Arrhythmogenic mechanisms in the isolated perfused hypokalaemic murine heart

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Abstract

Aim: Hypokalaemia is associated with a lethal form of ventricular tachycardia (VT), torsade de pointes, through pathophysiological mechanisms requiring clarification.

Methods: Left ventricular endocardial and epicardial monophasic action potentials were compared in isolated mouse hearts paced from the right ventricular epicardium perfused with hypokalaemic (3 and 4 mM [K+]o) solutions. Corresponding K+ currents were compared in whole-cell patch-clamped epicardial and endocardial myocytes.

Results: Hypokalaemia prolonged epicardial action potential durations (APD) from mean APD90s of 37.2 ± 1.7 ms (n = 7) to 58.4 ± 4.1 ms (n = 7) and 66.7 ± 2.1 ms (n = 11) at 5.2, 4 and 3 mM [K+]o respectively. Endocardial APD90s correspondingly increased from 51.6 ± 1.9 ms (n = 7) to 62.8 ± 2.8 ms (n = 7) and 62.9 ± 5.9 ms (n = 11) giving reductions in endocardial–epicardial differences, ΔAPD90, from 14.4 ± 2.6 to 4.4 ± 5.0 and ±3.4 ± 6.0 ms respectively. Early afterdepolarizations (EADs) occurred in epicardia in three of seven spontaneously beating hearts at 4 mM [K+]o with triggered beats followed by episodes of non-sustained VT in nine of 11 preparations at 3 mM. Programmed electrical stimulation never induced arrhythmic events in preparations perfused with normokalemic solutions yet induced VT in two of seven and nine of 11 preparations at 4 and 3 mM [K+]o respectively. Early outward K+ current correspondingly fell from 73.46 ± 8.45 to 61.16 ± 6.14 pA/pF in isolated epicardial but not endocardial myocytes (n = 9) (3 mM [K+]o).

Conclusions: Hypokalaemic mouse hearts recapitulate the clinical arrhythmogenic phenotype, demonstrating EADs and triggered beats that might initiate VT on the one hand and reduced transmural dispersion of repolarization reflected in ΔAPD90 suggesting arrhythmogenic substrate on the other.

Keywords arrhythmogenesis, hypokalaemia, mouse heart.

Cardiac K+ channels govern a range of important physiological functions that include heart rate and action potential (AP) waveform and duration (Tamargo et al. 2004). In particular, cardiac AP repolarization is regulated by a variety of K+ channel currents that include the transient outward current, Ito, the rapidly activating delayed rectifier current, IKr, and the inwardly rectifying current, IK1. Reductions in outward
K⁺ channel currents have been associated with impaired repolarization with a consequent increase in AP duration and a prolonged surface electrocardiographic QT interval (Choy et al. 1997).

Both increases and decreases in extracellular K⁺ ([K⁺]ₒ) have been associated with potentially life-threatening arrhythmias (Curtis et al. 1993). At the cellular level, low [K⁺]ₒ has been shown to reduce K⁺ currents and enhance the potency of agents that block K⁺ channels (Sanguinetti & Jurkiewicz 1992, Yang & Roden 1996). Hypokalaemia is a recognized risk factor alongside bradycardia for the development of torsade de pointes (TdP), a life-threatening form of ventricular tachycardia (VT), in which the QRS complexes appear to twist about the isoelectric line (Antzelevitch et al. 1996, Berthet et al. 1999, He & MacGregor 2001). Currently two theories, not necessarily exclusive, preside over the induction of TdP: (1) delayed repolarization, resulting from AP prolongation leads to early afterdepolarizations (EADs) that interrupt the otherwise smooth repolarization phase of the AP, and may give rise to salvos of premature triggered beats and TdP (Roden 2004). (2) Heterogeneous distribution of cardiac ion channel currents through the thickness of the ventricular wall creates a transmural dispersion of repolarization (TDR), which may exacerbate upon AP lengthening (Papadatos et al. 2002). Ordinarily, a TDR, and therefore a transmural gradient in refractoriness, plays an important role in the spread of repolarization throughout the ventricle (i.e. proceeding from the epicardium to the endocardium). Agents that affect action potential duration (APD) to differing extents across the ventricular wall, would result in altered APD transmural gradients, and hence refractoriness, both of which are potentially arrhythmogenic mechanisms (Janse & Wit 1989).

The multiple risk factor intervention trial (Cohen et al. 1987) reported a 28% increase in ventricular arrhythmias for every 1 mmol reduction in serum K⁺ amongst male hypertensive patients receiving diuretic therapy. Furthermore, corrections of serum K⁺ through intravenous or oral potassium administration have been reported to reduce the QT interval in long QT (LQT) patients, and may thus help prevent subsequent sudden cardiac death (SCD) (Choy et al. 1997, Etheridge et al. 2003).

The Nernst equation predicts that a reduction in [K⁺]ₒ should increase the driving force for outward current through K⁺ channels and therefore increase Iₖ. However, studies of Iₖ₉, Iₖᵣ, and Iₖ₁ in human atrial myocytes, guinea-pig myocytes and sheep cardiac purkinje fibres, respectively, have demonstrated that reduced [K⁺]ₒ actually decreases these K⁺ currents (Carmeliet 1982, Sanguinetti & Jurkiewicz 1992, Firek & Giles 1995). These findings could help explain the cardiac AP prolongation that has been observed at low [K⁺]ₒ, a well-recognized clinical phenomenon, that may play an important role in the genesis of arrhythmias such as TdP (Ginant et al. 1991; Yang & Roden 1996).

A reduction in [K⁺]ₒ is a common experimental manoeuvre employed in isolated cardiac tissue and whole-heart preparations when assessing the arrhythmic tendency of drugs implicated in acquired long QT syndrome (LQTS) or establishing indirect, pharmacological models of LQTS, or in assessing the pathogenesis of cardiac arrhythmias (Eckardt et al. 1998, Milberg et al. 2002, 2005). These studies have lowered [K⁺]ₒ in combination with the administration of a wide range of compounds thought to be implicated in the development of TdP. Furthermore, in many of these studies, it was actually necessary to reduce [K⁺]ₒ to induce arrhythmias, even in the presence of known arrhythmogenic agents (Milberg et al. 2005). This suggests that [K⁺]ₒ, is an important trigger for cardiac arrhythmias in its own right, yet such reports did not themselves assess the effects that reductions in [K⁺]ₒ, by itself may have upon the arrhythmic tendency of these cardiac preparations.

Despite studies documenting the effects of hypokalaemia upon Iₖ₉, Iₖᵣ and Iₖ₁ in isolated cardiac myocytes and tissue preparations and the established clinical association of hypokalaemia and TdP (Berthet et al. 1999), the existence of such a precise link has not yet been proven. Studies in the intact isolated heart have the advantage of containing all myocardial cell types whilst maintaining intercellular coupling, and could thus provide more physiologically relevant information regarding the induction and propagation of cardiac arrhythmia. The purpose of this study, therefore, was to determine the intrinsic arrhythmogenic effects of hypokalaemia in the isolated, Langendorff-perfused murine whole-heart model, and to assess if an increased arrhythmic state is accompanied by an altered transmural gradient of APD.

Methods

Experimental animals

The mice used in this study were kept in an animal house at room temperature and subjected to a consistent 12 h : 12 h light : dark cycle and fed with sterile rodent chow, having access to water at all times. Wild-type (WT) 129 background male and female mice aged 5–7 months were used in all experiments.

Langendorff-perfused preparation

The experiments used a Langendorff-perfused preparation that has been previously adapted for murine hearts (Balasubramaniam et al. 2003). Briefly, mice
were killed by cervical dislocation in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The heart was then quickly excised and submerged in ice-cold bicarbonate-buffered Krebs–Henseleit solution containing in mm: 119 NaCl, 25 NaHCO₃, 4 KCl, 1.2 KH₂PO₄, 1 MgCl₂, 1.8 CaCl₂, 10 glucose and 2 sodium pyruvate. The solution was bubbled with a 95% O₂–5% CO₂ mixture (British Oxygen Company, Manchester, UK). The aorta was cannulated under the buffer surface using a 21-gauge custom-made cannula, and was attached to the cannula needle using a micro-aneurysm clip (Harvard Apparatus, Edenbridge, UK). The preparation was then transferred to the perfusion apparatus, to which the cannula was attached, and perfusion commenced in a retrograde manner via the aorta with the abovementioned bicarbonate-buffered Krebs–Henseleit solution. Before entering the aorta, buffer was passed through 200 and 5 μm filters (Milipore, Watford, UK) and warmed to 37 °C by means of a water jacket and circulator (Model C-85A, Techne, Cambridge, UK). Perfusion was maintained at a constant flow rate of 2–2.5 mL min⁻¹ using a peristaltic pump (Watson–Marlow Bredel pumps model 505S, Falmouth, Cornwall, UK). Following the start of perfusion, healthy, experimentally viable hearts regained a pink colouration and spontaneous rhythmic contraction with warming. In 10% of experiments, hearts were discarded because of signs of ischaemia after cannulation and perfusion.

**Perfused heart electrophysiological measurements**

In the present experiments, a paired (1-mm inter-pole spacing) platinum stimulating electrode was placed on the basal surface of the right ventricular epicardium. Prior to experimental procedures, hearts were paced for 10 min at 8 Hz using 2-ms square-wave stimuli with amplitudes set to three times the excitation threshold (Grass S48 stimulator, Grass-Telefactor, Slough, UK).

Epicardial MAP recordings were obtained using a MAP electrode (Linton Instruments, Harvard Apparatus, UK) placed on the basal surface of the left ventricular epicardium. The epicardial MAP electrode was gradually positioned until a gentle but stable contact pressure was achieved. This resulted in a recording of MAP signals. For endocardial recordings, a small access window was created in the interventricular septum to gain access to left ventricular endocardium (Casimiro et al. 2001). A custom-made endocardial MAP electrode constructed from two twisted strands of Teflon-coated (0.25 mm diameter) silver wire (99.99% purity) (Advent Research Materials Ltd, Oxford, UK) that had been previously galvanically chlorided to eliminate DC offset, was positioned on to the left ventricular free wall under a stable contact pressure until MAP signals were achieved. MAPs were amplified, band-pass filtered (0.5 Hz to 1 kHz; Gould 2400S, Gould-Nicolet Technologies, Ilford, Essex, UK) and digitized (1401 plus MKII, Cambridge Electronic Design, Cambridge, UK). MAPs were extracted and analysed (SPIKE II version 4; Cambridge Electronic Design) to derive the precise duration of the digitized signals. The recordings were deemed reproducible and, hence of an acceptable standard for analysis if they had the following properties: a stable baseline, a rapid upstroke phase with consistent amplitude, a smooth contoured repolarization phase and a stable duration [MAP duration at 90% repolarization (APD₉₀) was reproducible within 2 ms under baseline conditions].

**Experimental protocol**

A standard pacing protocol (basic cycle length, BCL of 125 ms) that corresponded to physiological whole-animal heart rates (Papadatos et al. 2002) was initiated for periods of up to 20 min to measure APD at 50%, 70% and 90% repolarization. External pacing stimuli were subsequently withdrawn from all preparations, leading to a significantly reduced, intrinsic heart rate corresponding to a BCL of approximately 400 ms. Reduced heart rates are a known risk factor for the development of repolarization abnormalities such as EADs and triggered beats that may underlie the induction of VT (Roden & Hoffman 1985). Epicardial MAPs were recorded for periods of up to 20 min from isolated, perfused WT mouse hearts under intrinsic pacing conditions. Following this, programmed electrical stimulation (PES) of the heart was carried out using an adaptation of the corresponding clinical techniques (Saumarez & Grace 2000, Balasubramaniam et al. 2003). PES procedures began by applying standard pacing stimuli at a BCL of 125 ms for 25 s. Following this, a drive train of eight-paced beats (S1) again at a BCL of 125 ms preceded an extrastimulus (S2) every ninth beat. S1S2 intervals initially equalled the pacing interval and then were progressively reduced by 1 ms with each nine-beat cycle until ventricular refractoriness was reached, at which point the S2 stimulus elicited no MAP. BCL pacing protocols of 125 ms, corresponding to physiological whole-animal heart rates (Papadatos et al. 2002), were used in all paced experiments. Recordings were subsequently repeated following a 20-min wash-in of a reduced [K⁺]₀ perfusate, of either 4 or 3 mm.

We used two methods to quantify changes in transmural gradients of repolarization. Firstly, AAPD₉₀ was calculated from the difference between the mean...
endocardial and epicardial APD\textsubscript{90} values, giving positive results where the endocardial value exceeded the epicardial value, and negative results where the epicardial value was greater. Secondly, TDR was defined as the positive part of the APD\textsubscript{90} as described on earlier occasions (Kirchhof \textit{et al.} 1996). An EAD was defined as a positive deflection that interrupted the smooth repolarization phase of the AP. A triggered beat was similarly defined as a positive deflection in the smooth repolarization phase of the AP whose amplitude approximately matched the amplitude of the initial AP. Arrhythmias were defined as a ventricular tachyarrhythmia of more than five-cycle duration that was typically self-terminating. Following cannulation and subsequent perfusion of hearts, approximately 10\% of preparations were discarded because of signs of ischaemia.

**Isolation of single-ventricular myocytes**

Epicardial and endocardial myocytes were dissociated enzymatically from the left ventricle. Following cannulation, the heart was perfused in a retrograde fashion with Krebs–Henseleit buffer, warmed to 37°C by means of a water jacket and circulator (Techne model C-85A), at a rate of 2–2.5 mL/min for 5 min, until the heart regained a homogenous pink colouration and began contracting spontaneously. The heart was then perfused for 5 min with a nitrilotriacetic acid-based perfusion buffer containing (in mM): 125 NaCl, 4.75 KCl, 5 MgSO\textsubscript{4}, 10 HEPES, 5 sodium pyruvate, 20 glucose, 20 taurine and 4.5 nitrilotriacetic acid. Following this, the heart was perfused with a digestion buffer for 12–15 min containing (in mM): 125 NaCl, 4.75 KCl, 5 MgSO\textsubscript{4}, 10 HEPES, 5 sodium pyruvate, 20 glucose, 20 taurine, 0.6 CaCl\textsubscript{2} and 1 mg/mL collagenase type 2 (Worthington, UK), 1 mg/mL hyaluronidase (Sigma, Poole, UK). After this period, a small pair of 90\degree curved forceps was used to tear off a thin layer of left ventricular epicardial tissue. After epicardial tissue had been harvested, a surgical blade was used to create an incision along the length of the heart, to gain access to the left ventricular endocardial surface. As before, a pair of forceps was used to tear off thin sections of endocardial tissue. Epicardial and endocardial tissue samples were placed in separate tubes containing digestion buffer in addition to 1 mg/mL bovine serum albumin (Sigma) for 5 min before gentle trituration for a further 5 min in the same solution. Tissue samples were subsequently spun down in a centrifuge machine (1000 rpm for 3 min) before the supernatant from the epicardial and endocardial tissue tubes was discarded and replaced with a wash buffer containing (in mM): 135 NaCl, 1.1 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 5.4 KCl, 10 Hepes, 10 Glucose and pH was adjusted to 7.35 with NaOH. Epicardial and endocardial myocytes were stored in the aforementioned wash buffer and were studied within 4–6 h. Following initial perfusion of the heart, all subsequent steps were performed at room temperature.

**Single-cell electrophysiology**

Conventional whole-cell patch-clamp recording in voltage clamp mode were carried out using an Axopatch 200B amplifier (Axon Instruments, CA, USA) coupled to a Digidata series computer interface and controlled by pClamp software (Axon Instruments). Pipettes (1–4 M\Omega) were pulled from borosilicate glass capillaries (1.5 mm outer and 0.86 inner diameter, GC150–10; Harvard Apparatus Ltd). Extracellular buffer contained (in mM): 135 NaCl, 1.1 MgCl\textsubscript{2}, 1.8 CaCl\textsubscript{2}, 5.4 KCl, 10 Hepes, 10 Glucose and pH was adjusted to 7.35 with NaOH. Intracellular pipette saline contained (in mM): 130 KCl, 1 MgCl\textsubscript{2}, 10 Hepes, 5 Mg-ATP, 5 Na\textsubscript{2}-creatine phosphate and pH was adjusted to 7.2 with KOH. After formation of gigaseal, whole-cell configuration was achieved by applying gentle suction through pipette and ZAP. Up to 75\% series resistance compensation was achieved. Transient outward potassium currents and inward currents were triggered by applying a series of 10 mV incremental voltage pulses from −100 to 50 mV from a holding potential of −60 mV.

**Data analysis and statistics**

Single-cell electrophysiological data and whole-heart MAP data were initially imported into Microsoft Excel. All data are expressed as means ± SEM. For whole-heart data, comparisons were made using ANOVA (SPSS software) and for single-cell electrophysiological data comparisons were made using Student’s \textit{t}-test, with values of \textit{P} < 0.05 being considered significant.

**Results**

Hypokalaemia is a known risk factor for the development of a lethal form of VT termed torsade de pointes, although the underlying physiological mechanisms responsible for this remain unclear (Roden \textit{et al.} 1996). The experiments sought to investigate the intrinsic arrhythmogenicity induced by hypokalaemia by recording left ventricular epicardial and endocardial monophasic action potentials (MAPs) from isolated, perfused mouse hearts, and to determine whether arrhythmogenicity was associated with the occurrence of repolarization abnormalities such as EADs and triggered beats, an altered transmural gradient of repolarization or a combination of the two.
Stability of endocardial and epicardial MAP recordings

Experimental data were initially obtained from recordings of MAPs from isolated, perfused WT mouse hearts under normokalaemic conditions (5.2 mm [K⁺]₀) to establish the control phenotype. The procedures were then repeated following reductions in [K⁺]₀. Following cannulation and perfusion of the murine hearts, the electrophysiological parameters of MAP waveform morphology, amplitude and duration reached a steady state within 10 min. Following this stabilization period, MAP recordings and pacing thresholds remained highly reproducible throughout the experimental protocol. The MAPs recorded fulfilled the previously documented murine cardiac electrophysiological criteria in possessing a triangular morphology, a rapid upstroke phase, a smooth repolarization phase, and closely resembled murine ventricular MAPs from earlier studies (Guo et al. 1999) (Fig. 1). No MAP waveform repolarization abnormalities in either the epicardium or endocardium were ever seen under normokalemic (5.2 mm [K⁺]₀) conditions. Both epicardial and endocardial MAP amplitudes and durations remained highly stable throughout the duration of experimental recording procedures, with APD₉₀ values being reproducible within 2 ms under normokalemic conditions (Tables 1 & 2) in 25 separate preparations, further validating this experimental set-up.

Hypokalaemia modifies the regional heterogeneity of murine ventricular repolarization

The experiments then proceeded to investigate whether reductions in [K⁺]₀, affected the transmural gradient of repolarization in the intact, isolated, perfused mouse heart. Local activation time is the time measured from the point of electrical stimulus to the maximal amplitude of the AP repolarization time is obtained by the addition of local activation times to MAP duration; however, in the present study we only observed insignificant changes in local activation times in the presence of reduced [K⁺]₀ (data not shown). This finding is in keeping with a previous study in which perfusion of isolated rabbit hearts with amiodarone led to no significant increase or decrease in local activation times (Kirchhof et al. 2003). With this in mind, the present

| Parameter (ms) | 5.2 (n = 7) | 4 (n = 7) | 3 (n = 11) |
|----------------|-------------|-----------|------------|
| APD₅₀          | 7.8 ± 0.8   | 16.9 ± 3.6*| 19.5 ± 2.4*|
| APD₇₀          | 19.7 ± 2.3  | 31.4 ± 4.9 | 37.2 ± 2.9*|
| APD₉₀          | 37.2 ± 1.7  | 58.4 ± 4.1*| 66.7 ± 2.1*|

*P < 0.05 vs. baseline.

| Parameter (ms) | 5.2 (n = 7) | 4 (n = 7) | 3 (n = 11) |
|----------------|-------------|-----------|------------|
| APD₅₀          | 19.7 ± 1.3  | 19.7 ± 0.8| 17.2 ± 1.6 |
| APD₇₀          | 32.2 ± 1.6  | 32.7 ± 3.1| 34.7 ± 2.4 |
| APD₉₀          | 51.6 ± 1.9  | 62.8 ± 2.8*| 62.9 ± 5.9*|

*P < 0.05 vs. baseline.

Figure 1 Representative example of left ventricular endocardial and epicardial MAP recordings under control, normokalaemic conditions of 5.2 mm [K⁺]₀, in an isolated Langendorff-perfused WT mouse heart. Under control conditions, MAP waveform characteristics remained highly stable and reproducible throughout all recordings.
experiments measured changes in the transmural gradient of repolarization by first calculating a \( \Delta APD_{90} \) from the difference between the epicardial \( APD_{90} \) and the endocardial \( APD_{90} \); this gave a positive value where the endocardial \( APD_{90} \) exceeded the epicardial \( APD_{90} \), and a negative value if the opposite was the case. However, TDR was then obtained from the positive part of this \( \Delta APD_{90} \) as defined on earlier occasions (Kirchhof et al. 1996).

Fig. 2 shows representative epicardial and endocardial MAPs recorded from isolated, mouse hearts perfused with either normokalemic (5.2 mm [\( K^+ \]o]) (Fig. 2a) or hypokalemic (4 or 3 mm [\( K^+ \]o]) (Fig. 2b,c) physiological buffer solutions, at a BCL of 125 ms. Reductions in [\( K^+ \]o] to 4 and 3 mm did not significantly alter the endocardial \( APD_{50} \) \((n = 18)\) (Table 2). However, these reductions in [\( K^+ \]o] to 4 and 3 mm led to increases in epicardial \( APD_{50} \) values from 7.8 ± 0.8 to 16.9 ± 3.6 and 19.5 ± 2.4 ms respectively \((n = 18)\) (Table 1). Reduction of [\( K^+ \]o] from 5.2 to 4 mm led to significant increases in mean epicardial \( APD_{70} \) and \( APD_{90} \) values, from 19.7 ± 2.3 to 31.4 ± 4.9 ms and from 37.2 ± 1.7 to 58.4 ± 4.1 ms respectively \((P < 0.05)\) \((n = 7)\) (Table 1, Fig. 3a,b: clear columns). Endocardial MAP values were also affected. Admittedly mean endocardial \( APD_{70} \) values were not significantly affected by this initial reduction in [\( K^+ \]o] from 5.2 to 4 mm \((n = 7)\) (Table 2). Mean endocardial \( APD_{90} \) values were, however, significantly increased from 51.6 ± 1.9 to 62.8 ± 2.8 ms \((P < 0.05)\) \((n = 7)\) (Table 2, Fig. 3a,b: grey columns). These effects led to a marked reduction in both TDR and \( \Delta APD_{90} \) from 14.4 ± 2.6 ms under normokalemic conditions of 5.2 mm [\( K^+ \]o], to 4.4 ± 5.0 ms upon lowering [\( K^+ \]o] to 4 mm [\( K^+ \]o], that was attributable to a greater lengthening of the epicardial MAP over the endocardial MAP (Fig. 3a,b: black columns).

Further reductions in [\( K^+ \]o] from 4 to 3 mm similarly led to further prolongation of mean epicardial \( APD_{70} \) and \( APD_{90} \) values to 37.2 ± 2.9 and 66.7 ± 2.1 ms respectively \((n = 11)\) (Table 2, Fig. 3b,c: clear col-
A reduction from 4 to 3 mm $[K^+]_o$ led to no further significant change in endocardial APD$_{70}$ and APD$_{90}$ values ($n = 11$) (Table 1, Fig. 3b,c: grey columns). The preferential lengthening of epicardial APD$_{90}$ values over endocardial APD$_{90}$ values in isolated mouse hearts perfused with 3 mm $[K^+]_o$ led to a further reduction in TDR to 3.4 ms, and actually a negative $\Delta$APD$_{90}$ value of $-3.4 \pm 6.0$ ms, reflecting the greater epicardial compared with endocardial APD$_{90}$ (Fig. 2d, Fig. 3c: black column).

**Hypokalaemia induces repolarization abnormalities in spontaneously beating hearts**

Bradycardia is a known risk factor for TdP (Rodan & Hoffman 1985), and earlier studies have reported EADs and TdP in rabbit hearts following the perfusion of a range of drugs implicated in acquired LQTS under combined states of hypokalaemia and bradycardia (Eckardt et al. 1998, Milberg et al. 2002). Accordingly, following the standard pacing protocols to accurately measure APD at various stages of repolarization, extrinsic pacing was terminated in all preparations, leading to a pronounced decrease in heart rate. Under these conditions, no repolarization abnormalities were ever recorded following perfusion with a normokalemic physiological solution. Control epicardial intrinsic MAPs displayed a typical triangular morphology, with a smooth repolarization phase (Fig. 4a). However, following reductions in $[K^+]_o$ to 4 mm, under intrinsic pacing conditions, EADs were now recorded from three of seven hearts (Fig. 4b). In these unprovoked preparations, EADs presented as pronounced positive deflections occurring in the smooth repolarization phase of the AP. Further reductions in $[K^+]_o$ to 3 mm frequently lead to salvos of triggered beats that preceded periods of non-sustained VT (VT) in nine of 11 preparations (Fig. 4c).

**PES induces ventricular tachycardia in hypokalaemic mouse hearts**

The final experiments related the phenomena characterized above to an actual generation of arrhythmogenesis following a PES procedure. PES was used as an experimental tool to determine the arrhythmic susceptibility of isolated WT mouse hearts perfused with hypokalaemic (4 or 3 mm $[K^+]_o$) physiological buffer solutions. The PES procedures were directly adapted from clinical diagnostic techniques used to assess arrhythmogenic propensity in patients, for the current murine whole-heart model (Saumarez & Grace 2000, Balasubramaniam et al. 2003).

Short S1–S2 coupling intervals under normokalemic (5.2 mm $[K^+]_o$) baseline conditions elicited typical extrasystolic APs (Fig. 5a). Figure 5 illustrates epicardial MAP recordings from isolated, perfused WT mouse hearts subjected to PES following progressive reductions in $[K^+]_o$. PES repeatedly failed to induce VT in isolated WT hearts under normokalemic (5.2 mm $[K^+]_o$) baseline conditions ($n = 7$) (Fig. 5a). Reducing $[K^+]_o$ from 5.2 to 4 mm led to the induction of VT in only two of seven preparations subjected to PES (29% incidence), in close parallel with clinical case reports of cardiac arrhythmias from hypokalaemic patients (Cohen et al. 1987). Figure 5b, illustrates a heart in which was not induced following perfusion with 4 mm $[K^+]_o$). Upon further reduction of $[K^+]_o$ to 3 mm, triggered beats and non-sustained VT in nine of 11 preparations were seen during PES protocols (Fig. 5c).

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**Figure 3** Steady-state epicardial and endocardial APD measured at 90% repolarization (APD$_{90}$), and $\Delta$APD$_{90}$ values (white, grey and black columns respectively) under (a) control conditions (seven hearts), and following perfusion with hypokalaemic solutions (b) 4 mm (seven hearts) and (c) 3 mm $[K^+]_o$.
Patch-clamp study of the effects of hypokalaemia on transient outward and inward potassium currents from epicardial and endocardial cardiac myocytes

To compliment the whole-heart electrophysiological findings, the experiments proceeded to explore the effects of hypokalaemia at the single-cell level. Individual myocytes were selectively isolated from the left ventricular epicardial and endocardial surfaces as described in Methods. The whole-cell configuration of the patch-clamp technique was used to record repolarizing K⁺ channel currents in epicardial and endocardial myocytes in normokalemic and hypokalemic physiological buffer solutions.

To record a transient outward current (Ito), cells were voltage-clamped at -60 mV and depolarized to 50 mV for a 500-ms duration. Under normokalemic conditions, average amplitude of Ito as reflected in the early peak of the outward current, in epicardial cells was significantly greater than in endocardial cells (73.46 ± 8.45 and 32.87 ± 9.27 pA/pF, respectively, P < 0.05, n = 9) (Fig. 6a,b respectively). We additionally applied hyperpolarizing steps from a holding potential of -60 to -100 mV to record an inwardly rectifying K⁺ channel...
current ($I_{K1}$). Mean $I_{K1}$ density was not significantly different between epicardial and endocardial cells ($-10.18 \pm 0.28$ vs. $-9.62 \pm 1.65$ pA/pF, respectively, $P > 0.05, n = 9$) (Fig. 6a,b).

Under hypokalaemic conditions of 3 mM $[K^+]_o$, $I_{To}$ density was not significantly affected in endocardial cells under identical hypokalaemic conditions ($32.87 \pm 9.27$ and $31.09 \pm 8.03$ pA/pF, respectively, $P > 0.05, n = 5$) (Fig. 6b). Under hypokalaemic conditions $I_{K1}$ density was significantly reduced from $-10.18 \pm 0.28$ to $-3.66 \pm 0.77$ pA/pF in epicardial cells ($P < 0.05, n = 4$) (Fig. 6a). Similarly, hypokalaemia significantly reduced $I_{K1}$ in endocardial cells from $-9.62 \pm 1.65$ to $-2.93 \pm 0.35$ pA/pF ($P < 0.05, n = 4$) (Fig. 6b). However, reduction in $I_{K1}$ under hypokalaemic conditions in epicardial cells was not significantly different from endocardial cells ($-3.66 \pm 0.77$ vs. $2.93 \pm 0.35$ pA/pF; $P > 0.05$).

**Discussion**

Clinical findings suggest that hypokalaemia may have intrinsic arrhythmogenic effects but the underlying physiological mechanisms remain unclear. We accordingly sought to investigate the intrinsic arrhythmogenic effects of hypokalaemia in isolated, Langendorff-perfused wild-type (WT) mouse hearts for the first time by recording MAPs from endocardial and epicardial left ventricular sites. The mouse model has proven to offer a powerful tool for the study of arrhythmias and their associated risk factors in murine hearts harbouring specific cardiac ion channel mutations that are known to directly correspond to human LQTS subtypes (Papadatos et al. 2002, Balasubramaniam et al. 2003).
This study represents for the first time a quantitative description of the effects of hypokalaemia upon the occurrence of early EADs, VT and transmural changes in APD at the whole-heart and single-cell level. The results fully recapitulate clinical case reports of VT and TdP documented in hypokalaemic patients (Berthet et al. 1999, Kusano et al. 2001).

The present studies using Langendorff-perfused mouse hearts led to several new important conclusions. Firstly, we have confirmed for this isolated, perfused murine whole-heart model that under control conditions, endocardial MAPs were reproducibly longer in duration than epicardial MAPs. Our results confirm previous AP recordings from isolated murine myocytes (Guo et al. 1999), and from murine whole-heart preparations (Anumonwo et al. 2001, Casimiro et al. 2001, Knollmann et al. 2001).

In the present study, the greater APD of endocardial over epicardial MAPs led to a transmural APD gradient of 14.4 ms, which closely correlates with a previous study of in vivo murine MAP recordings (Liu et al. 2004). The transmural difference in APD across the ventricular wall is important in establishing normal TDR-refractoriness, which may help to prevent re-entrant arrhythmias. Alterations in the normal patterns of cardiac repolarization and refractoriness are known contributing factors to re-entrant arrhythmias (Janse & Wit 1989).

Secondly, it was successfully shown that a reduction in [K+]o leads to marked prolongation of epicardial and endocardial ventricular MAPs in mouse hearts, and a subsequent reduction in the transmural gradient of APD and therefore the TDR. To our knowledge this observation has not been reported on earlier occasions. Although a reduction in TDR is generally considered to reduce rather than increase the likelihood of arrhythmogenesis, one potential outcome of altered myocardial repolarization gradients would be an increased probability of repolarization gradient collision. This in turn would facilitate the generation of local conduction block and of consequent re-entrant arrhythmogenesis (Wolk et al. 1999, Rithalia et al. 2001). This could increase the susceptibility of the heart to arrhythmias initially induced by premature ventricular excitation through physiological phenomena such as EADs and triggered beats or via artificial premature excitation using PES. This first report of such phenomena in the isolated, perfused whole-heart model complements one previous study in isolated endocardial and epicardial rat myocytes that suggested that a reduction in TDR might
also be proarrhythmic (Rithalia et al. 2001). Our results in the intact myocardium directly demonstrate that a reduced transmural gradient in APD can afford a mechanism of proarrhythmia.

Thirdly, we have established that the reduced heart rate seen in spontaneously beating hypokalaemic hearts led to an increased propensity for the development of repolarization abnormalities, such as EADs and triggered beats, which preceded episodes of VT. Although reduction of outward K+ currents observed under hypokalaemic conditions cannot directly initiate arrhythmogenic mechanisms such as EADs, AP prolongation will ensue, which is a known arrhythmogenic mechanism of action (Clancy et al. 2003). Prolongation of repolarization through reductions in repolarizing K+ currents has been speculated to induce EADs through Ca2+ channel reactivation (Haverkamp et al. 2000). Increases in the time spent in the voltage window range for L-type Ca2+ channel reactivation through AP prolongation are likely to generate EADs, which may in turn give rise to salvos of premature potentials termed triggered beats (January et al. 2000, Fabritz et al. 2003). Bouchard et al. (2004) demonstrated that prolonged exposure of rabbit ventricular myocytes to hypokalaemic solutions led to fluctuations in membrane potential and subsequent oscillations in cell length. These oscillations were shown to be because of Ca2+ entry through L-type Ca2+ channels.

Intrinsically beating hearts perfused with a reduced [K+]o buffer of 4 mM, elicited EADs in three of seven preparations. Further reductions in [K+]o to 3 mM, elicited not only EADs but also triggered beats that were followed by episodes of non-sustained VT in nine of 11 preparations. These findings at the level of the intact heart correlate with earlier cellular studies. Such studies have shown that the occurrence of EADs is increased under low frequency pacing, implicating the L-type Ca2+ current as a necessary depolarizing charge carrier during the EAD which appears to predominate under slow stimulation rates (Damiano & Rosen 1984, Zeng & Rudy 1995).

Reduction of [K+]o to 3 mM therefore leads to a situation that contains high levels of EADs and triggered beats and the presence of both a further reduced TDR and a negative ΔAPD90 value, representing the first point at which epicardial exceeds endocardial APD90. This would be expected to lead to a proarrhythmic state, in which there is on the one hand an increased likelihood of EADs, and on the other in which an EAD is likely to give rise to development of VT only in the setting of markedly altered transmural gradients in APD. In this situation, induction of VT is considered to result from both a trigger and an appropriate substrate.

Finally, it was demonstrated that the occurrence of VT using PES correlated with reductions in both TDR and ΔAPD90, concomitant with a progressive reduction in [K+]o. The present results document that a reduction in [K+]o to 4 mM leads to a 29% incidence of VT, closely paralleling the clinical case study of Cohen et al. (1987), who reported a similar frequency of VT amongst hypertensive patients receiving diuretic therapy. This finding further validates the use of the intact, isolated, Langendorff-perfused mouse heart as an experimental set-up to accurately study human arrhythmogenicity. Following further reduction in [K+]o, to 3 mM, PES induced VT in nine of 11 preparations.

This study of arrhythmogenesis in the intact mouse heart complements previous studies on the molecular effects of hypokalaemia at the cellular level. Reductions in [K+]o have been shown to reduce the conductances of a number of K+ channels including the transient outward current (Ito) (Firek & Giles 1995), the rapidly activating delayed rectifier current (IK1) (Scamps & Carmeliet 1989, Yang & Roden 1996) and the inwardly rectifying current (Ikr) (Carmeliet 1982, Bouchard et al. 2004). Outward potassium current in response to depolarization (−60 to 50 mV) and inward rectifying current (Ikr) in response to hyperpolarization (−60 to −100 mV) were measured in normokalaemic (5.4 mM) and hypokalaemic (3 mM) buffer from both epicardial and endocardial myocytes.

Firstly, under normokalaemic conditions we recorded a greater earlier outward K+ current, attributable to a transient outward (Ito) current component, from epicardial compared with endocardial myocytes. Mouse cardiac repolarization is dominated by the rapidly activating Ito, K+ current (Nerbonne et al. 2001). Ito is differentially expressed in the murine ventricle, with higher protein levels found in the epicardium than the endocardium (Brunet et al. 2004). Such differences in the transmural expression of Ito are thought to account for the shorter APDs frequently reported at the murine ventricular epicardium compared with the endocardium (Knollmann et al. 2001). In the present study, this result compliments our MAP recordings from isolated perfused hearts, in which we demonstrated that APs recorded from the endocardial surface were greater in duration than APs recorded from the epicardial surface. Thus, the difference in Ito density between the epicardium and the endocardium may help explain why APs recorded from these two sites significantly differ in duration.

Secondly, under hypokalaemic conditions, we recorded a significant reduction of early outward current, attributable Ito in epicardial myocytes. Firek & Giles (1995) reported that reductions in [K+]o reduced Ito in human atrial myocytes. Reductions in Ito can prolong APD and increase Ca2+ entry via L-type Ca2+ channels.
(Fiset & Giles 2006). However, under similar conditions, early outward current was not significantly affected in the endocardial myocytes. Reductions in epicardial but not endocardial $I_{o}$ under hypokalaemic conditions correlates with preferential lengthening of epicardial compared with endocardial APD under hypokalaemic conditions reported in the present study at the whole-heart level. Furthermore, we documented a significant increase in the early repolarization phase, as reflected in epicardial APD$_{50}$, at the whole-heart level under hypokalaemic conditions of 3 mM $[K^{+}]_{o}$, supporting the notion that an early outward K$^{+}$ current, most likely $I_{o}$, is reduced in epicardial myocytes under hypokalaemic conditions. Thus, a reduction in epicardial $I_{o}$ under hypokalaemic conditions could account for preferential epicardial APD prolongation at 50%, 70% and 90% repolarization and could therefore be considered one of the primary mechanisms responsible for the change in the transmural gradient of repolarization, reflected by changes in ΔAPD$_{90}$ seen at the whole-heart level.

Thirdly, we also recorded K$^{+}$ current through $I_{K1}$ by applying hyperpolarizing steps. Under normokalaemic conditions, inward $I_{K1}$ was not significantly different between epicardial and endocardial myocytes. Under hypokalaemic conditions, inward $I_{K1}$ current was significantly reduced by equal extents in both epicardial and endocardial myocytes. Therefore, it is unlikely that a differential reduction in $I_{K1}$ between epicardial and endocardial myocytes may significantly contribute to an altered transmural gradient of repolarization.

Hypokalaemia induces hyperpolarization of the cell membrane, which inhibits $I_{K1}$ (Carmeliet 1992, Bouchar et al. 2004). $I_{K1}$ is the main current responsible for setting the resting membrane potential in mammalian heart cells and it can also contribute to the late phase of repolarization (Nichols et al. 1996). Inhibition of outward-going $I_{K1}$ via hypokalaemia-induced hyperpolarization of the cardiac cell membrane would therefore be expected to prolong the later phases of cardiac AP repolarization. Such effects could account for the significant epicardial and endocardial AP prolongation at 90% repolarization in isolated hearts perfused with 3 mM $[K^{+}]_{o}$. Previously, genetically engineered mice lacking $I_{K1}$ exhibit cardiac AP prolongation (Zaritsky et al. 2000, 2001). However, we understand that this is not a definitive experimental approach to assess the effect of $I_{K1}$ in AP repolarization under hypokalaemic conditions as the physiological function of $I_{K1}$ is because of a smaller outwardly rectifying component of $I_{K1}$ current. Under the conditions of our patch-clamp experiments, however, a large early transient outward K$^{+}$ current will mask any outward $I_{K1}$ current.

The results from the whole-heart and single-cell electrophysiological studies strongly suggest that reductions in an early outward K$^{+}$ current, most likely to be $I_{o}$, is the primary ionic mechanism for the significant increase in epicardial APD at 50%, 70% and 90% repolarization and for the alteration in the transmural gradients of repolarization observed under hypokalaemic conditions. Previously, $I_{K1}$ has been shown to be similarly sensitive to reductions in $[K^{+}]_{o}$ through either increased channel inactivation kinetics (Yang et al. 1997) or through an increased inhibitory effect of Na$^{+}$ ions at an extracellular binding site of the human ether-a-go-go related gene (HERG) K$^{+}$ channel, which constitutes $I_{K1}$, as $[K^{+}]_{o}$ is lowered (Numaguchi et al. 2000). HERG K$^{+}$ channels rapidly activate from closed to open states during depolarization, but pass little outward current as they rapidly inactivate (Vandenb et al. 2001). Channels subsequently pass an outward current as they recover from inactivation during repolarization (Clancy et al. 2003). Thus the corresponding murine HERG K$^{+}$ channel (mERG) may contribute to the late phase of murine repolarization and reductions in $I_{K1}$ may be responsible for increased epicardial and endocardial APD$_{90}$ observed at the whole-heart level under hypokalaemic conditions. When recording outward K$^{+}$ currents at the single-cell level in hypokalaemic solutions, we recorded a significant reduction of outward current at early times in epicardial myocytes, more likely reflecting $I_{o}$ as opposed to $I_{K1}$. Nevertheless we have taken the care to emphasize early outward K$^{+}$ current as opposed to individual outward K$^{+}$ current components. At the whole-heart level we report significant epicardial AP prolongation at 3 mM $[K^{+}]_{o}$, occurring at early repolarization times, as reflected by increased APD$_{50}$, consistent with the single-cell findings and further supporting the notion that an early outward K$^{+}$ current, attributable to $I_{o}$, is reduced in the epicardium under hypokalaemic conditions.

However, this does not exclude the possibility of other mechanisms contributing to arrhythmogenesis under hypokalaemic conditions. Reductions in $[K^{+}]_{o}$ have been associated with electrogenic Na$^{+}$/K$^{+}$ ATPase pump inhibition (Eisner & Lederer 1979) and a subsequent increase in $[Na^{+}]_{i}$ (Boyett et al. 1986). Elevations in $[Na^{+}]_{i}$ may lead to an increase in $[Ca^{2+}]_{i}$, through inhibition of Ca$^{2+}$ extrusion via the Na$^{+}$–Ca$^{2+}$ exchanger (White & Terrar 1991). Nevertheless, the isolated, perfused heart electrophysiological data alongside the single-cell patch-clamp data in the present study strongly supports the notion that the reduction of early repolarizing K$^{+}$ currents selectively in the epicardium leading to AP prolongation and the subsequent induction of EADs, alongside alteration of transmural gradients of repolarization is the primary arrhythmogenic mechanism of action associated with hypokalaemia in the mouse heart.
In conclusion, analysis of epicardial and endocardial MAPs recorded from isolated, Langendorff-perfused, WT murine whole-heart preparations and patch-clamp K⁺ current measurements in isolated epicardial and endocardial myocytes under varying degrees of hypokalaemia has thus shed new light on the pathogenesis of VT under hypokalaemic conditions. Here we report for the first time episodes of EADs, triggered beats and VT in the setting of a reduced TDR recorded from intact, isolated mouse hearts perfused with hypokalaemic solutions, further highlighting the possible severe clinical consequences of relatively small reductions in serum potassium levels. Furthermore, at the single-cell level we report a significant reduction in early outward current in epicardial myocytes under hypokalaemic conditions, an effect that is likely to play an important role in the generation of altered transmural gradients of repolarization seen at the whole-heart level. These data suggest that treatment of even modest hypokalaemia is critical in preventing serious unwanted lethal cardiac events. Intervention of serum [K⁺] may prove to be beneficial in the prophylaxis of VT and TdP induced by hypokalaemia.

Conflict of interest

We report no conflict of interest.

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