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Ajmaline blocks I_{Na} and I_{Kr} without eliciting differences between Brugada syndrome patient and control human pluripotent stem cell-derived cardiac clusters

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ABSTRACT

The class Ia anti-arrhythmic drug ajmaline is used clinically to unmask latent type I ECG in Brugada syndrome (BrS) patients, although its mode of action is poorly characterised.

Our aims were to identify ajmaline's mode of action in human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes (CMs), and establish a simple BrS hiPSC platform to test whether differences in ajmaline response could be determined between BrS patients and controls.

Control hiPSCs were differentiated into spontaneously contracting cardiac clusters. It was found using multi electrode array (MEA) that ajmaline treatment significantly lengthened cluster activation-recovery interval. Patch clamping of single CMs isolated from clusters revealed that ajmaline can block both I_{Na} and I_{Kr}.

Following generation of hiPSC lines from BrS patients (absent of pathogenic *SCN5A* sodium channel mutations), analysis of hiPSC-CMs from patients and controls revealed that differentiation and action potential parameters were similar. Comparison of cardiac clusters by MEA showed that ajmaline lengthened activation-recovery interval consistently across all lines.

We conclude that ajmaline can block both depolarisation and repolarisation of hiPSC-CMs at the cellular level, but that a more refined integrated tissue model may be necessary to elicit differences in its effect between BrS patients and controls.

1. Introduction

Brugada syndrome (BrS) is a cardiac arrhythmic syndrome and can cause ventricular fibrillation (VF) and sudden cardiac death. BrS is predominantly characterised by right bundle branch block, elevated J-point and coved ST segment of an electrocardiogram (ECG), with fibrillation and premature ventricular contractions often originating from the right ventricular outflow tract (RVOT) (Brugada and Brugada, 1992; Morita et al., 2003). Two specific hypotheses have been proposed to account for the Brugada ECG pattern - the depolarisation hypothesis, highlighting the importance of right ventricular activation delays, and the repolarisation hypothesis, focusing on transmural differences in action potential duration (Meregalli et al., 2005).

The class IA anti-arrhythmic drug ajmaline is used as a diagnostic pharmacological challenge in suspected cases of BrS (Rolf et al., 2003), however the mechanism of action is not fully established, and there has long been debate about the precision of its diagnostic effect (Brugada et al., 2003). Some studies in non-human or non-cardiac cell lines have indicated that ajmaline inhibits various currents, including I_{Na}, I_{to} or I_{Kr} (Bébarová et al., 2005; Kiesecker et al., 2004). Furthermore,

Abbreviations: AP, Action Potential; APD, Action Potential Duration; Br1, Brugada Type 1 ECG; BrS, Brugada Syndrome; cFPD, Corrected Field Potential Duration (Bazett's formula); CM, Cardiomyocyte; ECG, Electrocardiogram; FP, Field Potential; FPD, Field Potential Duration; hPSC, Human Pluripotent Stem Cell; hiPSC, Human Induced Pluripotent Stem Cell; HDFs, Human Dermal Fibroblasts; MEA, Multi Electrode Array; NGS, Next Generation Sequencing; RMP, Resting Membrane Potential; VF, Ventricular Fibrillation.

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a study recently found that ajmaline elicited a Brugada type I ECG pattern in 27% of atrioventricular nodal re-entrant tachycardia patients and even in 5% of control individuals (Hasdemir et al., 2015). The exact pathophysiological mechanism whereby ajmaline provokes the BrS ECG phenotype is thus unclear.

It is clear that in some cases BrS can be hereditary, and in these cases occurs predominantly due to loss-of-function mutations in the cardiac sodium channel *SCN5A* (Mizusawa and Wilde, 2012). Mutations have also been identified in sodium channel beta subunits and in other ion channels such as the L-type Ca^{2+} channel, however these are rare (Mizusawa and Wilde, 2012). There is a diversity of other associated genes with rare variants of sometimes disputed contribution (Le Scouarnec et al., 2015), as well as combinations of common variants likely to be contributing as disease modifiers (Bezzina et al., 2013). In total, a monogenic aetiology can be reasonably supported in only ~25% of patients (Wilde and Behr, 2013). Thus in the majority of patients there is scant data supporting a strong genetic predisposition. Furthermore, in addition to electrophysiological effects at the single cell level there may be structural changes that are critical for disease pathogenesis (Catalano et al., 2009; Papavassiliu et al., 2010).

The developments in human somatic cell reprogramming to induced pluripotent stem cells (hiPSCs) (Okita et al., 2011; Takahashi et al., 2007) and targeted differentiation of human pluripotent stem cells (hPSCs) to cardiomyocytes (CMs) (Burridge et al., 2011; Minami et al., 2012) have hugely empowered in vitro modelling of hereditary cardiac disease. Progress has been made in establishing hiPSC models for certain arrhythmias such as Long QT syndrome (Matsa et al., 2011; Sala et al., 2016), however diseases such as BrS present a more variable phenotype and polygenic background, with the potential added complexity of *endo*-epicardial tissue heterogeneities.

Here we aimed to address some of these issues by generating a simple cardiac model for the action of ajmaline in a BrS context, comprised of differentiation and electrophysiological analysis of hPSC-cardiac clusters/CMs from BrS patients and controls. Specifically we focus on patients without mutations in the cardiac sodium channel *SCN5A*, reflecting the predominant clinical population, and how they respond to ajmaline challenge.

2. Materials and methods

2.1. Consent and ethics

Work with human embryonic stem cells (hESCs) was reviewed and approved by the UK's Steering Committee For The Stem Cell Bank And For The Use Of Stem Cell Lines (reference number SCSC13–25). Use of patient samples following informed consent was approved by the UK's National Research Ethics Service (13/LO/0224).

2.2. Brugada subject identification

BrS subjects were identified from specialist inherited arrhythmia clinics, recruiting individuals with a history of BrS and out-of-hospital cardiac arrest or family members of sudden arrhythmic death syndrome (SADS) victims, who met the diagnostic criteria according to the 2013 inherited arrhythmia consensus document with either a spontaneous resting type 1 Brugada ECG pattern or positive ajmaline challenge test on screening (Priori et al., 2014).

2.3. Next generation sequencing (NGS) of patient and control genomic DNA

Following phenol:chloroform extraction of genomic DNA from blood samples, DNA libraries were prepared using the Illumina TruSight Cardio system (Pua et al., 2016) according to manufacturer's laboratory protocols, and sequenced on the Illumina NextSeq. Twelve genes in which variants had been previously reported as causative of BrS were analysed, together with a further 26 genes reportedly linked to other inherited arrhythmia syndromes (Table 2). Rare protein-altering variants were identified as those occurring at a frequency of ≤0.0001 (1 in 10,000) both in the ExAC database and in a cohort of healthy volunteers sequenced on the same platform (Lek et al., 2016). For further details please see Supplementary methods.

2.4. Reprogramming and maintenance of hPSCs

Human dermal fibroblast (HDF) cultures were derived from 4 mm skin punch biopsies from BrS patient and control individuals following overnight digestion with collagenase I (Sigma, USA). After two to five passages, HDFs were reprogrammed by lentiviral transduction of a polycistronic vector hSTEMCCA (Somers et al., 2010) or nucleofection of three plasmids containing reprogramming factors (Okita et al., 2011). Colonies were isolated and expanded clonally in DMEM:F12 medium containing 20% knock-out serum replacement (Gibco, UK) and 20 ng/ml FGF2 (Peprotech, USA) on mitotically inactivated mouse embryonic fibroblasts (MEFs), and characterised for pluripotency. hPSC lines were generally maintained and expanded on MEFs, and transitioned onto Matrigel in mTeSR for several passages prior to differentiation.

2.5. Cardiac differentiation of hPSCs

We adapted the protocol published by Burridge and colleagues (Burridge et al., 2011). hPSCs were enzymatically dissociated with Accutase (Thermo Fisher, USA) and plated at a density of 1.5–2 $\times 10^6$/75 cm^2 flask pre-coated with Matrigel (Corning, USA) in mTeSR ™1 (Stem Cell Technologies, Canada) + 5 μM Y-27632 (Tocris, UK). The next day (differentiation day 0–1, "D0"), cells were dissociated and resuspended at a density of 7×10^4 cells/ml in RPMI-Growth Factor (RGF) medium (for media formulation please see Supplementary table S2). Cells were pipetted into conical bottom 96-well plates (100 μl per well), and centrifuged at 950*g* for 5 mins to aggregate the cells into clusters. On D2, medium was exchanged with 100 μl RPMI-Serum (RS) medium, and on D3 this was again replaced with fresh RS medium but containing SB431542 (Tocris, UK). On D4, medium was changed for 150 μl RPMI-ITS (RI) medium containing inhibitors KY02111 (Tocris) and XAV939 (Sigma, UK) (Minami et al., 2012) and clusters were transferred to round bottom 96-well plates. From D6 and henceforth RI medium without inhibitors was used to maintain the clusters, with medium changed every three days. In some experiments, clusters were transferred and pooled into 6-well plates from D14. For metabolic enrichment of CMs (Tohyama et al., 2013), cardiac clusters were maintained in RI comprised of RPMI without glucose (Gibco) and 4 mM sodium lactate (Sigma) between D14–21.

2.6. Multi electrode array (MEA) analysis of hPSC-cardiac clusters

At weeks 6–8 of differentiation, two or three spontaneously contracting clusters per analysis were loaded onto gelatin or Matrigel-coated 60PedotMEA200/30iR-Au MEAs (containing 60 gold coated electrodes, 200 μm spaced) (Multi Channel Systems, Germany) in RI medium containing 20% FCS (to promote attachment). After two days, clusters were analysed using the MEA2100 system with an HS60 headstage, and signals were recorded using MC_Rack software (all from Multi Channel Systems). Sampling frequency was 10,000 Hz, and channels with a detectable signal in the range of ± 50 μV were selected and recorded. Basal medium of RPMI containing pen/strep and 5% FCS +/− drug dose was superfused across clusters at a rate of ~1.5 ml/min, with the headstage and superfused medium maintained at 37 °C throughout. One minute baseline recordings were taken after at least 15 mins superfusion of basal medium, and all subsequent 1 min drug dose recordings were taken following 6 mins superfusion. Drugs tested had stock solutions as follows: ajmaline (Carinopharm GmBH, Germany) at 15.32 mM in phosphate saline solution (ultrapure