Gap junction inhibition by heptanol increases ventricular arrhythmogenicity by reducing conduction velocity without affecting repolarization properties or myocardial refractoriness in Langendorff-perfused mouse hearts

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Abstract. In the current study, arrhythmogenic effects of the gap junction inhibitor heptanol (0.05 mM) were examined in Langendorff-perfused mouse hearts. Monophasic action potential recordings were obtained from the left ventricular epicardium during right ventricular pacing. Regular activity was observed both prior and subsequent to application of heptanol in all of the 12 hearts studied during 8 Hz pacing. By contrast, induced ventricular tachycardia (VT) was observed after heptanol treatment in 6/12 hearts using a S1S2 protocol (Fisher's exact test; P<0.05). The arrhythmogenic effects of heptanol were associated with increased activation latencies from 13.2±0.6 to 19.4±1.3 msec (analysis of variance; P<0.001) and reduced conduction velocities (CVs) from 0.23±0.01 to 0.16±0.01 msec (analysis of variance; P<0.001) in an absence of alterations in action potential durations (ADPs) at x=90% (38.0±1.0 vs. 38.3±1.8 msec), 70% (16.8±1.0 vs. 19.5±0.9 msec), 50% (9.2±0.8 vs. 10.1±0.6 msec) or 30% (4.8±0.5 vs. 6.3±0.6 msec) repolarization (APD_x) or in effective refractory period (ERPs) (39.6±1.9 vs. 40.6±3.0 msec) (all P>0.05). Consequently, excitation wavelengths (λ; CV x ERP) were reduced from 9.1±0.6 to 6.5±0.6 mm (P<0.01), however critical intervals for re-excitation (APD_{90} - ERP) were unaltered (-1.1±2.4 vs. -2.3±1.8 msec; P>0.05). Together, these observations demonstrate for the first time, to the best of our knowledge, that inhibition of gap junctions alone using a low heptanol concentration (0.05 mM) was able to reduce CV, which alone was sufficient to permit the induction of VT using premature stimulation by reducing λ, which therefore appears central in the determination of arrhythmic tendency.

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Introduction

An orderly spread of action potentials through the heart is critical for normal electrical function and its disruption can lead to cardiac arrhythmias (1). Experiments in pre-clinical models have advanced understanding of the electrophysiological mechanisms underlying arrhythmogenesis using genetic and pharmacological approaches (1-24). Experiments in mouse models have highlighted the role of gap junctions in ventricular conduction and arrhythmogenesis, however the results have been controversial. Heterozygous $Cx43^{+/-}$ mice were demonstrated to exhibit at 45-50% reduction in Cx43 expression, however, the degree of conduction velocity (CV) slowing was variable: CV was either unchanged (25-30) or reduced by 23-44% (31-33). Additional experiments used a pharmacological approach, demonstrating ventricular arrhythmogenesis associated with reduced CV using 2 mM heptanol (7). This agent inhibits gap junctions specifically at concentrations up to 1-2 mM (34,35) however at ≥2 mM additionally inhibits sodium channels (34,36). The extent to which the conduction defects and arrhythmogenesis observed could be attributed to loss of gap junction coupling alone remains to be fully elucidated.

Therefore, the aims of the present study were to examine the possible role of abnormal gap junction function in ventricular arrhythmogenesis, by applying heptanol at a low concentration that specifically targets gap junctions (0.05 mM). At this concentration, it was identified that heptanol did not elicit spontaneous arrhythmias during regular pacing, however increased the incidence of ventricular tachycardia induced by a S1S2 protocol. This was associated with increased activation latencies in an absence of alterations in either action potential durations (APDs) or effective refractory periods (ERPs). The observations of the present study suggest that loss of gap

junction function alone is sufficient to produce conduction slowing and ventricular arrhythmogenesis.

Materials and methods

Solutions. The experiments described in this study used Krebs-Henseleit solution (composition in 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 7.4) that had been bicarbonate-buffered and bubbled with 95% O_2/5% CO_2 (37). Heptanol (0.82 g ml^{-1}; Sigma-Aldrich, Haverhill, UK) is soluble in aqueous solutions up to 9 mM (https://www.rsc.org/merck-index), and was diluted using Krebs-Henseleit solution to produce a final concentration of 0.05 mM.

Preparation of Langendorff-perfused mouse hearts. A total of 13 wild-type mice of genetic background 129 (5 and 7 months of age, 3 male, 10 female; weight, 39.2±1.9 g) were used in the current study. The animals were maintained at room temperature (21±1˚C) and were subjected to a 12:12 h light/dark cycle with free access to sterile rodent chow and water in an animal facility. The experiments described here were compliant with the UK Animals (Scientific Procedures) Act 1986. The present study was approved by the Animal Welfare and Ethical Review Body at University of Cambridge. (Cambridge, UK) The procedures for the preparation of Langendorff-perfused mouse hearts were as follows: Mice were sacrificed by cervical dislocation in accordance with Sections 1 (c) and 2 of Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The hearts were rapidly excised and immediately submerged in ice-cold Krebs-Henseleit solution. Cannulation of the aorta was achieved using a tailor-made 21-gauge cannula that had been prefilled with ice-cold buffer. Using a micro-aneurysm clip (Harvard Apparatus, Cambridge, UK), the heart was securely attached to the perfusion system. Retrograde perfusion was initiated at a rate of 2-2.5 ml min^{-1} using a peristaltic pump (Model 505S Bredel Pump; Watson-Marlow, Ltd., Falmouth, UK) with the perfusate passing through 200 and 5 μm filters successively, and heated to 37˚C using a water jacket and circulator prior to reaching the aorta. The hearts that regained their pink colour and spontaneous rhythmic activity were studied further (approximately 90%). The remaining 10% were discarded. Perfusion took place for a further 20 min to minimise any residual effects of catecholamine released endogenously, prior to electrophysiological analysis of the hearts.

Stimulation protocols. Electrical stimulation was achieved using paired platinum electrodes (1 mm interpole distance) placed at the right ventricular epicardium. This took place at 8 Hz, using square wave pulses 2 msec in duration, with a stimulation voltage set to three times the diastolic threshold (Grass S48 Square Pulse Stimulator; Grass-Telefactor; Astro Med, Inc., Slough, UK) immediately subsequent to the start of perfusion. The S1S2 protocol was used to assess arrhythmogenicity and identify re-entrant substrates. This consisted of a drive train of 8 regularly paced S1 stimuli separated by a 125 msec basic cycle length (BCL), followed by premature S2 extra stimuli every ninth stimulus. The S1S2 interval was first set to 125 msec and then successively reduced by 1 msec with each nine stimulus cycles until arrhythmic activity was initiated or refractoriness was reached, whereupon the S2 stimulus elicited no ventricular response.

Recording procedures. Monophasic action potential (MAP) recordings from the left ventricular epicardium were obtained using a MAP electrode (Linton Instruments; Harvard Apparatus). MAPs from the left ventricular endocardium were obtained using a custom-made MAP electrode that was made from two strands of 0.25 mm Teflon-coated silver wire (99.99% purity; Advent Research Materials, Ltd., Witney, UK). To eliminate direct current offset, the electrode tips were galvanically chlorided. The stimulating and recording electrodes were maintained at constant positions, with an inter-electrode distance of 3 mm. This allowed CVs to be determined from the activation latencies. All recordings were performed using a BCL of 125 msec (8 Hz) to exclude rate-dependent differences in APDs. MAPs were pre-amplified using a NL100AK head stage, amplified with a NL104A amplifier and band pass filtered between 0.5 Hz and 1 kHz using a NL125/6 filter (Neurolog; Digitimer, Ltd., Welwyn Garden City, UK) and then digitized (1401plus MKII; Cambridge Electronic Design, Ltd., Cambridge, UK) at 5 kHz. They were then analysed using Spike2 version 5.11 software (Cambridge Electronic Design, Ltd.). MAP waveforms that did not match the previous established stringent criteria for MAP signals were rejected (38). They must have stable baselines, fast upstrokes, with no inflections or negative spikes, and a rapid first phase of repolarization. 0% repolarization was measured at the peak of the MAP and 100% repolarization was measured at the point of return of the potential to baseline (38-40).

The following parameters were obtained from the experimental records: i) Activation latency, defined as the time difference between the stimulus and the peak of the MAP; ii) CV, as the ratio of the inter-electrode distance to the activation latency. As the latter distance was kept constant, CVs were inversely proportional to the corresponding activation latencies; iii) APD_x, the time difference between the peak of the MAP and x=30, 50, 70 and 90% repolarization; iv) ERP, defined as the longest S1S2 interval at which the S2 extra stimulus failed to initiate a ventricular signal during programmed electrical stimulation; v) excitation wavelength, λ, given by CV x ERP; vi) critical intervals for re-excitation given by APD_{90} - ERP.

Statistical analysis. All values are expressed as the mean ± standard error. Categorical data were compared with Fisher's exact test (one-tailed) using OriginPro version 8 (OriginLab Corporation, Northampton, MA, USA). Different experimental groups were compared by one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference. $P<0.05$, 0.01 and 0.001 were denoted by *, ** and ***, respectively.

Results

Ventricular arrhythmogenicity and its association with action potential activation and recovery properties were examined prior and subsequent to introduction of 0.05 mM heptanol in Langendorff-perfused mouse hearts. The right ventricular

epicardium was electrically stimulated using either regular 8 Hz or S1S2 pacing (2,3,5,7,12). MAP recordings were obtained from the left ventricular epicardium. The stimulating and recording electrodes were maintained at a constant distance of 3 mm, which permitted CVs to be estimated from the respective activation latencies. Ventricular tachycardia (VT) was defined as a series of ≥5 action potentials with coupling intervals closer than the BCL.

Heptanol at 0.05 mM exerts ventricular pro-arrhythmic effects during S1S2, however not during regular pacing. The initial experiments conducted during regular pacing demonstrated consistent ventricular activity in the absence of spontaneous arrhythmias in all of the 12 hearts studied, whether prior or subsequent to introduction of 0.05 mM heptanol, or following removal of heptanol from the perfusate (Fig. 1). The second set of experiments then applied a S1S2 pacing protocol, which imposed extra systolic S2 stimuli following trains of regular S1 pacing stimuli. The S1S2 interval was initially at the BCL and subsequently reduced by 1 msec with each cycle until the S2 stimuli produced either arrhythmic activity or refractoriness. The latter indicating that the ERP was reached. None of the hearts studied demonstrated evidence of inducible arrhythmias prior to application of the test agent (Fig. 2A). By contrast, it was possible to induce VT subsequent to application of heptanol (Fig. 2B). The incidences of inducible VT prior and subsequent to introduction of heptanol, and following its withdrawal from the perfusing solution are summarized in Fig. 2C, indicating that heptanol exerted significant pro-arrhythmic effects, as the extra stimuli were able to induce VT in 6/12 hearts ($^{*}P<0.05$; Fisher's exact test).

Pro-arrhythmic effects of heptanol were associated with reduced CVs in an absence of alterations in APDs or ERPs. Previous studies in mouse models have associated increased arrhythmogenicity with reduced CVs, prolonged or shortened APDs and reduce ERPs (2,3,5,7,12). These values were therefore obtained from the experimental recordings described above. Thus, heptanol increased activation latencies from 13.2±0.6 to 19.4±1.3 msec (Fig. 3A; analysis of variance; P<0.001) and reduced CVs from 0.23±0.01 to 0.16±0.01 msec (Fig. 3B; P<0.001), without altering APD_{90} (38.0±1.0 vs. 38.3±1.8 msec; Fig. 3C), APD_{70} (16.8±1.0 vs. 19.5±0.9 msec; Fig. 3D), APD_{50} (9.2±0.8 vs. 10.1±0.6 msec; Fig. 3E), APD_{30} (4.8±0.5 vs. 6.3±0.6 msec; Fig. 3F) or ERPs (39.6±1.9 vs. 40.6±3.0 msec; Fig. 3G).

Pro-arrhythmic effects of heptanol were associated with reduced excitation wavelengths despite unaltered critical intervals. Reductions in excitation wavelengths (λ; CV x ERP) and increases in critical intervals for re-excitation (APD_{90} - ERP) have been associated with increased arrhythmogenicity (7,41). Accordingly, these parameters were calculated for the hearts used in the current study. Heptanol reduced λ from 9.1±0.6 to 6.5±0.6 mm (Fig. 4A; P<0.01) without altering critical intervals (-1.1±2.4 vs. -2.3±1.8 msec; Fig. 4B).

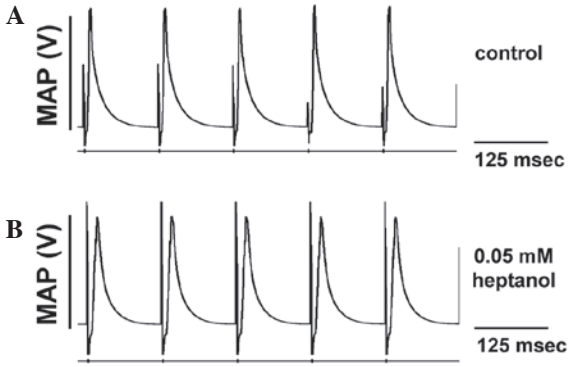

Figure 1. Stable MAPs obtained during regular 8 Hz pacing (A) prior to and (B) subsequent to introduction of 0.05 mM heptanol. MAP, monophasic action potential.

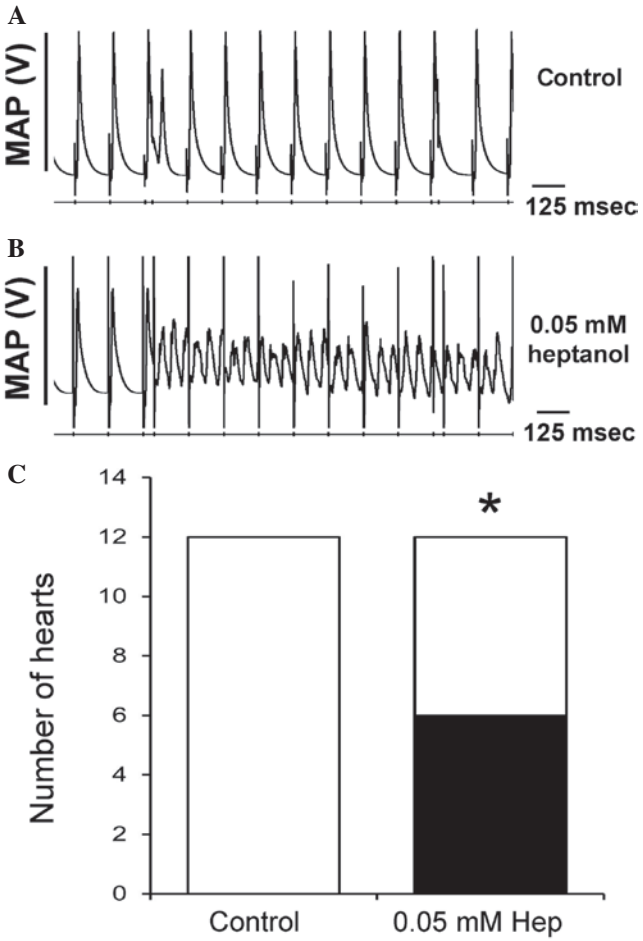

Figure 2. (A) Refractory outcomes observed prior to the introduction of the test agent and (B) the induced ventricular tachycardia following introduction of 0.05 mM Hep during S1S2 pacing. (C) Number of hearts showing refractory (clear bar) or arrhythmic (black bar) outcomes. $^{*}P<0.05$ vs. control. Hep, heptanol; MAP, monophasic action potential.

Discussion

Sudden cardiac death (SCD) is a significant problem and is responsible for around 60,000 deaths in the UK (42), 200,000 deaths in the US (43) and 4-5 million deaths globally (44) per year. It has been suggested that SCD arises from the development of malignant ventricular arrhythmias, the electrophysiological mechanisms of which remain to be fully understood. Mouse hearts have been used to study arrhythmogenesis as they are amenable to both genetic and pharmacological manipulation.

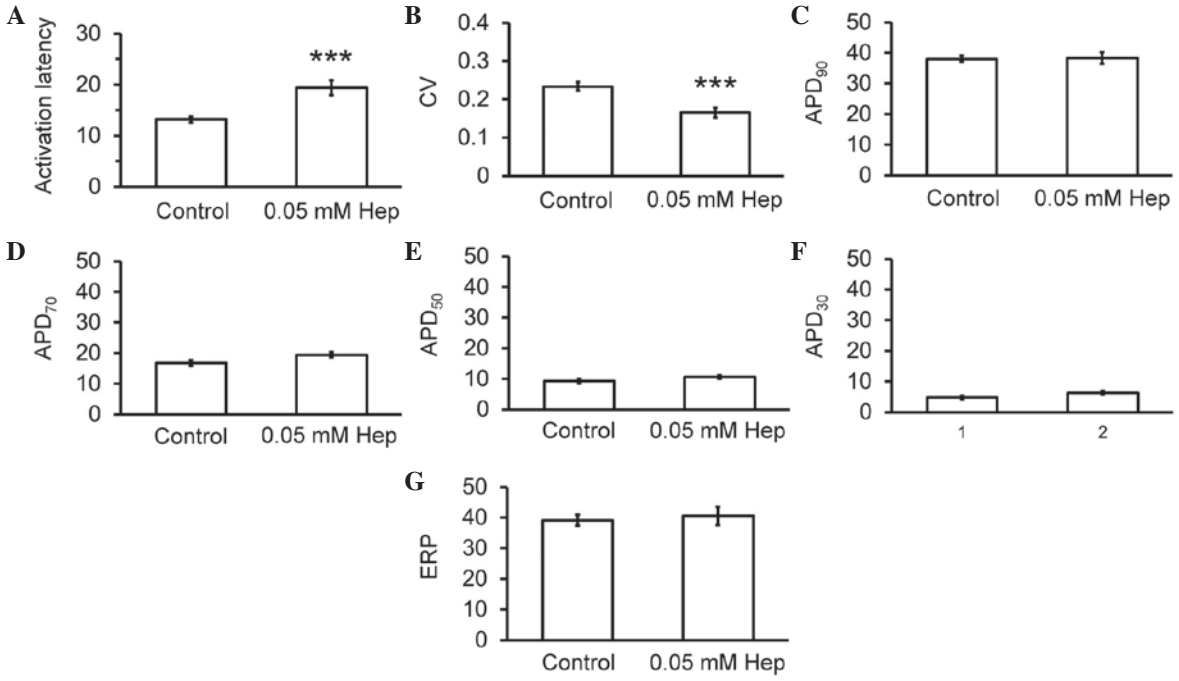

Figure 3. Hep (0.05 mM) increased (A) activation latencies and (B) reduced CV values without affecting APD values at (C) 90%, (D) 70%, (E) 50% or (F) 30% repolarization (APD_x), or (G) ERPs. ***P<0.001; Hep, heptanol; CV, conduction velocity; APD, action potential duration; ERP, effective refractory period.

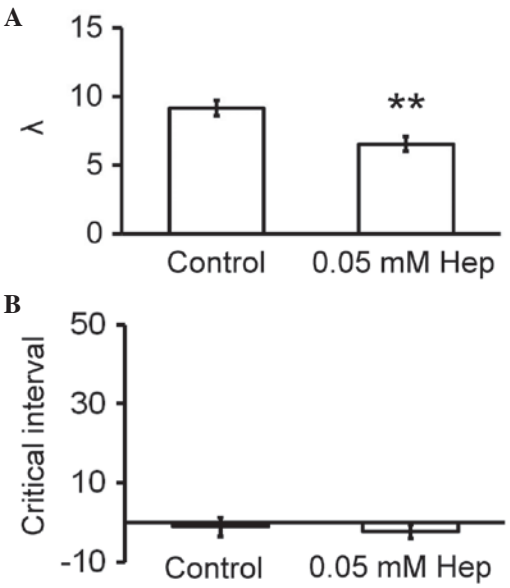

Figure 4. Hep (0.05 mM) (A) reduced excitation wavelengths (λ; CV x ERP), (B) without altering critical intervals for re-excitation (CI, APD_{90} - ERP). **P<0.01 vs. control. Hep, heptanol; CV, conduction velocity; ERP, effective refractory period.

Propagation of the action potentials through the working myocardium depends on sodium channel activation followed by gap junction conduction. Gap junctions are hexameric proteins made of connexins mediate intercellular coupling by allowing passive electrotonic spread of ions and of larger molecules (45). Their resistance contributes to axial resistance and modulates CV (46,47). Cx43 is the isoform present in ventricles, and the effects of loss of Cx43 on ventricular conduction and arrhythmogenesis have been extensively studied in mouse models (25-33,48,49), however, with significant disagreement between the results of these studies (28). Thus, cardiac-restricted Cx43 inactivation followed by crossing with Cre recombinase produced mosaic mice, in which Cx43 was observed to be educed by up to 95% when compared with wild-type mice (48). Additional experiments identified that heterozygous $Cx43^{+/-}$ mice exhibited a 45-50% reduction in Cx43 expression. In these mice, CV was either unchanged (25-30) or reduced by 23-44% (31-33). These studies suggest different parameters, including interstitial volume (50), width of the perinexus, intracellular calcium concentrations, perfusate composition and osmolarity (28), have additional effects on cardiac conduction. Pharmacological methods have additionally been used to study the role of gap junctions in arrhythmogenesis. A previous study reported that 2 mM heptanol exerted significant pro-arrhythmic effects by reducing CVs without influencing APDs, however increased ERPs (7). These alterations led to reduced excitation wavelength (λ; CV x ERP), which is consistent with the increased likelihood of re-entry. Heptanol is an agent that reversibly inhibits gap junctions at concentrations up to 1 mM and also sodium channels at concentrations ≥2 mM (34,36). It was therefore not possible to determine the relative contributions of gap junction uncoupling vs. reduced sodium channel function in the reduction of CV and the ventricular arrhythmogenesis observed. Furthermore, 2 mM heptanol produced not only CV slowing, however additionally increased ERPs. The latter observation is consistent with its effects on sodium channel kinetics of producing a depolarizing shift of the activation curve, and a hyperpolarizing shift of the inactivation curve, which would reduce the sodium window current (36). Increasing ERP alone is suggested to be anti-arrhythmic via the increase in λ, regional increases in ERP could theoretically predispose to re-entry by producing refractory obstacles

around which action potentials can circulate, and areas of unidirectional conduction block (51).

Therefore, the present experiments were conducted to determine whether heptanol at a concentration that specifically inhibits gap junctions (0.05 mM) (34,36) could produce pro-arrhythmic effects. Its application resulted in an increased incidence of inducible, however not spontaneous, arrhythmias, which was associated with increased activation latencies and reduced CVs, in an absence of alterations in APDs or ERPs. Together, these alterations led to a reduced excitation wavelength (λ) despite leaving critical intervals unaltered. These results are consistent with previous observations that inhibition of gap junctions and sodium channels at 2 mM heptanol resulted in a greater degree of CV slowing compared with the low concentration used in the current study, and increased ERPs. Under these conditions, spontaneous and provoked VT were observed. In the present study, gap junction inhibition alone using 0.05 mM heptanol did not elicit spontaneous VT during regular pacing.

As the aim of this study was to examine the effects of reducing gap junction coupling, it was therefore appropriate to use the MAP method. This method has been extensively used to study cardiac electrophysiology in animal systems (8,52-59). For future experiments, the measurement of magnetic signals may be beneficial. It has been previously demonstrated to useful for characterizing cardiac structural abnormalities (60-62), and observed that functional mapping could be achieved using magnetocardiography in mouse models. Thus, it is suggested that its use in assessing abnormal cardiac electrophysiology in mice warrants future investigation (63-66).

In conclusion, the current study demonstrated that gap junction inhibition by heptanol alone was sufficient to reduce CV without affecting APD or ERP, and the consequent reduction in λ was suggested to be responsible for the arrhythmogenesis observed.

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