471:225–229. [PubMed: 21240260]
15. Matsa E, Rajamohan D, Dick E, Young L, Mellor I, Staniforth A, et al. Drug evaluation in cardiomyocytes derived from human induced pluripotent stem cells carrying a long QT syndrome type 2 mutation. *Eur Heart J.* 2011; 32:952–962. [PubMed: 21367833]
16. Lahti AL, Kujala VJ, Chapman H, Koivisto AP, Pekkanen-Mattila M, Kerkela E, et al. Model for long QT syndrome type 2 using human iPS cells demonstrates arrhythmogenic characteristics in cell culture. *Dis Model Mech.* 2012; 5:220–230. [PubMed: 22052944]
17. Davis RP, Casini S, van den Berg CW, Hoekstra M, Remme CA, Dambrot C, et al. Cardiomyocytes derived from pluripotent stem cells recapitulate electrophysiological characteristics of an overlap syndrome of cardiac sodium channel disease. *Circulation.* 2012; 125:3079–3091. [PubMed: 22647976]
18. Yazawa M, Hsueh B, Jia X, Pasca AM, Bernstein JA, Hallmayer J, et al. Using induced pluripotent stem cells to investigate cardiac phenotypes in Timothy syndrome. *Nature.* 2011; 471:230–234. [PubMed: 21307850]
19. Novak A, Barad L, Zeevi-Levin N, Shick R, Shtrichman R, Lorber A, et al. Cardiomyocytes generated from CPVT D307H patients are arrhythmogenic in response to beta-adrenergic stimulation. *J Cell Mol Med.* 2012; 16:468–482. [PubMed: 22050625]
20. Jung CB, Moretti A, Schnitzler M, Iop L, Storch U, Bellin M, et al. Dantrolene rescues arrhythmogenic RYR2 defect in a patient-specific stem cell model of catecholaminergic polymorphic ventricular tachycardia. *EMBO Mol Med.* 2012; 4:180–191. [PubMed: 22174035]
21. Fatima A, Xu G, Shao K, Papadopoulos S, Lehmann M, rnaiz-Cot JJ, et al. In vitro modeling of ryanodine receptor 2 dysfunction using human induced pluripotent stem cells. *Cell Physiol Biochem.* 2011; 28:579–592. [PubMed: 22178870]

22. Doss MX, Di Diego JM, Goodrow RJ, Wu Y, Cordeiro JM, Nesterenko VV, et al. Maximum diastolic potential of human induced pluripotent stem cell-derived cardiomyocytes depends critically on IKr. *PLoS One*. 2012; 7:e40288. [PubMed: 22815737]
23. Sicouri S, Cordeiro JM, Di Diego JM, Nesterenko VV, Goodrow RJ, Treat J, et al. Characteristics of the transient outward K⁺ current and its contribution to the action potential in hiPSC-derived cardiomyocytes. *Program and abstracts of NYSTEM 2012: Windows of Opportunity*. 2012:23.
24. Kattman SJ, Huber TL, Keller GM. Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev Cell*. 2006; 11:723–732. [PubMed: 17084363]
25. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, et al. Human cardiovascular progenitor cells develop from a KDR + embryonic-stem-cell-derived population. *Nature*. 2008; 453:524–528. [PubMed: 18432194]
26. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, Kamp TJ, et al. High purity human-induced pluripotent stem cell-derived cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am J Physiol Heart Circ Physiol*. 2011; 301:H2006–H2017. [PubMed: 21890694]
27. Dumaine R, Cordeiro JM. Comparison of K⁺ currents in cardiac Purkinje cells isolated from rabbit and dog. *J Mol Cell Cardiol*. 2007; 42:378–389. [PubMed: 17184792]
28. Calloe K, Soltysinska E, Jespersen T, Lundby A, Antzelevitch C, Olesen SP, et al. Differential effects of the transient outward K⁺ current activator NS5806 in the canine left ventricle. *J Mol Cell Cardiol*. 2010; 48:191–200. [PubMed: 19632239]
29. Luo CH, Rudy Y. A dynamic model of the cardiac ventricular action potential. I. Simulations of ionic currents and concentration changes. *Circ Res*. 1994; 74:1071–1096. [PubMed: 7514509]
30. Zeng J, Laurita KR, Rosenbaum DS, Rudy Y. Two components of the delayed rectifier K⁺ current in ventricular myocytes of the guinea pig type. Theoretical formulation and their role in repolarization. *Circ Res*. 1995; 77:140–152. [PubMed: 7788872]
31. Dumaine R, Towbin JA, Brugada P, Vatta M, Nesterenko DV, Nesterenko VV, et al. Ionic mechanisms responsible for the electrocardiographic phenotype of the Brugada syndrome are temperature dependent. *Circ Res*. 1999; 85:803–809. [PubMed: 10532948]
32. Di Diego JM, Cordeiro JM, Goodrow RJ, Fish JM, Zygmunt AC, Perez GJ, et al. Ionic and cellular basis for the predominance of the Brugada syndrome phenotype in males. *Circulation*. 2002; 106:2004–2011. [PubMed: 12370227]
33. Han W, Zhang L, Schram G, Nattel S. Properties of potassium currents in Purkinje cells of failing human hearts. *Am J Physiol Heart Circ Physiol*. 2002; 283:H2495–H2503. [PubMed: 12388306]
34. Shimoni Y, Clark RB, Giles WR. Role of an inwardly rectifying potassium current in rabbit ventricular action potential. *J Physiol*. 1992; 448:709–727. [PubMed: 1593485]
35. Cordeiro JM, Spitzer KW, Giles WR. Repolarizing K⁺ currents in rabbit heart Purkinje cells. *J Physiol*. 1998; 508(Pt 3):811–823. [PubMed: 9518735]
36. Lundby A, Jespersen T, Schmitt N, Grunnet M, Olesen SP, Cordeiro JM, et al. Effect of the I(to) activator NS5806 on cloned K(V)4 channels depends on the accessory protein KChIP2. *Br J Pharmacol*. 2010; 160:2028–2044. [PubMed: 20649599]
37. Tan SC, Carr CA, Yeoh KK, Schofield CJ, Davies KE, Clarke K. Identification of valid housekeeping genes for quantitative RT-PCR analysis of cardiosphere-derived cells preconditioned under hypoxia or with prolyl-4-hydroxylase inhibitors. *Mol Biol Rep*. 2012; 39:4857–4867. [PubMed: 22065248]
38. Everaert BR, Boulet GA, Timmermans JP, Vrints CJ. Importance of suitable reference gene selection for quantitative real-time PCR: special reference to mouse myocardial infarction studies. *PLoS One*. 2011; 6:1–8.
39. Nabauer M, Beuckelmann DJ, Uberfuhr P, Steinbeck G. Regional differences in current density and rate-dependent properties of the transient outward current in subepicardial and subendocardial myocytes of human left ventricle. *Circulation*. 1996; 93:168–177. [PubMed: 8616924]
40. Calloe K, Nof E, Jespersen T, Olesen SP, Di Diego JM, Chlus N, et al. Comparison of the effects of the transient outward potassium channel activator NS5806 on canine atrial and ventricular cardiomyocytes. *J Cardiovasc Electrophysiol*. 2011; 22:1057–1066. [PubMed: 21457383]

Fig. 1.

A: Action potential (AP) recordings obtained from a beating cluster (BC) exhibiting a small phase 1 repolarization (notch). Note the relatively large V_{max} (177 V/s). Only 6 out of 155 BCs exhibited a notch. B: Effect of 4-aminopyridine (4-AP) on APs recorded from a BC. Top traces: Control. Middle traces: 1 mM 4-AP abolished phase 1 repolarization suggesting a Ca^{2+} -independent transient outward current (I_{to}) was responsible for the small notch. Lower traces: Washout of 4-AP.

Fig. 2.

Characteristics of transient outward current (I_{to}). A: Representative traces of I_{to} recorded from human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) in response to the voltage clamp protocol shown at top of figure. B: Mean I-V relation hiPSC-CM. C: Time constants of decay (τ) obtained by fitting a single exponential equation to the decaying phase of the currents are plotted as a function of voltage (Panel C).

Fig. 3.

Voltage-dependence of inactivation and time course of recovery from inactivation of an transient outward current (I_{to}) in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). A and B: Representative traces showing time course of reactivation and voltage dependence of inactivation of I_{to} . C: Time course of recovery of I_{to} from inactivation. D: Boltzmann curve showing voltage dependence of inactivation.

Fig. 4.

Rate-dependence of transient outward current (I_{to}). A: Representative traces showing changes in I_{to} in response to various pacing rates. The cell was activated 10 times at various cycle lengths and every pulse is shown. The red trace illustrates the first pulse after a prolonged (>15 s) rest period whereas the black traces show the magnitude of I_{to} recorded during pulses 2–10. An increase in the pacing rate results in a progressive decrease in the size of I_{to} . B: Mean data showing reduction in I_{to} as a function of pacing rate.

Fig. 5.

Effect of BaCl_2 on an isolated canine right ventricular endocardial (Endo) and epicardial (Epi) tissue-slices. A: Control recordings (basic cycle length (BCL) = 1000 msec). B: Effect of 300 μM BaCl_2 (20–30 min) to block the inwardly rectifying K^+ current (I_{K1}). Note the presence of phase 1 repolarization in Endo and of spike and dome morphology (notch) in Epi under control conditions (A) and the lack thereof following 20 min of superfusion with barium. C: Computer model Luo–Rudy II (LRII) of action potentials (APs) and currents responsible for the morphology of the AP in adult native cardiomyocytes. The two left panels show APs simulated in the absence (Endo) and presence of I_{to} (Epi) D: Computer simulation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM)-

like APs with maximal I_{K1} conductance reduced to 10% of normal. The right most panels in both C and D illustrate ion channel currents contributing to the APs under these conditions. Note that the vertical scale is 10 times larger and time scale is 10 times shorter in C, highlighting the ion distinctions between hiPSC-CM and adult native cardiomyocytes.

Fig. 6. Cable model of a beating cluster with linear distribution of the inwardly rectifying K^+ current (I_{K1}) current. Top panel: Propagating action potentials (APs) were simulated in a short 4 mm cable comprised of 41 right ventricular epicardial (Epi) cells (Luo-Rudy II (LRII) plus modified transient outward current model). The maximal conductance of I_{K1} current in the cable was linearly distributed in the range between 0% (cell No.1) and 30% (cell No.41). Cells between No.1 and No.15 exhibit intrinsic automaticity due to low I_{K1} density. Cells in the rest of the cable are not automatically active and are excited by propagating action potential. In this model, the phase 1 repolarization due to modified I_{to} was observed in cells between No.31 and No.41. Bottom panel: The shape of the spike-and-dome in cell No.35 shown in larger scale (c.f., Figs. 1).

Fig. 7.

Epicardial (Epi) action potentials (APs) simulated using the single cell model with the full expression of the inwardly rectifying K^+ current (I_{K1}) current (100%) but with different kinetics of the transient outward current (I_{to}). Upper panels: Effect of cycle length on the shape of Epi APs simulated using original I_{to} model (normal I_{to} ; top panels) and modified slow I_{to} model (bottom panels). Lower panels: Effects of rates of inactivation (γ) and recovery (γ) of I_{to} on the shape of the Epi AP simulated in the presence of the fully-expressed I_{K1} . (A): normal inactivation and normal recovery rates (adult cardiomyocyte (CM) I_{to}). (B): normal inactivation and slow recovery rates. (C): slow inactivation and normal recovery rates. (D): slow inactivation and slow recovery rates (human induced

pluripotent stem cell-derived (hiPSC)-CM I_{to}). Equations for normal (Eq. S3) and modified (Eq. S3m) recovery rates and for normal (Eq. S4) and modified (Eq. S4m) inactivation rates are shown in on-line Supplement. Insets show the time course of I_{to} current during the corresponding simulated AP. The density of I_{to} current was $0.5 \text{ mS}/\mu\text{F}$ in all four models.

Fig. 8.

Effect of the transient outward current (I_{to}) agonist NS5806 on I_{to} in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM). A: Representative I_{to} traces recorded under control conditions using the voltage protocol shown. B: 10 μ M NS5806. C: Mean I-V relation for peak I_{to} in absence and presence of NS5806.

Fig. 9.

Expression profile of I_{to} -related genes obtained using real-time PCR in beating clusters (BCs, left, our data) and in normal human right ventricle (RV, right, data from Gaborit et al. from reference [8] with permission). Data presented relative to *GAPDH* expression $\times 100$. Values for genes expression in human RV were re-normalized to *GAPDH* using published expression ratio of *GAPDH* to *HPRT* ≈ 61 [37,38] Mean $\pm$ S.E.M.

Table 1

Action potential (AP) parameters derived from 6 beating clusters (BCs) that displayed phase 1 repolarization (Notch) and from 149 BCs that did not. cAPD90-B (Bazett's correction: $ADP90/(CL)^{1/2}$); cAPD90-H (Hodge's: $ADP90 + 1.75 \times [\text{heart rate} - 60]$); cAPD90-Fri (Fridericia's: $ADP90/(CL)^{1/3}$).

	Without a notch (149)	With a notch (6)
Spontaneous Rate (bpm)	60.4 ± 2.6	62.7 ± 16.9
Cycle length (ms)	1494 ± 144	1632 ± 566
AP amplitude (mV)	101.8 ± 0.9	109.2 ± 1.7
MDP (mV)	-67.0 ± 0.8	-87.8 ± 1.4 ^a
V/max (V/s)	29.3 ± 1.7	141.8 ± 18.5 ^a
Notch magnitude (mV)	0.0 ± 0.0	6.4 ± 1.0 ^a
APD30 (ms)	161.4 ± 6.2	152.7 ± 53.1
APD40 (ms)	188.8 ± 7.4	178.1 ± 58.9
APD50 (ms)	211.0 ± 8.2	210.9 ± 69.3
APD70 (ms)	238.5 ± 8.6	229.4 ± 60.6
APD80 (ms)	254.0 ± 8.8	247.5 ± 64.6
APD90 (ms)	277.3 ± 9.0	296.9 ± 87.5
Bazett's-APD90 (ms)	251.4 ± 6.4	226.7 ± 33.9
Hodge's-APD90 (ms)	278.0 ± 7.5	301.6 ± 62.4
Fridericia's-APD90 (ms)	257.1 ± 6.5	244.2 ± 47.5
APD50/APD90 (RO)	0.75 ± 0.01	0.66 ± 0.05 ^b
APD30-40/APD70-80 (RO)	1.94 ± 0.09	1.41 ± 0.19
Age (days)	57.1 ± 25.3	45.3 ± 21.3
Age range (days)	11–119	27–74
Ventricular-like	n = 83 (56%)	n = 1 (17%)
Atrial-like	n = 66 (44%)	n = 5 (83%)

^a p < 0.001,

^b p < 0.05 (vs. Without a Notch).

ADP: action potential duration; MDP: maximum diastolic potential.

Table 2

Action potential (AP) parameters recorded from 13 beating clusters before and after the addition of 1 mM 4-aminopyridine (4-AP) to the superfusate, a concentration known to block Ito. 4/13 action potentials displayed a relatively small phase 1 and notch, which were completely eliminated with 4-AP (AP with a notch (n = 4). Of note, an AP notch was observed in only 6/155 preparations studied (3.9%).

	APs without a notch (n = 9)		APs with a notch (n = 4)	
	Control	4-AP (1 mM)	Control	4-AP (1 mM)
Spontaneous Rate (bpm)	67.8 ± 13.5	55.1 ± 9.5	83.7 ± 16.4	90.9 ± 13.8
AP amplitude (mV)	97.2 ± 2.5	101.9 ± 3.2 ^b	107.5 ± 2.1 ^b	108.2 ± 1.0
MDP (mV)	-66.3 ± 4.6	-65.6 ± 4.0	-86.6 ± 1.9	-83.7 ± 3.7
V _{max} (V/s)	30.9 ± 8.4	28.3 ± 6.9	129.9 ± 26.2 ^c	121.4 ± 23.9 ^d
Notch magnitude (mV)	0.0 ± 0.0	0.0 ± 0.0	7.3 ± 1.2 ^c	0.0 ± 0.0
APD ₉₀ (ms)	206.0 ± 20.8	300.4 ± 24.4 ^a	194.0 ± 70.3	258.0 ± 80.9 ^b
APD ₅₀ (ms)	148.7 ± 17.3	223.4 ± 19.1 ^a	122.9 ± 51.0	186.9 ± 54.0 ^b
Bazett's-APD ₉₀ (ms)	203.5 ± 28.3	272.9 ± 28.5 ^a	200.5 ± 43.6	296.4 ± 67.0 ^b
Hodge's-APD ₉₀ (ms)	219.6 ± 26.4	291.7 ± 23.9 ^a	235.5 ± 47.2	312.1 ± 64.7 ^a
Friederica's-APD ₉₀ (ms)	202.0 ± 23.1	279.7 ± 24.3 ^a	196.9 ± 51.5	282.2 ± 71.6 ^b
APD ₅₀ /APD ₉₀ (RO)	0.7 ± 0.0	0.7 ± 0.0	0.6 ± 0.1 ^b	0.7 ± 0.0
APD ₃₀₋₄₀ /APD ₇₀₋₈₀ (RO)	1.1 ± 0.1		1.2 ± 0.2	

AP without a notch (n = 9):

^a p < 0.001,

^b p < 0.005 vs. control.

AP without a notch (n = 4):

^a p < 0.01,

^b p < 0.05 vs. control.

^c p < 0.001 vs. control without a notch.

^d p < 0.001 vs. 4-AP without a notch.

APD: action potential duration; MDP: maximum diastolic potential.