Alternans in genetically modified Langendorff-perfused murine hearts modeling catecholaminergic polymorphic ventricular tachycardia

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INTRODUCTION

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is a hereditary arrhythmic disorder characterized by ventricular tachyarrhythmias precipitated by exercise, stress or catecholamine infusion (Wehrens et al., 2003). Such arrhythmias may potentially lead to ventricular fibrillation, resulting in syncope and sudden cardiac death (Priori et al., 2001). CPVT is a highly lethal condition, with Kaplan Meyer analyses confirming mortality rates of 30–33% by age 35 (Priori et al., 2001). Current therapies are unsatisfactory but include regular administration of β-adrenoceptor antagonists (Lehnart et al., 1995) and insertion of automatic implantable cardioverter defibrillators (Priori et al., 2001).

CPVT is associated with mutations in the cardiac ryanodine receptor (RyR2) (Marks et al., 2002). Six distinct RyR2 mutations, occurring in the absence of structural abnormalities, echocardiographic evidence of cardiac failure or QT prolongation, have been reported to result in CPVT (Laitinen et al., 2001; Priori et al., 2001). Of these, a hereditary P2328S mutation has been shown to alter FKBP12.6 binding following PKA phosphorylation in HEK293 cells (Lehnart et al., 2004). Mice genetically modified to lack FKBP12.6 and proposed as a model for CPVT demonstrate arrhythmia in association with alternans in action potential duration (APD) (Lehnart et al., 2006); both these phenomena could be prevented by pharmacological maneuvers expected to increase the binding affinity of FKBP12.6 for RyR2. Such associations between alternans and ventricular arrhythmia are well established in both historical and contemporary literature (Lewis, 1911; Gold et al., 2000; Weiss et al., 2006), with electrocardiographic alternans having been reported in carriers of mutations associated with CPVT (Aizawa et al., 2006).

The present study extends correlations between alternans and arrhythmia (Weiss et al., 2006) to genetically modified hearts carrying the P2328S mutation and thus representing a true genetic model of CPVT (Goddard et al., 2008). It thus: (1) confirms previous reports of elevated incidences of arrhythmia in RyR2-P2328S hearts as compared to wild-type (WT) controls (Goddard et al.,...
2008); (2) correlates such arrhythmogenicity with a tendency to exhibit alternans, in agreement with observations both in humans with CPVT (Aizawa et al., 2006) and in murine genetic models of other hereditary arrhythmic syndromes and (3) demonstrates that treatment with adrenergic agonists and antagonists, known to be pro- and anti-arrhythmic respectively in humans with CPVT (Leenhardt et al., 1995), suppresses and accentuates alternans as might be expected. It then proceeds to demonstrate that such alternans occurs in the absence of changes in either (4) mean action potential properties, (5) refractory periods or (6) restitution curve characteristics, in sharp contrast to findings in those other murine models (Sabir et al., 2008a).

**MATERIALS AND METHODS**

Genetically modified mice heterozygous (RyR2<sup>p/s</sup>) and homozygous (RyR2<sup>s/s</sup>) for the P2328S mutation in the central region of the RyR2 gene (Goddard et al., 2008), along with WT controls, were housed in an animal facility at 21 ± 1°C with 12:1 light/dark cycles. All mice were fed sterile chow (RM3 Maintenance Diet, SDS, Witham, Essex, UK) and given free access to water. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Bicarbonate-buffered Krebs–Henseleit solutions (119 mM NaCl, 25 mM NaHCO<sub>3</sub>, 4 mM KCl, 1.2 mM KH<sub>2</sub>P<sub>O</sub>4, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 10 mM glucose and 2 mM Na-pyruvate) were used. These were bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> (British Oxygen Company, Manchester, UK) to achieve a physiological pH of 7.4 and prevent the precipitation of calcium carbonate. Drug-containing solutions were prepared by adding epinephrine (Sigma-Aldrich, Poole, UK) to final concentrations of either 100 nM or 1 μM as stated and propranolol (Sigma-Aldrich, Poole, UK) to a final concentration of 100 nM, as used on previous occasions (Tosaka et al., 2003; Thomas et al., 2007a).

A Langendorff-perfusion protocol previously validated for use with murine hearts (Balasubramaniam et al., 2003) was used. In brief, mice were killed by cervical dislocation (Schedule 1: UK Animals [Scientific Procedures] Act 1986). Hearts were then quickly excised and placed in ice-cold bicarbonate-buffered Krebs–Henseleit solution. Short sections of aorta were then rapidly cannulated under the surface of the ice-cold buffer solution and attached using an aneurysm clip (Harvard Apparatus, Edendbridge, Kent, UK) to a custom-made 21-gauge cannula. Fresh Krebs–Henseleit solution was passed through 200 and 5 μm filters (Millipore, Watford, UK) and warmed to 37°C using a water jacket and circulator (Technic model C-85A, Cambridge, UK). A peristaltic pump (Watson-Marlow Bredel model 505S, Falmouth, Cornwall, UK) in constant flow mode was then used to perfuse hearts with this solution at 10 mL/min while stimulation was performed. Signals were amplified, band-pass filtered (0.5 Hz to 1 kHz: Neurolog, Digitimer, Welwyn Garden City, Hertfordshire, UK) and digitized at a sampling frequency of 5 kHz (micro1401, Cambridge Electronic Design, Cambridge, UK). Data analysis was performed using Spike II (Cambridge Electronic Design, Cambridge, UK).

A bipolar platinum stimulating electrode (1 mm inter-pole spacing) was placed against the basal surface of the right ventricular epicardium. Square-wave stimuli (Grass S48 stimulator, Grass-Telefactor, Slough, UK) of 2-ms duration and amplitudes of twice the diastolic excitation threshold were initially applied to hearts at a constant baseline cycle length (BCL) of 125 ms. Recordings met quality assurance criteria defined previously, with one action potential occurring in response to each stimulus, each action potential demonstrating a rapid upstroke phase that reached a consistent amplitude and a smooth re-polarization phase and the baseline remaining stable between action potentials (Knollmann et al., 2001; Fabritz et al., 2003). APD was quantified at 90% re-polarization (APD<sub>90</sub>), APD<sub>100</sub> being defined as the peak upstroke potential difference and APD<sub>100</sub> as the diastolic potential difference. Notably, inclusion or exclusion of the upstroke phase did not make a significant (P > 0.05) difference to results under any set of experimental conditions studied. Before subsequent recordings were made hearts were then exposed to test solutions for 10 min while stimulation was continued.

MAPs were recorded during regular stimulation at a BCL of 85, 95, 105, 115, and 125 ms and the data obtained used to assess the magnitude of alternans present. Such alternans was defined as a sequence of ten or more action potentials the duration of which were shorter then longer (or vice versa) than in the previous beat. A detection margin of 0.2 ms was used, reflecting the sampling sensitivity of the Spike II data acquisition software utilized. An _extrasystolic stimulation protocol_ (Saumarez and Grace, 2000) previously adapted for use in murine hearts (Head et al., 2005) was then applied. This comprised regular stimuli delivered at a BCL of 125 ms interrupted by extrasystolic stimuli imposed after every eighth regular stimulus at delays progressively decremented with each successive stimulus cycle in 1 ms steps from the BCL. Such cycles were repeated until the extrasystolic stimuli either initiated arrhythmia or failed to initiate a MAP, the ventricular effective refractory period (VERP) having been reached. Hearts were then subjected to an adapted _dynamic pacing protocol_ (Koller et al., 1998; Sabir et al., 2008b). This comprised cycles each of 100 stimuli, initially delivered at a BCL of 175 ms. BCL was decremented in 5 ms steps with each successive stimulus cycle until a reproducible sequence of consistently shaped MAP waveforms was no longer obtained. This dynamic pacing protocol was also used to induce arrhythmia, with arrhythmia being defined as a > 5 s period of morphologically irregular waveforms continuing in the absence of stimulation.

Recordings were made in the absence of pharmacological agents, following addition of epinephrine, following addition of propranolol, and following addition of both epinephrine and propranolol. Hearts were exposed to reagent-containing solutions for 10 min during stimulation at a constant BCL of 125 ms before data were collected.

All data are presented as means ± standard errors of the means and include the numbers of hearts studied. Comparisons between data sets used analysis of variance (ANOVA) and the Chi-squared test to make multiple comparisons between continuous and discrete data respectively (significance thresholds set at P ≤ 0.05). A Levenberg–Marquardt algorithm (OriginPro 7.5, OriginLab, Northampton, MA, USA) was used for curve fitting.
RESULTS

The experiments first confirmed the presence or absence of arrhythmogenic phenomena in intact Langendorff-perfused WT murine hearts, as well as in hearts heterozygous (RyR2<sup>p/s</sup>) and homozygous (RyR2<sup>s/s</sup>) for a mutation known to result in CPVT in humans. This confirmed and extended previous reports of an increase in incidence of arrhythmia with gene dosage in RyR2-P2328S mice (Goddard et al., 2008), as well as the pro-arrhythmic and anti-arrhythmic effects of epinephrine and propranolol respectively.

The experiments then demonstrated alternans in RyR2-P2328S hearts for the first time, a finding in full agreement with clinical reports (Aizawa et al., 2006). Figure 3 plots alternans magnitude, the mean difference in APD (quantified at 90% re-polarization, APD<sub>90</sub>) between successive odd- and even-numbered action potentials, against BCL. Hearts were studied before (diamonds) and after addition of 100 nM (squares) or 1 μM (triangles) epinephrine both before (Figure 3, top panel) and after (Figure 3, bottom panel) introduction of propranolol. Alternans magnitude tended to increase with decreasing BCL in agreement with previous findings (Walker et al., 2003), yielding significantly larger values at the shortest (85 ms) compared to the longest (125 ms, <i>P</i> < 0.05) BCLs studied under all conditions. All subsequent comparisons between alternans magnitudes were made using data obtained at a BCL of 85 ms.

WT hearts demonstrated alternans only at the shortest BCL studied, as in previous reports (Sabir et al., 2008b). Exposure to epinephrine, whether at concentration of 100 nM or 1 μM, significantly (<i>P</i> < 0.05) increased alternans magnitude. Before
Alternans magnitude in pooled data from arrhythmic hearts was significantly greater than in hearts that did not demonstrate arrhythmia (4.2 ± 0.5 ms, n = 55 vs. 2.8 ± 0.3 ms, n = 126; P < 0.05), confirming an association between alternans and arrhythmia under the experimental conditions studied.

pharmacological manipulation, RyR2<sup>−/−</sup> hearts demonstrated alternans magnitudes statistically indistinguishable from values obtained in WTs. Following treatment with epinephrine, however, alternans magnitude was significantly (P < 0.05) greater than seen in equivalent groups of WT hearts. RyR2<sup>−/−</sup> hearts showed significantly (P < 0.05) greater alternans magnitudes than either WT or RyR2<sup>p/s</sup> hearts, irrespective of the pharmacological conditions studied. Treatment with epinephrine again increased alternans magnitude, with values being significantly (P < 0.05) larger than found in WT or RyR2<sup>p/s</sup> hearts. While treatment with propranolol tended to increase alternans magnitude when applied to WT hearts in the absence of epinephrine, it significantly (P < 0.05) decreased alternans magnitude in all other cases.

Alternans magnitude in pooled data from arrhythmic hearts was significantly greater than in hearts that did not demonstrate arrhythmia (4.2 ± 0.5 ms, n = 55 vs. 2.8 ± 0.3 ms, n = 126; P < 0.05), confirming an association between alternans and arrhythmia under the experimental conditions studied.
Alternans in a murine genetic model of CPVT

Alternans and arrhythmia in murine models of a number of hereditary (Thomas et al., 2007a,b) and acquired (Sabir et al., 2007) arrhythmic disorders have been associated with changes in APD, VERP and restitution curve slopes. The present experiments thus proceeded to study these parameters, the latter two for the first time in RyR2-P2328S mutant hearts.

FIGURE 3 | Relationships between alternans magnitude and baseline cycle length. Relationships between alternans magnitude, defined as the difference between APD at 90% re-polarization (APD90) for successive odd- and even-numbered action potentials, and BCL in WT (A), RyR2p/s (B) and RyR2s/s (C) hearts treated with control solution (diamonds) and with solutions containing 100 nM epinephrine (squares) and 1 μM epinephrine (triangles). The top panel shows traces obtained in the absence of propranolol; the bottom panel shows traces obtained in the presence of 100 nM propranolol.
First, APD$_{90}$ was recorded through a wide range of BCLs before and after exposure to epinephrine and to propranolol, separately and in combination. APD$_{90}$ increased with increasing BCL, as previously established (Sabir et al., 2007) but did not differ significantly between WT, RyR2<sup>−/−</sup> and RyR2<sup>+/−</sup> hearts at any BCL. Furthermore, epinephrine treatment left APD$_{90}$ unchanged apart from in two cases in WT (P < 0.05), but not RyR2<sup>−/−</sup> or RyR2<sup>+/−</sup>, hearts. Finally, propranolol did not significantly affect APD$_{90}$ at any BCL. These observations fully agree with previous observations of electrocardiographic QT interval in patients with CPVT (Priori et al., 2001).

Secondly, VERP values were obtained under the full range of experimental conditions studied using an extrasystolic stimulation protocol. Neither these genetic nor pharmacological manipulations resulted in significant changes in VERP in any case.

Thirdly, recent studies had associated alternans and arrhythmia in murine hearts with increases in the slopes of restitution curves relating APD$_{90}$ to the preceding diastolic interval (DI), given by the difference between the preceding BCL and APD$_{90}$ (Sabir et al., 2008a,b) (illustrated in Figure 4).

Such curves were constructed using APD$_{90}$ and DI data obtained during the dynamic pacing protocol. They could not be derived from parameters recorded during treatment with 1 μM epinephrine as the high incidence of arrhythmia did not permit acquisition of the appropriate data. Their slopes were assessed from fits to a simple, mono-exponential growth function of the form:

\[ y = y_0 + A(1-e^{-x/\tau}) \]  

(1) (Sabir et al., 2008b)

\( y \) representing APD$_{90}$, \( x \) representing DI and \( y_0 \), \( A \) and \( \tau \) being constants obtained by least-squares fitting to the experimental values of APD$_{90}$ and DI. Fits were made using a least-squares method.

Figure 5 shows an example of such a curve obtained from WT hearts under control conditions.

The corresponding slopes are then given by:

\[ \frac{dy}{dx} = \frac{A}{\tau}e^{-x/\tau} \]  

(2)

Figure 6 summarizes the maximum values of slopes obtained at the shortest BCLs studied.

Maximum slopes obtained from WT hearts under control conditions closely agreed with previous data (Sabir et al., 2008a,b). There was no significant difference in restitution curve slope between any experimental groups, however. Furthermore, pooled data from arrhythmic hearts gave a maximum slope that was not significantly different from that obtained from non-arrhythmic hearts (0.4 ± 0.2, \( n = 55 \) vs. 0.5 ± 0.2, \( n = 126 \)). Thus alternans and arrhythmia were not associated with significant changes in the slopes of restitution curves. Thus, in contrast to the results of a previous study carried out in models of other hereditary arrhythmic syndromes (Sabir et al., 2008a), alternans and arrhythmia in this setting cannot be explained by alterations in the slopes of restitution curves.

DISCUSSION

Catecholaminergic polymorphic ventricular tachycardia is a hereditary arrhythmic disorder attributed to mutations that affect the cardiac ryanodine receptor (RyR2) (Priori et al., 2001). Ventricular arrhythmia in CPVT patients has been associated with alterations in APD, alternans (Aizawa et al., 2006), a link already well established in other situations (Lewis, 1911; Gold et al., 2000; Weiss et al., 2006). The present study sought to determine if genetically modified mice incorporating a P2328S mutation in the RyR2 gene (Goddard et al., 2008), previously established to result in CPVT in some patients (Laitinen et al., 2001; Priori et al., 2001), exhibit alternans. It then proceeded to determine if such alternans and arrhythmia were associated with changes in action potential or refractory properties, or in the slopes of restitution curves (Nolasco and Dahlen, 1968), as is the case in murine models of other human arrhythmic syndromes (Thomas et al., 2007a; Sabir et al., 2008a,b).

**FIGURE 4** Cartoon representation of parameters used to construct restitution curves.

**FIGURE 5** Sample restitution curve. Sample curve obtained from WT hearts in the absence of pharmacological manipulations. Data were fitted with a curve of the form \( y = y_0 + A(1-e^{-x/\tau}) \) where \( y_0 = -11.9 \pm 3.6, A = 60.0 \pm 2.6, \tau = 57.8 \pm 6.4, \chi^2 = 0.98 (n = 7) \).
MAP recordings were obtained from the epicardia of Langendorff-perfused hearts both heterozygous (RyR2<sup>p/s</sup>) and homozygous (RyR2<sup>s/s</sup>) for the mutation, as well as from WT controls.

Previous studies conducted on genetically modified murine hearts modeling CPVT have used a range of maneuvers to demonstrate the presence of an arrhythmogenic phenotype. For example, Cerrone et al. (2005) used a combination of isoprenaline and caffeine. Goddard et al. (2008) later reported the qualitative presence of an arrhythmogenic phenotype in catecholamine-treated hearts incorporating the RyR2-P2328S mutation during the application of both regular and extrasystolic stimulation protocols. In the present study arrhythmogenicity was assessed during periods of rapid pacing as part of a dynamic pacing protocol. Results confirmed the arrhythmogenic effect of this mutation, as well as demonstrating a pro-arrhythmic effect from epinephrine and an anti-arrhythmic effect from propranolol, all in full agreement with expectations from clinical studies (Priori et al., 2002; Postma et al., 2005).

The experiments then sought to study alternans in these mice. Under all genetic and pharmacological conditions studied alternans magnitude, defined as the difference in APD between successive odd- and even-numbered action potentials, increased as BCL was decreased. This is in full agreement with previous work (Walker et al., 2003). In general, treatment with epinephrine increased, while treatment with propranolol decreased, alternans magnitude. Furthermore, RyR2<sup>P2328S</sup> hearts demonstrated significantly larger alternans magnitudes than RyR2<sup>p/s</sup> hearts or WT controls, in fitting with the increased incidences of arrhythmia observed. This represents both the first experimental demonstration of alternans occurring in the murine heart as a result of exposure to catecholamines and the first demonstration of alternans in any experimental model of CPVT. Notably alternans magnitude in pooled data from arrhythmic hearts was significantly greater than that from hearts that did not demonstrate arrhythmia.

Subsequent experiments examined a possible basis for alternans in this setting. A large number of previous studies (Karagueuzian et al., 1993; Lee et al., 1996; Koller et al., 1998, 2000; Taggart et al., 2003; Pak et al., 2004) have associated alternans with steeply sloping restitution curves relating APD to the preceding DI (Nolasco and Dahlen, 1968). Furthermore, such steeply sloping restitution curves have been associated with arrhythmogenicity in humans with the Brugada syndrome (Narayan et al., 2007), another hereditary arrhythmic syndrome (Antzelevitch et al., 2003). Furthermore, similar associations have been made in murine genetic models of both the Brugada syndrome and the congenital long-QT syndrome type 3, as well as in hypokalemic murine hearts (Sabir et al., 2008a,b). In this study, however, neither the effect of RyR2-P2328S mutation, epinephrine nor propranolol were associated with changes in the slopes of restitution curves. Furthermore, the maximum slopes of restitution curves did not exceed unity, an established criterion for arrhythmogenesis (Nolasco and Dahlen, 1968), under any set of experimental conditions studied. Notably alternans has previously been reported in the absence of steeply sloping restitution curves (Pruvot et al., 2004; Goldhaber et al., 2005). In these cases alternations in [Ca<sup>2+</sup>], has been suggested to affect both the inward
current carried by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and L-type Ca\textsuperscript{2+} channel inactivation, thereby resulting in alternation in APD (Weiss et al., 2006). Such a mechanism has been suggested to underlie alternans in chronic heart failure, where hyperphosphorylation of RyR2 results in increased Ca\textsuperscript{2+} leak (Wehrens et al., 2005). Given that mutations underlying CPVT ultimately result in increased Ca\textsuperscript{2+} leak from the RyR2 (Lehnart et al., 2004), a similar mechanism may explain the alternans reported here.

We thus establish that genetically modified murine hearts modeling CPVT exhibit alternans and that this phenomenon is exacerbated by epinephrine suppressible by propranolol, fully reflecting clinical findings (Lehnart et al., 1995; Wehrens et al., 2003). The presence of such alternans is not, however, associated with changes in APD, VERP or the slopes of restitution curves. CPVT has not previously been associated with consistent electrocardiographic features that might predict arrhythmic risk (Sumitomo et al., 2003). It is possible that the presence of alternans may represent a useful marker in patients suspected of harboring this highly lethal condition.

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