Transient alterations in transmural repolarization gradients and arrhythmogenicity in hypokalaemic Langendorff-perfused murine hearts

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Clinical hypokalaemia is associated with acquired electrocardiographic QT prolongation and arrhythmic activity initiated by premature ventricular depolarizations and suppressed by lidocaine (lignocaine). Nevertheless, regular (S1) pacing at a 125 ms interstimulus interval resulted in stable waveforms and rhythm studied using epicardial and endocardial monophasic action potential (MAP) electrodes in Langendorff-perfused murine hearts whether under normokalaemic (5.2 mM K^+) or hypokalaemic (3.0 mM K^+) conditions, in both the presence and absence of lidocaine (10 μM). Furthermore, the transmural gradient in repolarization time, known to be altered in the congenital long-QT syndromes, and reflected in the difference between endocardial and epicardial MAP duration at 90% repolarization (ΔAPD_{90}), did not differ significantly ($P > 0.05$) between normokalaemic (5.5 ± 4.5 ms, $n = 8$, five hearts), hypokalaemic ($n = 8$, five hearts), or lidocaine-treated normokalaemic ($n = 8$, five hearts) or hypokalaemic ($n = 8$, five hearts) hearts. However, premature ventricular depolarizations occurring in response to extrasystolic (S2) stimulation delivered at S1S2 intervals between 0 and 22 ± 6 ms following recovery from refractoriness initiated arrhythmic activity specifically in hypokalaemic ($n = 8$, five hearts) as opposed to normokalaemic ($n = 25$, 14 hearts), or lidocaine-treated hypokalaemic ($n = 8$, five hearts) or normokalaemic hearts ($n = 8$, five hearts). This was associated with sharp but transient reversals in ΔAPD_{90} in MAPs initiated within the 250 ms interval directly succeeding premature ventricular depolarizations, from 3.3 ± 5.6 ms to -31.8 ± 11.8 ms ($P < 0.05$) when they were initiated immediately after recovery from refractoriness. In contrast the corresponding latency differences consistently remained close to the normokalaemic value (-1.6 ± 1.4 ms, $P > 0.05$). These findings empirically associate arrhythmogenesis in hypokalaemic hearts with transient alterations in transmural repolarization gradients resulting from premature ventricular depolarizations. This is in contrast to sustained alterations in transmural repolarization gradients present on regular stimulation in long-QT syndrome models.

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The pathophysiological state of hypokalaemia is associated both with acquired electrocardiographic T-wave inversion and marked prolongation of the QT interval reflecting increased ventricular action potential duration, and is known to predispose to ventricular arrhythmic activity (Zipes & Braunwald, 2005). In the murine heart, action potential prolongation under hypokalaemic conditions has recently been attributed to reductions in the repolarizing K^+ currents I_{to} (transient outward current) and I_{K1} (inwardly rectifying current) (Killeen *et al.* 2007). Arrhythmic activity associated with increased ventricular action potential duration is also a characteristic physiological feature of the congenital long-QT syndromes (LQTS) (Moss & Kass, 2005). This heterogeneous group of disorders result from autosomal dominant mutations in cardiac ion channels and the disorders are characterized clinically by syncope and sudden cardiac death resulting from ventricular arrhythmic activity (Moss & Kass, 2005).

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Arrhythmic activity in situations including thiazide-induced hypokalaemia (Whelton, 1984), LQTS (Noda *et al.* 2004), the Brugada syndrome (Yan & Antzelevitch, 1999) and myocardial ischaemia (El-Sherif *et al.* 1976; Naito *et al.* 1982) frequently follows premature ventricular depolarizations (PVDs). These are especially arrhythmogenic in both humans (Smirk, 1949; Ruberman *et al.* 1977; Ruberman *et al.* 1981) and animals (Naito *et al.* 1982; Kiryu *et al.* 1999) when they occur soon after the previous action potential and result in an electrocardiographic R-wave being superposed on the preceding T-wave. However, neither the electrophysiological basis for this phenomenon nor the antiarrhythmic effect of class 1b antiarrhythmic drugs, such as lidocaine (Rehnqvist *et al.* 1984), in such conditions has been explored in detail.

PVDs initiated by extrasystolic stimulation are known to exhibit altered repolarization kinetics (Boyett & Jewell, 1978) and to result in alterations in the repolarizaton kinetics of subsequent action potentials, part of the phenomenon of *postextrasystolic potentiation* (Bass, 1975; Schouten *et al.* 1990). Furthermore, PVDs display spatial heterogeneities in action potential repolarization (Allessie *et al.* 1976; Kuo *et al.* 1983). Such heterogeneities have been observed across the surfaces of both the epicardium (Laurita *et al.* 1998) and endocardium (Kobayashi *et al.* 1992), as well as between epicardial and endocardial cells in both isolated myocytes (Litovsky & Antzelevitch, 1989) and whole-heart preparations (Weissenburger *et al.* 2000; Wolk *et al.* 2002). This has been attributed variously to effects on Na^+ or K^+ channel activation (Schouten *et al.* 1990), as well as alterations in Ca^{2+} homeostasis whether through effects on L-type Ca^{2+} channels (Sun *et al.* 1997), the Na^+–Ca^{2+} exchanger, possibly through effects on the Na^+–K^+-ATPase (Janvier *et al.* 1997), or sarcoplasmic reticular Ca^{2+} stores (Isenberg & Han, 1994). At all events the consequent PVDs are well established as triggers for arrhythmic activity (Naito *et al.* 1982).

Sustained increases in the magnitudes of transmural repolarization gradients have frequently been associated with arrhythmogenesis in the setting of action potential prolongation. This has been demonstrated in the congenital LQTS types 1 (Shimizu & Antzelevitch, 1998), 2 (Shimizu & Antzelevitch, 1997; Akar *et al.* 2002) and 3 (Shimizu & Antzelevitch, 1997; Milberg *et al.* 2005), as well as following exposure to sodium pentobarbital (Shimizu *et al.* 1999) and cisapride (Di Diego *et al.* 2003). Further, manoeuvres which reduce the magnitude of such repolarization gradients have been shown to exert an antiarrhythmic effect (Shimizu & Antzelevitch, 2000; Antzelevitch *et al.* 2004), even if they further increase action potential duration (Shimizu *et al.* 1999).

Prompted by these previous reports, we tested the hypothesis that transient alterations in transmural repolarization gradients following PVDs might be *associated* with the pro-arrhythmic effect of hypokalaemia and the antiarrhythmic effect of lidocaine in Langendorff-perfused murine hearts.

Methods

Experimental animals

Mice were housed in an animal facility at $21 \pm 1^{\circ}$C with 12 h light/dark cycles. Animals were fed sterile chow (RM3 Maintenance Diet, SDS, Witham, Essex, UK) and had free access to water. Male wild-type 129 Sv mice aged 3–6 months were used in experiments. All procedures complied with UK Home Office regulations (Animals (Scientific Procedures) Act 1986).

Preparation

A Langendorff-perfusion protocol previously adapted for murine hearts (Balasubramaniam *et al.* 2003) was used. The mice were killed by cervical dislocation (Schedule 1, UK Animals (Scientific Procedures) Act 1986). In brief, hearts were then quickly excised and placed in ice-cold bicarbonate-buffered Krebs–Henseleit solution (mM: NaCl 119, $NaHCO_3$ 25, KCl 4, KH_2PO_4 1.2, $MgCl_2$ 1, $CaCl_2$ 1.8, glucose 10 and sodium pyruvate 2; pH adjusted to 7.4) bubbled with 95% O_2–5% CO_2 (British Oxygen Company, Manchester, UK). A short section of aorta was cannulated under the surface of the solution and attached to a custom-made 21 gauge cannula filled with the same solution using an aneurysm clip. Fresh Krebs–Henseleit solution was then passed through 200 μm and 5 μm filters (Millipore, Watford, UK) and warmed to 37°C using a water jacket and circulator (Techne model C-85A; Cambridge, UK) before being used for constant-flow retrograde perfusion at 2–2.5 ml min^{-1} using a peristaltic pump (Watson-Marlow Bredel model 505S; Falmouth, Cornwall, UK). Hearts were regarded as suitable for experimentation if, on warming, they regained a healthy pink colour and began to contract spontaneously.

Electrophysiological measurements

A bipolar platinum stimulating electrode (1 mm interpole spacing) was placed on the basal surface of the right ventricular epicardium. Hearts were stimulated at an interstimulus interval of 125 ms, close to the expected *in vivo* electrocardiographic RR interval of 131.4 ± 32.8 ms (VanderBrink *et al.* 2000) and faster than the intrinsic rate, using 2 ms duration square-wave stimuli. Our experiments sought to study the effects of PVDs on the subsequent action potentials. This required a choice of stimulus parameters that would consistently produce PVDs and subsequent action potentials, all of which are all-or-none events. This was achieved by a applying stimuli at twice

diastolic threshold which was in turn consistent with previous studies (Milberg *et al.* 2002, 2005; Papadatos *et al.* 2002; Kirchhof *et al.* 2003; Knollmann *et al.* 2006) using a Grass S48 stimulator (Grass-Telefactor, Slough, UK) for at least 10 min before recording began to allow a steady state to be established.

Epicardial monophasic action potential (MAP) electrodes (Hugo Sachs; Harvard Apparatus, Edenbridge, Kent, UK) were placed on the basal region of the left ventricular epicardium. In addition, a small access window was created in the interventricular septum in order to allow access to the left ventricular endocardium (Casimiro *et al.* 2001). A custom-made endocardial MAP electrode composing two twisted strands of high-purity Teflon-coated 0.25 mm diameter silver wire (Advent Research Materials Ltd, Eynsham, Oxford, UK) was constructed. The Teflon coat was removed from the distal 1 mm of the electrode which was then galvanically chlorided to eliminate DC offset, inserted and placed against the septal endocardial surface. MAPs were amplified, band-pass filtered (0.5 Hz to 1 kHz, Gould 2400S; Gould-Nicolet Technologies, Ilford, Essex, UK) and digitized at a sampling frequency of 5 kHz, giving a Nyquist frequency of 2.5 kHz (temporal resolution of 200 μs) using a 1401plus analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK). Analysis of MAPs was performed using Spike II software (Cambridge Electronic Design).

Experimental protocol

Electrical stimulation was carried out at an interstimulus interval of 125 ms and using an adaptation of previously described clinical techniques (Saumarez & Grace, 2000; Balasubramaniam *et al.* 2003; Head *et al.* 2005). This extrasystolic (S2) stimulation protocol imposed successive cycles beginning with two pacing stimuli (S0 and S1) separated by 125 ms, the S2 stimulus itself (open arrows), then five further pacing stimuli (S3–S7) again spaced by 125 ms (Fig. 1*A*) to investigate changes in the nature of the MAPs that succeeded the S2 stimulus, as well as to provide an 875 ms conditioning period between the delivery of successive S2 stimuli. The S1S2 interval was decremented by 1 ms with each successive cycle from an initial value of 120 ms. Cycles were continued until either the S2 stimulus

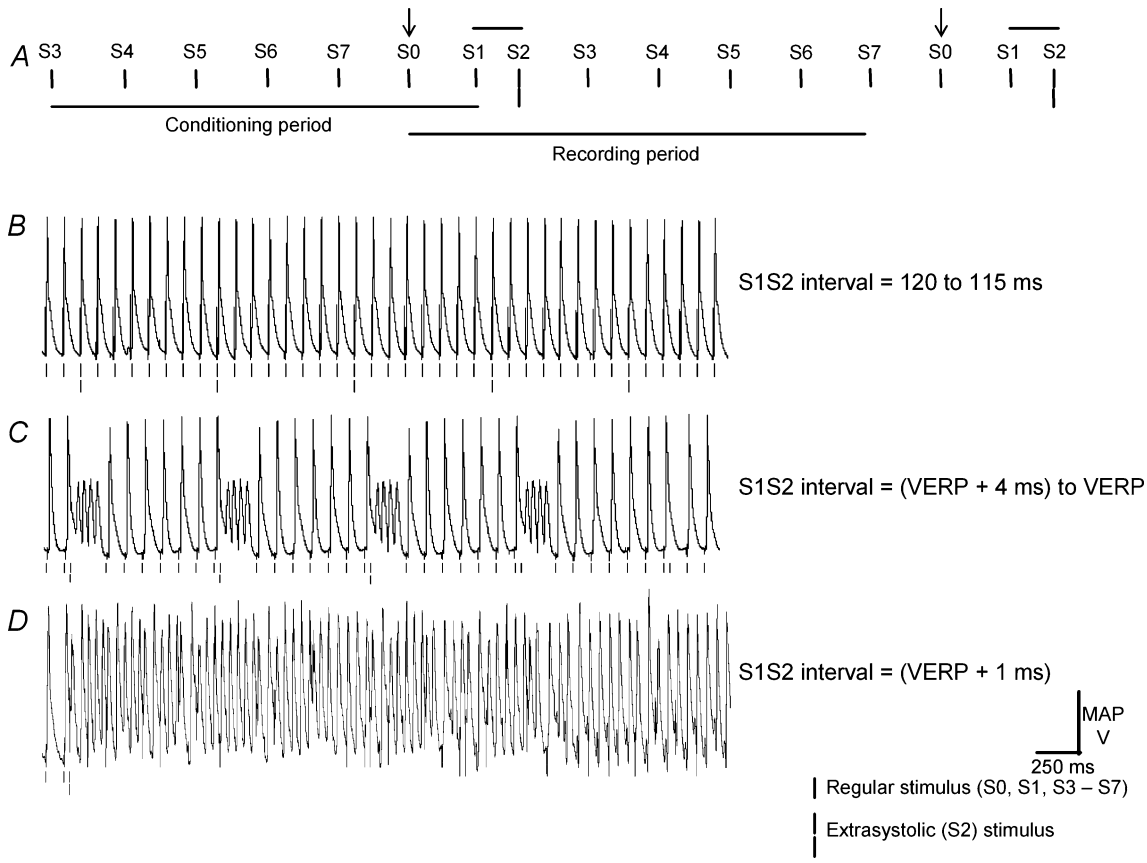

Figure 1. Initiation of arrhythmic activity by premature ventricular depolarizations occurring in response to extrasystolic (S2) stimulation in hypokalaemic hearts
A, extrasystolic (S2) stimulation protocol. Epicardial monophasic action potential (MAP) recordings from a heart exposed to hypokalaemic (3.0 mM K^+) test solution for 20 min at S1S2 intervals that were decremented between 120 and 115 ms (*B*), and to intervals just greater and including the ventricular effective refractory period (VERP) (*C*).

appeared to initiate arrhythmic activity as confirmed by the demonstration of morphologically irregular waveforms during an imposed 250 ms pause, or the ventricular effective refractory period (VERP) following the previous action potential was reached whereupon the S2 stimulus failed to elicit a MAP.

Recordings were made after 10, 15 or 20 min exposure to test solutions. The constituents of normokalaemic (5.2 mM K^+) test (Krebs–Henseleit) solution are described above. In hypokalaemic test solutions [K^+] was lowered to 3.0 mM by reduction of KCl content. Both these solutions were also prepared with the addition of lidocaine (10 μM; Sigma-Aldrich, Poole, UK).

Data analysis

Data are expressed as means ± S.E.M., with n being the number of repetitions. Comparisons were made using ANOVA (StatsPlus 3.5; Analystsoft) with a significance threshold set at $P \leq 0.05$.

Results

The experiments investigated the effect of hypokalaemia, proarrhythmic in clinical situations, and its modification by the class 1b antiarrhythmic agent lidocaine in isolated Langendorff-perfused murine hearts. Hearts were initially paced at a regular 125 ms interstimulus interval using bipolar platinum electrodes placed against the right ventricular epicardial surface while exposed for 20 min to normokalaemic (5.2 mM K^+), hypokalaemic (3.0 mM K^+), normokalaemic lidocaine-containing (10 μM) or hypokalaemic lidocaine-containing test solutions, respectively. Monophasic action potentials (MAPs) were recorded as regular stimulation was continued over the subsequent 20 min using basal left ventricular epicardial and endocardial electrodes. Action potential duration was quantified at 90% repolarization (APD_{90}), APD_0 being defined as the peak upstroke potential difference and APD_{100} as the diastolic potential difference. Inclusion or exclusion of the upstroke phase did not make a significant difference ($P > 0.05$) to results in any case. MAPs demonstrated stable durations with each stimulus eliciting a single MAP which showed a stable baseline, rapid upstroke phase that reached a consistent amplitude and a smooth repolarization phase (cf. Knollmann *et al.* 2001; Fabritz *et al.* 2003). Neither epicardial nor endocardial APD_{90} differed significantly between the first and last 5 min of the recording period after exposure to any test solution, confirming stable recording conditions.

Initiation of arrhythmic activity by extrasystolic stimulation

Experiments began by establishing steady initial conditions with 25 s of regular stimulation at a 125 ms cycle length. PVDs were then initiated by extrasystolic (S2) stimuli in a protocol adapted from a programmed electrical stimulation procedure previously used to assess arrhythmic tendency in clinical situations (Saumarez & Grace, 2000; Balasubramaniam *et al.* 2003; Head *et al.* 2005) and described in the Methods (Fig. 1*A*).

Figure 1*B*, *C* and *D* shows typical results. Single vertical lines indicate the timing of regular stimuli and double lines indicate S2 stimuli. Hearts exposed to hypokalaemic test solution for 10 or 15 min maintained a stable rhythm whether on regular stimulation at an interstimulus interval of 125 ms or on application of the S2 stimulation protocol in 36 out of 36 cases (26 out of 26 hearts). After 20 min exposure to hypokalaemic test solution, hearts maintained a stable rhythm on regular stimulation and on S2 stimulation at long S1S2 intervals (Fig. 1*B*). However, when S2 stimuli were delivered at S1S2 intervals of between the ventricular effective refractory period (VERP, filled arrow) and VERP + (14 ± 5) ms, arrhythmic activity was initiated in 15 out of 15 cases (in seven out of seven hearts, Fig. 1*C*) and became sustained in 13 out of 15 cases (in six out of seven hearts, Fig. 1*D*). This was seen under hypokalaemic conditions only.

Arrhythmogenicity is specific to hypokalaemic hearts

The arrhythmogenic effect of PVDs occurring in response to S2 stimulation soon after recovery from refractoriness was specific to hypokalaemic hearts and could be prevented by exposure to lidocaine. Figure 2 shows typical results obtained at a long S1S2 interval (115 ms, upper traces in each pair) and at an S1S2 interval resulting in the delivery of an S2 stimulus immediately after recovery from refractoriness (early S2 stimulation, lower traces in each pair). When this stimulation protocol was also applied to hearts exposed to normokalaemic solution a stable rhythm was maintained regardless of S1S2 interval ($n = 25$, 13 hearts, Fig. 2*A*). In contrast, hearts exposed to hypokalaemic test solution showed arrhythmic activity following PVDs initiated by early S2 stimuli in 15 out of 15 cases (seven out of seven hearts, Fig. 2*B*). Normokalaemic hearts exposed to lidocaine never showed arrhythmic activity ($n = 8$, four hearts, Fig. 2*C*). Furthermore, arrhythmic activity was prevented by addition of lidocaine even with hypokalaemic test solution ($n = 15$, seven hearts, Fig. 2*D*).

PVDs occurring in response to early S2 stimuli alter transmural repolarization gradients

Arrhythmic activity in models showing QT prolongation has been associated with large transmural repolarization gradients even during regular stimulation (Shimizu & Antzelevitch, 1997, 1998; Shimizu *et al.* 1999;

Akar *et al.* 2002; Di Diego *et al.* 2003; Milberg *et al.* 2005). The possibility that altered transmural repolarization gradients of this kind could be involved in arrhythmogenesis in hypokalaemic murine hearts and the detailed circumstances in which such alterations might occur were investigated.

We proceeded to examine transmural repolarization gradients by determining stimulation to depolarization latencies and APD_{90} values in the epicardia and endocardia of hearts exposed to the four test solutions. The time taken for depolarization to propagate across the myocardial wall, Δ(latency), was calculated as endocardial latency minus epicardial latency. Similarly, the time taken for repolarization to spread across the myocardial wall, ΔAPD_{90}, was calculated as endocardial APD_{90} minus epicardial APD_{90}. Summed together, ΔAPD_{90} and Δ(latency) provided a measure of the difference in time between stimulation and action potential repolarization between endocardium and epicardium.

Application of the S2 stimulation protocol to hearts exposed to hypokalaemic test solution for 20 min reliably precipitated arrhythmic activity and thus precluded quantitative analysis of transmural repolarization gradients. Accordingly, recordings were made after 15 min rather than 20 min exposure to test solutions. At this time a stable rhythm still persisted following the application of both regular and S2 stimulation protocols in all cases. There was no significant difference ($P > 0.05$) in Δ(latency) or ΔAPD_{90} between hearts exposed to normokalaemic (-0.7 ± 2.3 and 5.5 ± 4.5 ms, respectively) and other test solutions when subjected to regular stimulation at an interstimulus interval of 125 ms. However, contrasting results were obtained when hearts were subjected to the S2 stimulation protocol. Figure 3 illustrates typical examples of the resulting epicardial (upper traces in each pair) and endocardial (lower traces in each pair) recordings. MAPs occurring in response to S2 and S3 stimuli showed noticeably different waveforms that contrasted with the consistent waveforms obtained

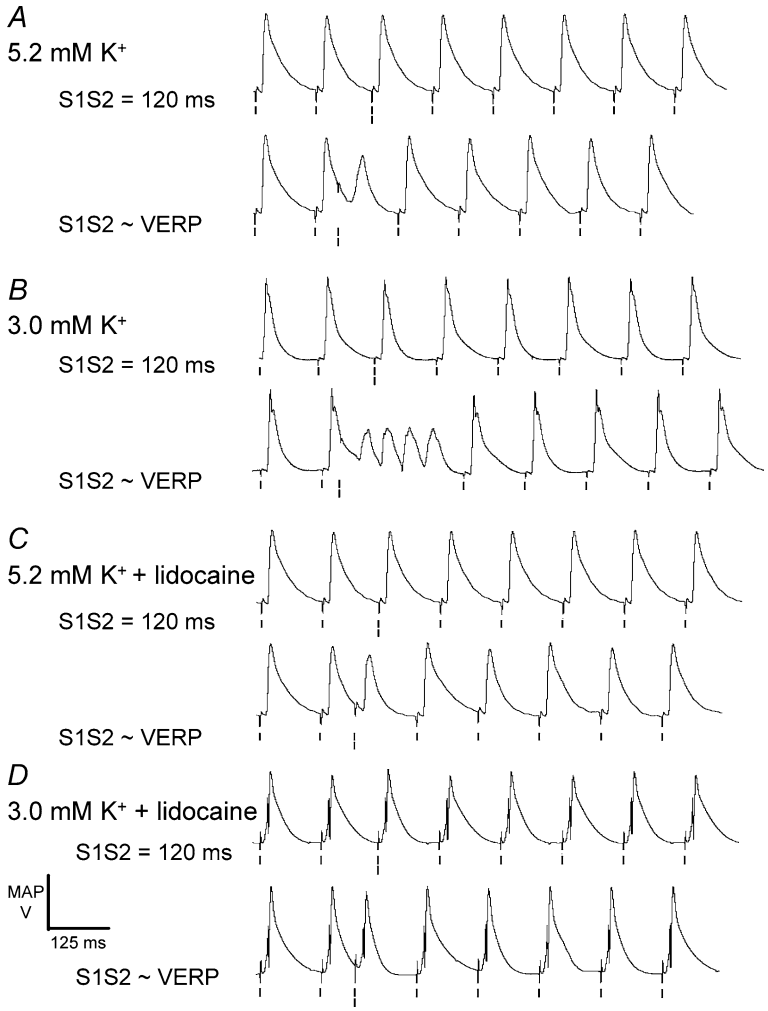

Figure 2. Specificity of arrhythmogenicity to hypokalaemic hearts
Epicardial MAP recordings resulting from application of the S2 stimulation protocol at S1S2 intervals of 120 ms (upper traces in each pair) and close to the ventricular effective refractory period (VERP) (lower traces in each pair) in hearts exposed to normokalaemic (5.2 mM K^+, *A*) and hypokalaemic (3.0 mM K^+, *B*) test solutions, and normokalaemic (*C*) and hypokalaemic (*D*) test solutions containing lidocaine (10 μM), for 20 min. Single vertical lines indicate the timing of S0–S1 and S3–S7 stimuli and double lines indicate the timing of S2 stimuli.

in response to other stimuli. Nevertheless, latency and Δ(latency) values obtained from hearts exposed to the four test solutions and subjected to such stimulation were statistically indistinguishable ($P > 0.05$) from values obtained with regular stimulation.

Transmural repolarization gradients alter with S1S2 interval in hypokalaemic hearts

Figure 4*A*–*D* gives typical results of a closer, quantitative exploration of changes in these kinetics of MAPs evoked by S1–S4 stimuli. The figure shows epicardial APD_{90} (filled circles), endocardial APD_{90} (filled squares) and ΔAPD_{90} (open circles) at S1S2 intervals ranging between the VERP (arrows) and 120 ms, a positive ΔAPD_{90} indicating spread of repolarization from epicardium to endocardium and a negative ΔAPD_{90} indicating the reverse. ΔAPD_{90} was consistently positive in hearts subjected to regular stimulation irrespective of the test solution, as reflected in the ΔAPD_{90} values at the longest S1S2 interval in Fig. 4. In hearts exposed to normokalaemic test solution, progressively shortening the S1S2 interval proportionally decreased epicardial and endocardial APD_{90} values, leaving ΔAPD_{90} constant and positive. However, in MAPs following the subsequent S3 stimulus endocardial APD_{90} decreased more sharply than did epicardial APD_{90} with decreasing S1S2 interval. The two sets of points crossed to give negative ΔAPD_{90} values at S1S2 intervals between the VERP and (VERP + 18 ± 6 ms) ($n = 8$, five hearts, VERP = 61 ms in the heart shown). Finally after the S4 stimulus, epicardial and endocardial APD_{90} values and ΔAPD_{90} had recovered to their values obtained with regular stimulation. Perfusion with hypokalaemic test solution (Fig. 4*B*) accentuated these changes, S2 and S3 stimulation resulting in steeper decreases in endocardial as compared to epicardial APD_{90} with decreasing S1S2 interval. The two sets of points intersected to give a negative ΔAPD_{90} at a wider range of S1S2 intervals between the VERP and (VERP + 22 ± 6 ms) ($n = 8$, five hearts, VERP = 50 ms in the heart shown), that agreed with the range of S1S2 intervals that resulted in arrhythmic activity after longer durations (20 min) of hypokalaemia

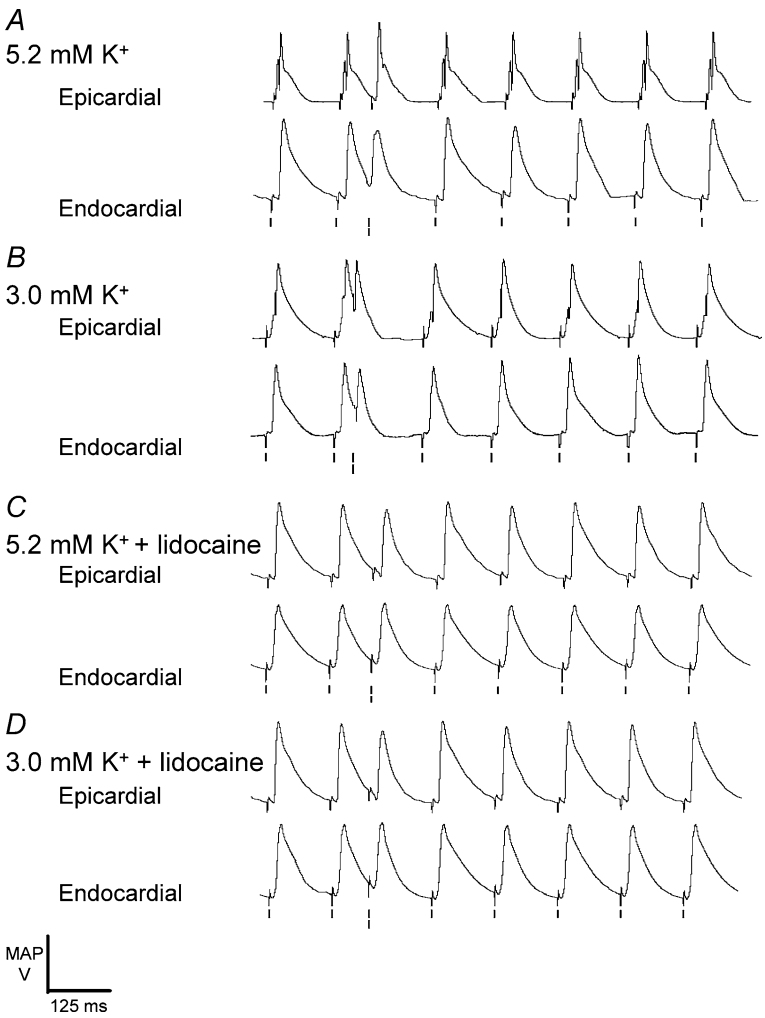

Figure 3. Epicardial and endocardial MAPs following application of the S2 stimulation protocol at S1S2 intervals just greater than the ventricular effective refractory period
Epicardial (upper traces in each pair) and endocardial (lower traces in each pair) monophasic action potential recordings from hearts exposed to normokalaemic (*A*) and hypokalaemic (*B*) test solutions, and normokalaemic (*C*) and hypokalaemic (*D*) test solutions containing lidocaine, for 15 min. Single vertical lines indicate the timing of S0–S1 and S3–S7 stimuli, and double lines indicate the timing of S2 stimuli.

(horizontal thick lines in Fig. 4*B*). Nevertheless, after the S4 stimulus both epicardial and endocardial APD_{90} values and ΔAPD_{90} again had fully recovered.

In parallel with its abolition of arrhythmic activity (Fig. 2), exposure to lidocaine resulted in ΔAPD_{90} remaining positive throughout the S2 stimulation protocol in both normokalaemic ($n = 8$, five hearts, Fig. 4*C*) and hypokalaemic ($n = 8$, five hearts, Fig. 4*D*) hearts despite its decreasing at S1S2 intervals just greater than the VERP (81 ms in Fig. 4*C*, and 78 ms in Fig. 4*D*).

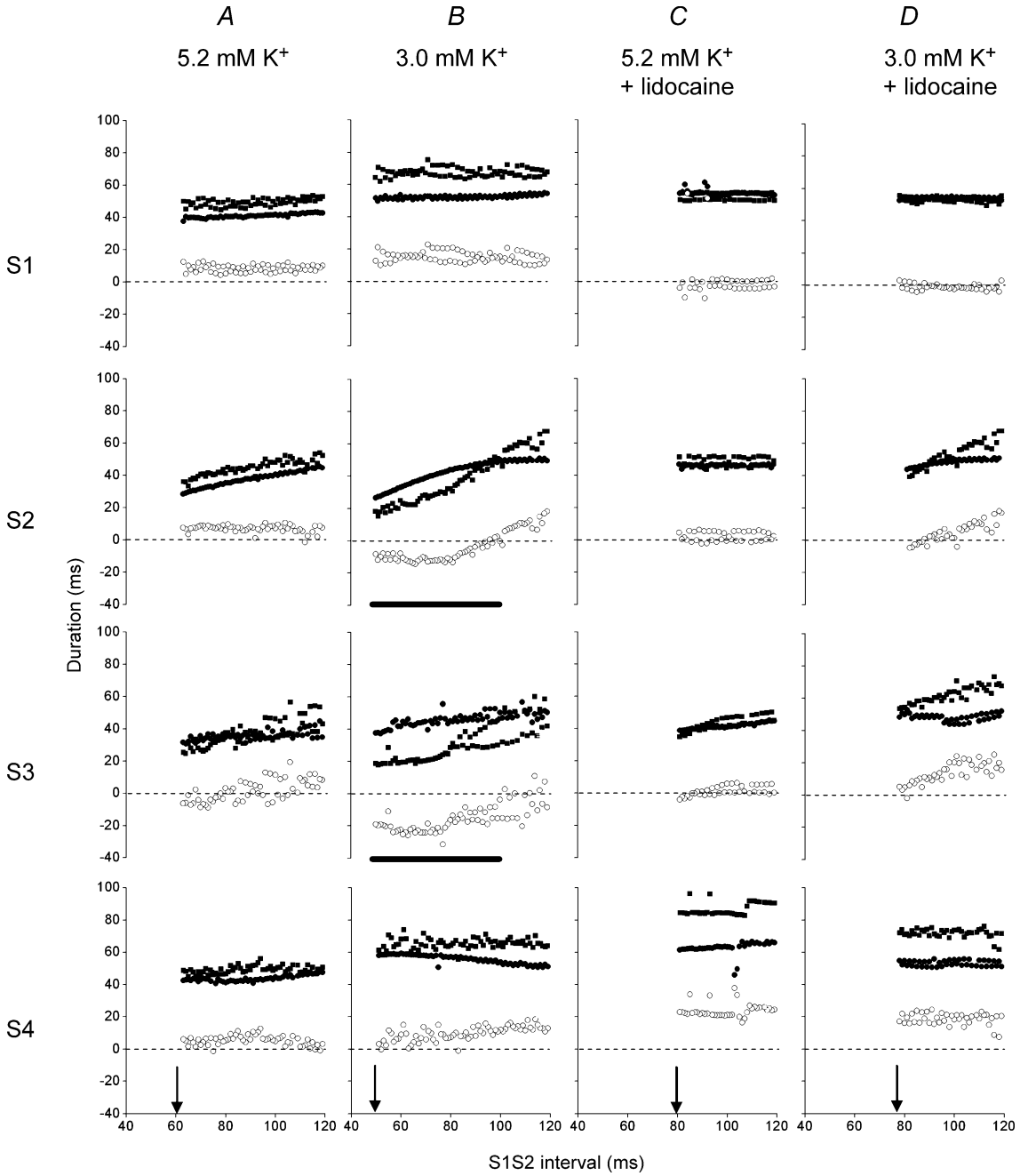

Figure 4. Transient alterations in transmural repolarization gradients at varying S1S2 intervals
Epicardial APD_{90} (filled circles), endocardial APD_{90} (filled squares) and ΔAPD_{90} (open circles) of monophasic action potentials obtained after control (S1) extrasystolic (S2) and subsequent (S3, S4) stimulation at S1S2 intervals progressively from 120 ms to the ventricular effective refractory period (filled arrows) in hearts exposed to normokalaemic (*A*) and hypokalaemic (*B*) test solutions, and normokalaemic (*C*) and hypokalaemic (*D*) test solutions containing lidocaine. Thick horizontal lines indicate significantly negative ΔAPD_{90} in *B* (S2 and S3).

PVDs resulting from extrasystolic stimulation produce transient alterations in ΔAPD_{90}

These findings prompted a further, quantitative, analysis of Δ(latency) and ΔAPD_{90} in a population of hearts specifically studied at the shortest S1S2 interval compatible with the generation of a MAP, the point at which both maximal changes in ΔAPD_{90} and arrhythmogenesis were observed as illustrated in Fig. 2. Figure 5 indicates mean epicardial latency and APD_{90} obtained with regular pacing by continuous lines, endocardial latency and APD_{90} by dotted lines, and Δ(latency) and ΔAPD_{90} by dashed lines; the corresponding mean values obtained following the S0–S7 stimuli of the final cycle of the S2 stimulation

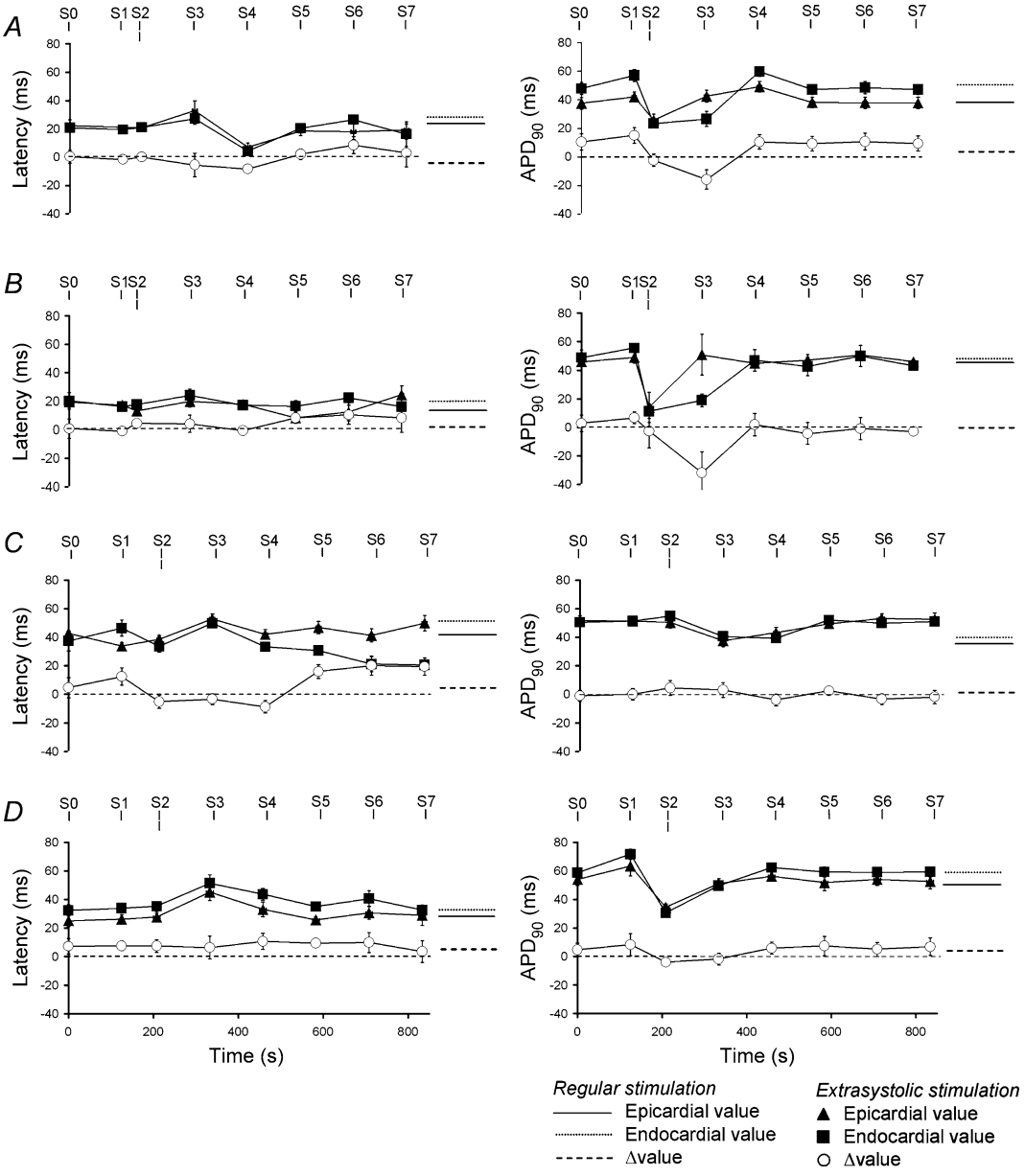

Figure 5. Transient alterations in ΔAPD_{90} following S2 stimulation close to refractoriness
Effect of regular stimulation at a 125 ms interstimulus interval and of S2 stimulation at an S1S2 interval just greater than the ventricular effective refractory period on the difference between endocardial and epicardial latencies, Δ(latency) and on the difference between endocardial and epicardial action potential duration at 90% repolarization, ΔAPD_{90} after 15 min exposure to normokalaemic (*A*) and hypokalaemic (*B*) test solutions, and normokalaemic (*C*) and hypokalaemic (*D*) test solutions containing lidocaine. Epicardial ΔAPD_{90} on regular stimulation (continuous lines) and on S2 stimulation (triangles), endocardial APD_{90} on regular stimulation (dotted lines) and on S2 stimulation (squares), and ΔAPD_{90} on regular stimulation (dashed lines) and on S2 stimulation (circles). Lines indicate the timing of stimuli.

protocol in which early S2 stimuli were delivered are indicated by triangles, squares and circles, respectively. This demonstrates for the first time transient changes in transmural repolarization gradients following PVDs occurring in response to extrasystolic simulation, taking place in the absence of alterations in latencies.

First, latencies and Δ(latency) were consistent throughout both types of stimulation (Fig. 5, left). Regular stimulation after exposure to normokalaemic test solution initiated MAPs with a 21.0 ± 0.8 ms epicardial and a 19.4 ± 1.2 ms endocardial latency giving a Δ(latency) of -1.6 ± 1.4 ms ($n = 25$, 14 hearts). The latter did not change significantly ($P > 0.05$) after exposure to hypokalaemic test solution ($n = 8$, five hearts), lidocaine-containing normokalaemic ($n = 8$, five hearts) or hypokalaemic ($n = 8$, five hearts) test solutions. Furthermore, the S2 stimulation protocol gave statistically identical MAP latencies following S0–S7 stimulation, all of which were indistinguishable from those obtained on regular stimulation ($P > 0.05$).

Second, a statistical analysis of changes in ΔAPD_{90} (Fig. 5, right) following extrasystolic stimulation confirmed and extended the findings presented in Fig. 4. When normokalaemic hearts were subjected to regular stimulation, epicardial APD_{90} (42.0 ± 3.6 ms) was significantly shorter than endocardial APD_{90} (57.0 ± 4.3 ms, $P < 0.05$) giving a baseline ΔAPD_{90} of 15.0 ± 5.6 ms (Fig. 5*A*, right), all in close agreement with values previously reported in mice (Knollmann *et al.* 2001; Liu *et al.* 2004). Both S0 and S7 stimulation gave similar results, reflecting stable APD_{90} at the beginning and end of each cycle. However, when PVDs were elicited by early S2 stimuli (S1S2 interval $= 45 \pm 6$ ms) both epicardial and endocardial APD_{90} decreased significantly ($P < 0.05$), epicardial APD_{90} falling to 25.7 ± 4.3 ms and endocardial APD_{90} falling to 23.4 ± 9.9 ms, giving a ΔAPD_{90} of -2.3 ± 4.4 ms. This change was accentuated in MAPs following subsequent S3 stimulation, epicardial APD_{90} recovering to its value after S0 stimulation (42.5 ± 4.2 ms) while endocardial APD_{90} remained significantly shortened (26.6 ± 5.3 ms), resulting in the reversal of ΔAPD_{90} to -15.9 ± 6.8 ms ($P < 0.05$). After S4 and subsequent stimulation, all MAP parameters had fully recovered to their steady-state values, as indicated in Fig. 4*A*.

Hypokalaemia accentuated these effects. On regular stimulation, epicardial APD_{90} had increased to 52.4 ± 1.7 ms, not differing significantly from endocardial APD_{90} (55.7 ± 5.3 ms, $P > 0.05$), giving a ΔAPD_{90} of 3.3 ± 5.6 ms (Fig. 5*B*, right). Early S2 stimuli delivered at an S1S2 interval of 35 ± 4 ms resulted in PVDs with reduced epicardial (14.1 ± 3.9 ms) and endocardial APD_{90} values (11.5 ± 5.2 ms), giving a ΔAPD_{90} of -2.7 ± 4.2 ms. In contrast, MAPs obtained in response to subsequent S3 stimulation showed an epicardial APD_{90} that had fully recovered (50.9 ± 10.5 ms). However, endocardial APD90 remained significantly shortened (24.1 ± 4.5 ms), resulting in a significant and negative ΔAPD_{90} (-31.8 ± 11.8 ms, $P < 0.01$). Once again, APD_{90} values and ΔAPD_{90} after S4 and subsequent stimulation had fully recovered, as indicated in Fig. 4*B*. Thus exposure to hypokalaemic test solution significantly increased the magnitude of the negative ΔAPD_{90} obtained on such S3 stimulation, in fitting with the initiation of arrhythmic activity on early S2 stimulation after 20 min hypokalaemia.

These delayed effects of PVDs resulting from early S2 stimulation on ΔAPD_{90} were entirely abolished by exposure to lidocaine. On regular stimulation addition of lidocaine to the normokalaemic test solution resulted in proportionate increases in epicardial (50.3 ± 4.0 ms) and endocardial (54.9 ± 5.2 ms) APD_{90} ($P < 0.05$) and had no effect on ΔAPD_{90} (4.6 ± 5.2 ms, Fig. 5*C*, right). APD_{90} and ΔAPD_{90} values of MAPs were unchanged during the S2 stimulation protocol (S1S2 interval for early S2 stimuli $= 89 \pm 6$ ms). On regular stimulation, addition of lidocaine to the hypokalaemic test solution again increased both epicardial (50.3 ± 4.0 ms) and endocardial (54.9 ± 5.2 ms) APD_{90} but had no effect on ΔAPD_{90} (4.6 ± 5.2 ms, Fig. 5*D*, right). These values remained unchanged during the S2 stimulation protocol (S1S2 interval for early S2 stimulation $= 84 \pm 8$ ms), again compatible with the antiarrhythmic effect of lidocaine in hearts exposed to hypokalaemic test solution for 20 min.

Reversals in ΔAPD_{90} are reflected in altered epicardial and endocardial MAP waveforms

Figure 6 compares in detail Δ(latency) (Fig. 6*Aa–d*, left) and ΔAPD_{90} (Fig. 6*Ba–d*, left) values in hypokalaemic hearts (*b*, $n = 8$, five hearts) where the transient transmural repolarization gradients were at their most prominent with corresponding findings from hearts exposed to normokalaemic (*a*, $n = 25$, 14 hearts), and lidocaine-containing normokalaemic (*c*, $n = 8$, five hearts) and hypokalaemic (*d*, $n = 8$, five hearts) test solutions. Δ(latency) values were neither significantly different from zero nor from each other (Fig. 6*A*, left). In contrast, the asterisk indicates that the ΔAPD_{90} value in hypokalaemic hearts is significantly more negative ($P < 0.05$) than those obtained in each of the remaining cases *a*, *c* and *d*. These latter findings correlate with the comparison of the corresponding epicardial and endocardial MAP waveforms (Fig. 6*A* and *B*, right). Thus the vertical lines pass through not only peak amplitudes of the endocardial MAPs evoked by S1–S4 stimuli, but also those of the epicardial MAPs (Fig. 6*A*, right). In contrast the vertical lines drawn through the APD_{90} values of endocardial MAPs evoked by S3, but not S2 or S4, stimuli transect the epicardial MAPs while the epicardium was still significantly depolarized.

Taken together, these findings thus demonstrate that PVDs occurring in response to early S2 stimuli result in the *transient* reversal of ΔAPD_{90}. The resulting altered transmural repolarization gradients otherwise resemble those described in the LQTS models cited above.

Discussion

Clinical hypokalaemia predisposes to ventricular arrhythmias associated with T-wave inversion and prolongation of the electrocardiographic QT interval reflecting increased ventricular action potential duration (Zipes & Braunwald, 2005). The latter has already been attributed to decreased $[K^+]_o$ reducing repolarizing currents through altered ion channel gating, despite increased outward K^+ gradients that would otherwise have been expected to increase these currents (Yang & Roden, 1996; Killeen *et al.* 2007).

Arrhythmogenicity in the context of QT prolongation has previously been associated with sustained increases in the magnitude of transmural repolarization gradients (Shimizu & Antzelevitch, 1997, 1998; Shimizu *et al.* 1999; Akar *et al.* 2002; Di Diego *et al.* 2003; Milberg *et al.* 2005). Manipulation of such gradients where they occur across the thickness of the myocardium by warming or cooling the epicardium has correspondingly been shown to modify T-wave polarity in dogs (Higuchi & Nakaya, 1984), in fitting with T-wave changes being an important indicator of arrhythmic tendency in humans (Zipes & Braunwald, 2005).

PVDs are implicated in the initiation of arrhythmic activity in a variety of situations including both hypokalaemia (Whelton, 1984) and the congenital LQTS (Noda *et al.* 2004). The present experiments explored the possibility that transient alterations in transmural repolarization gradients following PVDs initiated by extrasystolic (S2) stimuli might be associated with arrhythmogenesis in the hypokalaemic murine heart and further whether the clinically effective antiarrhythmic drug lidocaine (Rehnqvist *et al.* 1984) influenced such gradients. Hearts were studied following exposure times just shorter than those producing frank arrhythmogenesis that would otherwise preclude the quantitative measurement of monophasic action potential parameters.

First, these experiments established that any arrhythmogenicity does not reflect alterations in conduction velocity. This contrasts with findings in a mouse model of the Brugada syndrome where arrhythmogenicity is attributed to slowed conduction (Papadatos *et al.* 2002). Thus the difference between endocardial and epicardial stimulation to depolarization latency (Δ(latency)) did not differ significantly whether hearts were exposed to hypokalaemic test solution,

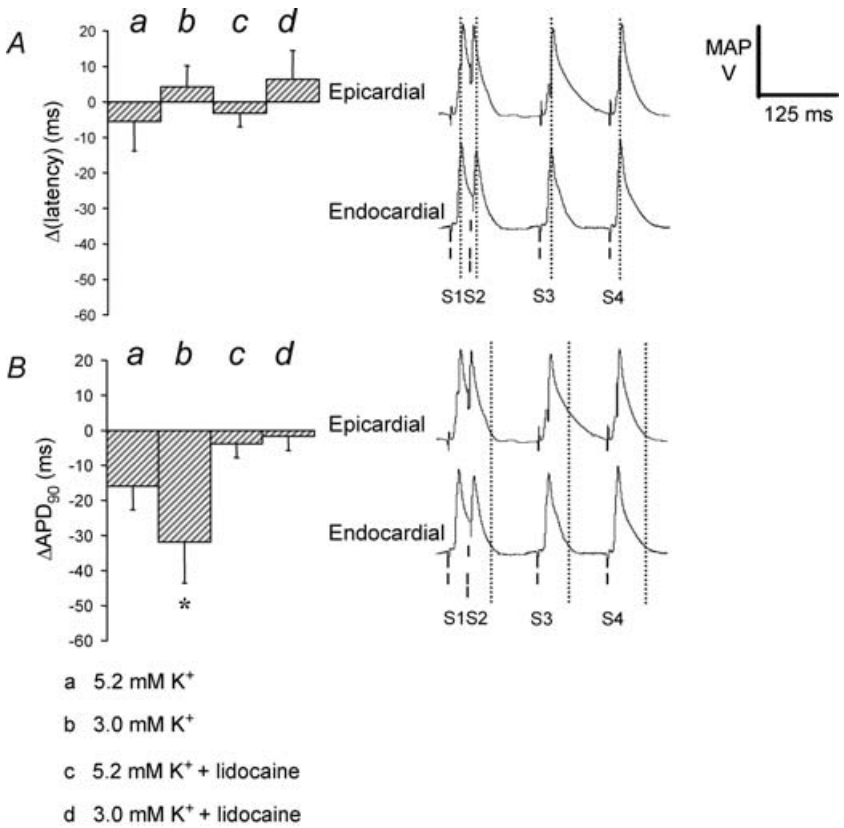

Figure 6. Reversals in ΔAPD_{90} as reflected in altered epicardial and endocardial MAP waveforms
Relationship between changes in the difference between endocardial and epicardial latencies, Δ(latency) (*A*, left) and the difference between endocardial and epicardial action potential durations at 90% repolarization, ΔAPD_{90} (*B*, left) in hearts subjected to S2 stimulation after 15 min exposure to normokalaemic (*a*) and hypokalaemic (*b*) test solutions, and normokalaemic (*c*) and hypokalaemic (*d*) test solutions containing lidocaine, and corresponding MAP waveforms. The asterisk indicates that the value in hearts exposed to hypokalaemic test solution is significantly ($P < 0.05$) more negative than that recorded in hearts exposed to all the other test solutions. In the right-hand panels, lines indicate the timings of S1–S4 stimuli. Vertical dotted lines are drawn through peak endocardial action potential amplitudes (*A*, right) and endocardial ΔAPD_{90} (*B*, right) in a single set of waveforms recorded from a heart exposed to hypokalaemic test solution for 15 min.

lidocaine, or both, when compared to normokalaemic controls.

Second, on regular stimulation under the conditions investigated, hearts did not show the transmural repolarization gradients described in LQTS (Shimizu & Antzelevitch, 1997, 1998; Akar *et al.* 2002; Milberg *et al.* 2005). Thus the difference between endocardial and epicardial depolarization to repolarization time (quantified at 90% repolarization, ΔAPD_{90}) was not statistically different from normokalaemic hearts.

Third, PVDs initiated by S2 stimuli delivered soon after recovered from refractoriness were shorter than action potentials evoked by regular stimuli. The possibility that these events rather represented motion artefacts is unlikely since they were reproducible between hearts, appeared identical in both electrodes and similar findings have been reported in previous studies (reviewed in Franz, 1999). This has previously been explained in terms of a combination of reduced inward currents owning to incomplete recovery of L-type Ca^{2+} channels (Sun *et al.* 1997), reduced Ca^{2+}-induced-Ca^{2+}-release from sarcoplasmic recticular stores (Isenberg & Han, 1994) and decreased currents through the Na^+–Ca^{2+} exchanger (Janvier *et al.* 1997). Regional heterogeneities in the degree of action potential shortening resulted in alterations in transmural repolarization gradients, reflected in the negative ΔAPD_{90} seen specifically in hypokalaemic hearts. This extends to isolated rodent hearts findings from canine heart preparations (Litovsky & Antzelevitch, 1989) and further correlates these with the initiation of arrhythmic activity observed after longer exposure times. In addition the finding that ΔAPD_{90} became most negative when PVDs were initiated by S2 stimuli delivered immediately after recovery from refractoriness is in fitting with the especially arrhythmogenic effect of PVDs which interrupt electrocardiographic R waves reported elsewhere (Smirk, 1949; Naito *et al.* 1982; Kiryu *et al.* 1999). In hypokalaemic hearts PVDs could be elicited by S2 stimuli delivered at shorter S1S2 intervals than in normokalaemic hearts. As predicted by the Nernst equation, experimental work confirms that decreasing extracellular $[K^+]$ hyperpolarizes feline ventricular myocyte membranes (Sperelakis *et al.* 1970). Such hyperpolarization increases the proportion of Na^+ channels available for activation in guinea pig ventricular myocytes (Gettes & Reuter, 1974), potentially explaining this finding.

Fourth, the present study went on to demonstrate for the first time in the intact murine heart that PVDs result in changes in the duration of subsequent (S3-evoked) action potentials. This observation has previously been reported in excised canine papillary muscle (Bass, 1975) and human ventricular trabeculae (Schouten *et al.* 1990) as a part of the phenomenon of *postextrasystolic potentiation*. In the ventricular trabeculae of the ferret such changes have been attributed to changes in the diastolic interval preceding the initiation of the S3-evoked action potential modifying Ca^{2+}-induced Ca^{2+} release with consequences for inward Na^+–Ca^{2+} exchanger activity and therefore action potential duration (Urthaler *et al.* 1994). Differences in Ca^{2+} handling between murine epicardial and endocardial myocytes may thus contribute to changes in ΔAPD_{90} following S3 stimulation (Dilly *et al.* 2006). Ultimately responses to S3 stimuli unmasked significant alterations in ΔAPD_{90}.

Fifth, exposure to lidocaine abolished all changes in ΔAPD_{90} resulting from PVDs, correlating with its anti-arrhythmic effect both in these preparations and in clinical situations (Rehnqvist *et al.* 1984). This effect of Na^+ channel block is in fitting with a previous report that increases in action potential duration in response to S3 stimulation are attenuated by reduction of extracellular $[Na^+]$ (Schouten *et al.* 1990). Finally this is emphasized by comparison of ΔAPD_{90} values with corresponding MAP waveforms.

Taken together, these findings suggest that PVDs initiated by S2 stimuli result in transient changes in transmural repolarization gradients in association with the initiation of arrhythmic activity in hypokalaemic murine hearts. During such arrhythmia the consequent rapid succession of depolarizations may then replicate further PVDs, contributing to its maintenance. At the very least, the possibility that transient changes in transmural repolarization gradients following PVDs might also be associated with arrhythmogenicity in LQTS now merits investigation.

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