Prolongation of minimal action potential duration in sustained fibrillation decreases complexity by transient destabilization

Brian O. Bingen1, Saïd F.A. Askar1, Martin J. Schalij1, Ivan V. Kazbanov2, Dirk L. Ypey1, Alexander V. Panfilov2, and Daniël A. Pijnappels1*

1Laboratory of Experimental Cardiology, Department of Cardiology, Leiden University Medical Center, P.O. Box 9600, Leiden 2300 RC, The Netherlands; and 2Department of Physics and Astronomy, Ghent University, Ghent, Belgium

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Aims
Sustained ventricular fibrillation (VF) is maintained by multiple stable rotors. Destabilization of sustained VF could be beneficial by affecting VF complexity (defined by the number of rotors). However, underlying mechanisms affecting VF stability are poorly understood. Therefore, the aim of this study was to correlate changes in arrhythmia complexity with changes in specific electrophysiological parameters, allowing a search for novel factors and underlying mechanisms affecting stability of sustained VF.

Methods and results
Neonatal rat ventricular cardiomyocyte monolayers and Langendorff-perfused adult rat hearts were exposed to increasing dosages of the gap junctional uncoupler 2-aminoethoxydiphenyl borate (2-APB) to induce arrhythmias. Ion channel blockers/openers were added to study effects on VF stability. Electrophysiological parameters were assessed by optical mapping and patch-clamp techniques. Arrhythmia complexity in cardiomyocyte cultures increased with increasing dosages of 2-APB (n > 38), leading to sustained VF: 0.0 ± 0.1 phase singularities/cm² in controls vs. 0.0 ± 0.1, 1.0 ± 0.9, 3.3 ± 3.2, 11.0 ± 10.1, and 54.3 ± 21.7 in 5, 10, 15, 20, and 25 μmol/L 2-APB, respectively.

Arrhythmia complexity inversely correlated with wavelength. Lengthening of wavelength during fibrillation could only be induced by agents (BaCl2/BayK8644) increasing the action potential duration (APD) at maximal activation frequencies (minimal APD); 123 ± 32%/117 ± 24% of control. Minimal APD prolongation led to transient VF destabilization, shown by critical wavefront collision leading to rotor termination, followed by significant decreases in VF complexity and activation frequency (52%/37%). These key findings were reproduced ex vivo in rat hearts (n = 6 per group).

Conclusion
These results show that stability of sustained fibrillation is regulated by minimal APD. Minimal APD prolongation leads to transient destabilization of fibrillation, ultimately decreasing VF complexity, thereby providing novel insights into anti-fibrillatory mechanisms.

Keywords
Ventricular fibrillation • Action potential duration • Gap junctions • Optical mapping • In vitro and ex vivo

1. Introduction
Ventricular fibrillation (VF) is the most common cause of sudden cardiac death.1 Treatment of VF has vastly improved over the past years, mainly through progress in engineering strategies that resulted in defibrillating devices. However, while defibrillators can have a significant effect on survival, the majority of VF victims are not defibrillator candidates and at least 50% have VF as their first symptom of heart problems.3 This is indicative of an unabated need to expand the current understanding of mechanisms underlying VF stability and termination.

One of the factors which can underpin the initiation of VF is gap junction remodelling. It is widely accepted that gap junctions are redistributed or downregulated following myocardial infarction, in cardiac

* Corresponding author. Tel: +31 71 7526 2020; fax: +31 71 7526 6809. Email: d.a.pijnappels@lumc.nl

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hypertrophy, and other causes of cardiomyopathy.\textsuperscript{3} Such reorganization of gap junctions is associated with the onset of malignant ventricular tacharyrhythmias.\textsuperscript{3–5}

After initiation, VF progresses through several distinct activation pattern phases, of which the hindmost are characterized by a reduction in the number of new rotor formations, reduced rotor meandering, and increased spatiotemporal periodicity,\textsuperscript{6–10} leading to a more organized and stable form of fibrillation. Affecting the stability of sustained VF may lead to a lower complexity of VF (estimated by the number of phase singularities per cm\textsuperscript{2}), but the underlying mechanisms are poorly understood. Traditionally, the fibrillatory aspect of conduction as well as arrhythmia complexity during early VF is believed to be determined by conduction velocity (CV), action potential duration (APD), APD restitution slope, and wavelength (the product of CV and APD).\textsuperscript{11–13} However, considering the distinctive VF activation patterns, the importance of these factors could differ significantly during the different phases of VF. Furthermore, while data on the first phases after VF initiation are abundant,\textsuperscript{11,14–16} data on sustained VF are scarce.\textsuperscript{5} Therefore, a new in vitro and ex vivo model of sustained VF was developed that enabled to correlate a systematic and controllable increase in arrhythmia complexity with changes in specific electrophysiological parameters. Subsequent pharmacological modification of key parameters was used to search for novel factors affecting the stability of sustained VF and thereby unravel the underlying anti-fibrillatory mechanisms.

2. Methods

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conform to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

A more detailed description can be found in the Supplementary material online.

2.1 Cell isolation and culture

Neonatal rat ventricular myocytes were isolated by collagenase digestion as described previously.\textsuperscript{17} Animals were anaesthetized with 4–5% isoflurane inhalation anaesthesia. Adequate anaesthesia was assured by the absence of reflexes prior to rapid heart excision. Ventricles were minced and digested using collagenase (Worthington, Lakewood, NJ, USA) and DNase (Sigma-Aldrich, St Louis, MO, USA). After isolation, cells were plated out isotropically on fibronectin-coated, round glass cover slips (15 mm) at a cell density of 2–8 × 10\textsuperscript{5} cells/well in 24-well plates (Corning Life Sciences, Amsterdam, the Netherlands). To prevent overgrowth of remaining cardiac fibroblasts, proliferation was inhibited by Mitomycin-C (Sigma-Aldrich) treatment at Day 1, as described previously.\textsuperscript{17} All cultures were refreshed daily with DMEM/HAM’s F10 in a 1:1 mixture with 5% HS and cultured in a humidified incubator at 37°C and 5% CO\textsubscript{2}.

2.2 Immunocytochemical analyses

Cultures were stained for Connexin40 (Sigma-Aldrich), Connexin43 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and Connexin45 (Santa Cruz Biotechnologies) to assess the presence of gap junctional proteins, and for active caspase-3 (Abcam, Cambridge, MA, USA) to assess the number of apoptotic cells. Images of cultures were taken and quantified using dedicated software (Image), National Institutes of Health, USA).

2.3 Optical mapping of myocardial cultures

At Day 4 of culture, propagation of action potentials was investigated on a whole-culture scale by optical mapping using di-4-anePES (Sigma-Aldrich) as voltage sensitive dye, as described previously.\textsuperscript{17} Cells were incubated with 2-APB in five different concentrations (5, 10, 15, 20, and 25 μM) for 20 ± 2 min, targeting Connexin43, Connexin45, and Connexin40\textsuperscript{18,19} to induce arrhythmias of increasing complexity, while vehicle-treated cultures were used as controls. Data analysis, construction of activation maps, and stripe analysis (e.g. plotting of optical signal amplitude against time, at the maximal diameter of a culture, or short and long axis of whole heart) were performed with specialized software (Brainvision Analyze 1101, Brainvision Inc., Tokyo, Japan) after pixel signals were averaged with eight of its nearest neighbours, minimizing noise-artefacts. CV in cultures with uniform or re-entrant activation patterns was calculated perpendicular to the activation wavefront, between two 3 by 3 pixel grids typically spaced 2–8 mm apart. CV, activation frequency, minimal APD (during maximal paced activation frequency), and 1 Hz APD were determined at six different locations equally distributed throughout the culture and averaged before further analysis. APD was determined at 80% of repolarization (APD\textsubscript{80}) because of the rat action potential shape. Wavelength was calculated by the product of average CV and an APD\textsubscript{80} (for uniform propagation) or re-entrant cycle length.\textsuperscript{15} Arrhythmia complexity was defined as the number of phase singularities per cm\textsuperscript{2}, determined by using the phase space method\textsuperscript{20} and correlated with CV, APD\textsubscript{80}, and wavelength in order to identify potential targets that can be modified to affect VF stability and thereby reduce VF complexity. As a result of the outcome of this correlation, appropriate drugs [3 μmol/L nitrendipine (Sigma-Aldrich), 1 mmol/L sotalol (Sigma-Aldrich), 0.5 mmol/L BaCl\textsubscript{2} (Merck, Darmstadt, Germany), or 1 μmol/L BayK8644 (Sigma-Aldrich)] were administered to 2-APB treated and control cultures to modify these targets and study the effects on arrhythmia complexity.

2.4 Assessment of arrhythmia complexity

Arrhythmia complexity in cardiomyocyte cultures was defined as the number of phase singularities per cm\textsuperscript{2}. To quantify the number of phase singularities, the phase space approach was used.\textsuperscript{20} Time series analyses using the empirical mode decomposition method and the Hilbert transform were used to determine the phase.\textsuperscript{21} Data were smoothed by Gaussian filtering with a spatial size of 5 pixels (~0.75 mm) and a temporal size of five frames (30 ms). Subsequently, a discrete Fourier transform was performed for each pixel over the full time series. By using the Fourier spectra, the dominant frequency was determined. After that a band-pass filter was applied to each time series removing low and high frequencies. Next, the local extrema for the time series were found using a half of the inverse dominant frequency as the window size. Local maxima (and local minima) were interconnected by a piece-wise linear curve and their mean curve was subtracted from the corresponding time series. Then by using the Hilbert transform of the resulting time series, the phase \(\varphi_j(t)\) for every pixel \((i,j)\) at the moment of time \(t\) was determined as

\[
\varphi_j(t) = \arctan\left(\frac{2\text{Im}[H]\left(I_i(t)\right)}{1+\text{Re}[H]\left(I_i(t)\right)}\right)
\]

where \(I_i(t)\) is the filtered and detrended intensity of the optical mapping signal for the pixel \((i,j)\) at the moment of time \(t\) and \(H\) denotes the Hilbert transform.

The topological charge of the area \(\Omega\) is determined by

\[
n(\Omega) = \frac{1}{2\pi} \oint_{\partial \Omega} \text{grad} \varphi \, dl
\]

where the integral is taken along the oriented boundary of \(\Omega\). For each pixel \((i,j)\), we defined \(\Omega_j\) as a square around it with the side of
3 pixels. Thus the integral was approximated by the sum of nine finite differences.

\[ n(W) = \frac{1}{M} \sum_{i=1}^{M} \left[ (\Phi_{i,j-1} - \Phi_{i,j+1}) + (\Phi_{i-1,j} - \Phi_{i+1,j}) + \cdots + (\Phi_{i-1,j-1} - \Phi_{i-1,j+1}) \right] \]

The overall algorithm was implemented in the OCaml programming language using the GTK+ toolkit for visualization.

Meandering of the phase singularities was defined as the maximal straight distance covered by the same phase singularity within 6 s of optical mapping.

### 2.5 Whole-cell patch-clamp

Whole-cell measurements were performed in spontaneously active cultures plated out in a density of \(4 \times 10^5\) cells/well in 24-well plates as described previously. At Day 4 of culture, current-clamp experiments were performed in CMCs at 25°C using an L/M-PC patch-clamp amplifier (3 kHz filtering) (List-Medical, Darmstadt, Germany). To study the effects of 2-APB on electrophysiological properties of CMCs, 25 \(\mu M\) 2-APB (Tocris Bioscience, Bristol, UK) was incubated for 20 min prior to measurements.

### 2.6 Ex vivo experiments

For ex vivo experiments, female adult Wistar rats of 6 ± 3 months were anesthetized through inhalation of 3–5% isoflurane and received 400 IE of heparin intraperitoneally. After confirmation of adequate anesthesia by absence of pain reflexes, beating hearts were rapidly excised and immediately submerged in cold Tyrode solution comprising (in mM) NaCl 130, CaCl\(_2\) 1.8, KCl 4.0, MgCl\(_2\) 1.0, NaHPO\(_4\) 1.2, NaHCO\(_3\) 24, and glucose 5.5 at pH 7.4. Subsequently, the aorta was cannulated and retrogradely perfused with Tyrode that was freshly oxygenated with carbogen (95% O\(_2\), 5% CO\(_2\)) and supplemented with 20 mM of 2,3-butanediol monoxime (BDM) to reduce motion artefacts, at a constant flow of 15 ± 2 mL/min at 37°C using a modified Langendorff apparatus (AD instruments, Spechbach, Germany). Hearts were stained with 2 \(\mu M\) di-4-anepp by a 10 mL bolus injection into the bubble trap. The optical mapping camera was positioned facing the ventral surface of the heart, viewing equal portions of the left and the right ventricle during mapping. All hearts exhibited spontaneous sinus rhythm during initial acclimatization. Arrhythmia complexity was defined as the number of separate wavefronts present at the epicardial surface of the heart. The targets that were shown to affect arrhythmia complexity in vitro were modified by administration of 0.5 \(\mu M\) BaCl\(_2\) to the perfusate for 10 min prior to measurements to confirm their ex vivo effects on arrhythmia complexity.

### 2.7 Statistical analysis

Statistical analyses were performed using SPSS11.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were compared with one-way ANOVA test with Bonferroni post hoc correction if appropriate and expressed as mean ± SD. Comparison between two groups was performed using Student’s t-test. Before and after comparisons were performed with a paired t-test. Differences were considered statistically significant if \(P < 0.05\). Non-linear regression curves were constructed by using a robust exponential two phase decay curve fit. Accuracy of these curves was expressed as the robust standard deviation of the residuals (RSDR).

### 3. Results

#### 3.1 Cell culture characterization and the effect of gap junctional uncoupling by 2-APB

Immunocytochemical analysis of cultures by collagen-I and α-actin double-staining, suitable for distinction between fibroblasts and CMCs, showed that cultures consisted of 17.6 ± 3.1% fibroblasts \((n = 6)\) (Supplementary material online, Figure S1A). Fibroblasts were homogeneously spread across the culture. In addition, cultures showed expression of Connexin43 and Connexin45 as well as heterogeneous expression of Connexin40 in between CMCs, which are the targets for 2-APB, as judged by immunocytochemical staining (Supplementary material online, Figure S1B–D).

#### 3.2 2-APB causes stable multiorotor tachyarrhythmias, resembling sustained VF, in a dose-dependent relation

During optical mapping, spontaneously active control cultures typically showed uniform and fast conduction (Figure 1A). However, after incubation with 2-APB, cultures showed a strong increase in the incidence of spontaneous re-entrant tachyarrhythmias (Figure 1A and B). Furthermore, we observed a significant increase in the complexity of tachyarrhythmias, as judged by the number of phase singularities per square centimetre with increasing dosages of 2-APB (Figure 1A and C and Supplementary material online, Movie S1). As a consequence of the increasing incidence of re-entry with increasing 2-APB dosages (activation is higher during reentrant activation when compared with spontaneous uniform activation), average activation frequency was significantly increased after incubation with increasing dosages of 2-APB (Figure 1D). CV was dose-dependently decreased by treatment with 2-APB (Figure 1E). The decrease in CV remained apparent even when determined only during re-entrant activation (Figure 1E, hatched subsets). Despite the high complexity of the tachyarrhythmias observed, the arrhythmias showed a high degree of stability that resembled sustained VF. In more detail, after initiation of re-entry by treatment with 2-APB cultures showed a minimal extent of rotor meandering, which further decreased significantly with increasing 2-APB dosages (Supplementary material online, Figure S3B). In addition, a relatively low number of fibrillating cultures showed new rotor formations, while all cultures showed minimal dispersion in optical signal amplitude as well as re-entrant cycle length (Supplementary material online, Figure S3C–E), exemplified by stripe analysis of optical mapping recordings (Figure 1F).

#### 3.3 Arrhythmia complexity increase is strongly related to wavelength shortening

To identify factors associated with increased arrhythmia complexity, the relationship between several electrophysiological parameters and complexity were investigated at variable 2-APB concentrations. As increasing 2-APB concentrations dose-dependently increased complexity, while at the same time decreasing CV, expectedly CV showed a strong hyperbolic-like relationship with complexity (RSDR = 1.9) (Figure 2A). Furthermore, \(APD_{80}\) showed a weak inverse correlation with complexity in the low-complexity range.
Figure 1 (A) Activation maps (6 ms isochrone spacing) of control cultures and 5–25 μmol/L 2-APB treated cultures. (B) Quantification of re-entry incidence, (C) complexity, (D) average activation frequency (both uniform and reentrant conduction included), and (E) CV in control cultures and 5–25 μmol/L 2-APB treated cultures (n = 38, n = 38, n = 39, n = 61, and n = 39, respectively). Hatched subsets indicate average CV during re-entry in control and 5 μmol/L 2-APB treated cultures (n = 1 and 1). *P < 0.05 vs. control. (F) Typical line scan analysis across the diameter of a culture treated with 20 μmol/L 2-APB (dotted line indicates rotor position).

Figure 2 Relationship between (A) CV and complexity, (B) APD₈₀ and complexity, (C) wavelength and complexity, and (D) 1/[wavelength]² and complexity in control and 2-APB treated cultures (5–25 μmol/L).
(Figure 2B). However, as at the highest 2-APB concentration the beating frequency decreases (Figure 1D), APD increases in cultures treated with 25 μmol/L 2-APB. This slightly decreased the negative correlation between APD and complexity, while greatly increasing variation in APD. As wavelength is the product of CV and APD, wavelength shortening strongly related to complexity increases (RSDR = 0.4) (Figure 2C). Together, these results support that inversely, complexity may be strongly diminished by increasing wavelength.

3.4 Effects of pharmacological ion current modulators on 1 Hz and minimal APD

To test whether arrhythmia complexity can be diminished by increasing wavelength, several ion channel modulators were selected, which according to their mechanism of action should have an effect on APD and wavelength. However, as fast activation, during fibrillation, can have an effect on activation and inactivation status of targeted ion channels, the effect of the selected ion channel modulators might differ between fibrillation and normal uniform activation. Therefore, we assessed the effect of pharmacological ion channel modulation on APD at 1 Hz electrical activation and at the minimal diastolic interval during 1–10 Hz pacing (measuring minimal APD) in the absence of re-entrant circuits. As expected, nitrendipine, which inhibits I_CaL, significantly shortened the 1 Hz APD by 28% (to 72 ± 12%, P < 0.05 vs. control) (Figure 3A and I), as well as minimal APD (to 84 ± 14%, P < 0.05 vs. control) (Figure 3E and J). Treatment with sotalol and BaCl2 slowed repolarization and thus prolonged 1 Hz APD (to 117 ± 12% P < 0.05 and 162 ± 25% P < 0.05 vs. control, respectively) (Figure 3B, C, and I). However, the effect of sotalol on minimal APD was not significant (Figure 3F and J), while BaCl2 still had a significantly prolonging effect on APD during 10 Hz pacing (to 145 ± 9%, P < 0.05 vs. control) (Figure 3G and J). Additionally, activation of I_CaL by Bayk8644 increased both 1 Hz (to 168 ± 13% P < 0.05 vs. control) (Figure 3D and I) and minimal APD (to 133.7 ± 9.6%, P < 0.05 vs. control) (Figure 3H and J) significantly.

3.5 Different effects of ion channel modulators on 2-APB-induced fibrillation

In order to correlate the effects of selected ion channel modulators on minimal APD with their effects on the characteristics of fibrillation (i.e. APD, wavelength, stability, activation frequency, and complexity), tachyarrhythmic cultures induced by 2-APB were treated with nitrendipine, sotalol, BaCl2, and BayK8644 (n > 24 per agent).

In line with the previous, electrically stimulated experiments, APD80 was significantly decreased by nitrendipine throughout all 2-APB dosages, while APD was increased by both BaCl2 and BayK8644. In
contrast, sotalol did not affect APD significantly (Figure 4A, Supplementary material online, Table S1).

Wavelength was decreased significantly by nitrendipine treatment in the lowest concentration of 2-APB, although there was no significant effect in the other 2-APB dosages. In contrast, wavelength was significantly increased after BaCl$_2$ and BayK8644 treatment while sotalol did not significantly affect wavelength (Figure 4B, Supplementary material online, Table S2).

Interestingly, decreasing the APD with nitrendipine increased activation frequency of tachyarrhythmias. Conversely, lengthening of APD decreased activation frequency as seen after treatment with BaCl$_2$ and BayK8644 throughout all 2-APB dosages (Figure 4C, Supplementary material online, Table S3). Sotalol induced a small but significant decrease in activation frequency in 15 and 20 μmol/L 2-APB treated cultures only.

The average complexity of conduction did not significantly alter after nitrendipine, while sotalol only had a small but significant effect on complexity in 25 μmol/L 2-APB treated cultures. However, in BaCl$_2$ and BayK8644 treated cultures, arrhythmia complexity was significantly lowered throughout all 2-APB concentrations except for the less complex arrhythmias after 10 μmol/L 2-APB (Figure 4D, Supplementary material online, Table S4). These results are indicative of the importance of minimal APD in decreasing and increasing complexity, through specific ion channel modulation.

### 3.6 Mechanism of decrease in arrhythmia complexity by increase in minimal APD

Optical mapping through a permeable membrane during minimal APD prolongation by BayK8644 and BaCl$_2$ again showed that both substances decrease the number of rotors in vitro (Figure 5A). Prior to the addition of BaCl$_2$ or BayK8644, optical signal amplitude, spatial rotor distribution, and rotor cycle lengths were highly stable (Figure 5B and D left). During incubation of BaCl$_2$ or BayK8644, transient instability in optical signal amplitude, spatial rotor distribution, and rotor cycle length was induced (Figure 5B and D middle). Subsequently, a new equilibrium was formed, with increased optical signal amplitude, decreased number of rotors and stable but increased rotor cycle lengths (Figure 5B, D right and Supplementary material online, Figure S4A and B). During incubation of BaCl$_2$ and BayK8644, this transient instability, which was mediated by an increase in wavelength, led to termination of neighbouring rotors. Rotor termination resulted from critical collisions of wavefronts propagated from two different rotors, after which activation of that particular tissue is taken over by a separate pre-existing rotor, decreasing the total number of rotors (Figure 5C and D and Supplementary material online, Movie S2).

### 3.7 2-APB in adult rat heart ventricles

To investigate the functional implications of in vitro findings on the intact heart, Langendorff-perfused adult rat hearts were subjected to ex vivo optical mapping. Baseline activation frequency before addition of BDM to the oxygenated Tyrode perfusate was 4.50 ± 0.5 Hz. After addition of BDM sinus rhythm remained stable for at least 1 h at an average activation frequency of 2.5 ± 0.4 Hz (Supplementary material online, Figure S3A and B). Perfusion with oxygenated Tyrode supplemented with 5 μmol/L 2-APB for 20 min slowed conduction from 54.1 ± 3.2 cm/s to 27.8 ± 2.7 cm/s (n = 6, P < 0.05). No arrhythmias developed at this dosage and sinus rhythm was maintained at 2.5 ± 0.5 Hz (Supplementary material online, Figure S3A–C, P > 0.05 vs. control). Perfusion of hearts with 10 μmol/L 2-APB caused VT in all hearts (2.2 ± 0.2 wavefronts at 5.4 ± 3.3 Hz, n = 6), whereas complexity of arrhythmias increased to fibrillation with 20 μmol/L 2-APB (5.0 ± 1.1 wavefronts at 11.0 ± 0.9 Hz, n = 6) (Supplementary material online, Figure S3A and C). Complexity of arrhythmias stabilized within 10 min of incubation with 2-APB.
3.8 Minimal APD determines arrhythmia complexity in adult rat heart ventricles

To investigate whether BaCl$_2$ lowered arrhythmia complexity ex vivo as in vitro, rat hearts were first perfused with 20 μmol/L 2-APB until fibrillation was present and stable for at least 5 min. Then, 500 μmol/L of BaCl$_2$ was added to the perfusate consisting of tyrode with 20 μmol/L 2-APB. Typically within 5 min, BaCl$_2$ decreased arrhythmia complexity by 71.4% compared with controls (Figure 6A, C, and D, n = 6). Also, activation frequency decreased from 9.98 ± 0.9 to 2.8 ± 0.3 Hz by BaCl$_2$ (Figure 6A and C). Importantly, BaCl$_2$ treatment significantly increased minimal APD$_{90}$ to 265.4 ± 35.1% of control hearts (Figure 6B, n = 6). Together these results show that, similar to in vitro experiments, arrhythmia complexity can be decreased by increasing minimal APD ex vivo.

4. Discussion

The key findings of this study are (i) incubation with 2-APB induces re-entrant tachyarrhythmias in myocardial cultures and adult rat hearts, which are maintained by multiple stable and co-existing rotors, resembling sustained VF, (ii) The complexity of these arrhythmias increases exponentially with increasing dosages of 2-APB,
allowing a systematic study of arrhythmia complexity in vitro and ex vivo, (iii) increasing arrhythmia complexity during fibrillation is associated with a shortening of the average wavelength and APD, (iv) hence, complexity and activation frequency during fibrillation could be decreased pharmacologically by transient destabilization of sustained VF through prolongation of minimal APD in vitro and ex vivo, regardless of ionic mechanism.

4.1 The importance of wavelength in re-entrant tachyarrhythmias

Since the introduction of the circus movement re-entry theory and the leading circle concept, it has been established that if the wavelength of a given re-entrant circuit exceeds the path-length, re-entry cannot be sustained as a consequence of a vanishing excitable gap. Hence, wavelength prolongation has traditionally been viewed as an important anti-arrhythmic strategy. Elaborating on this theory, we now demonstrated that wavelength prolongation affects coexistence of multiple neighbouring rotors, although rotor termination by prolongation of wavelength in single rotor tachyarrhythmias appeared to be more complicated (Figure 4D; 10 μmol/L 2-APB).

The slope in the relationship between wavelength and complexity is steepest in the low-complexity range (<2 rotors) (Figure 2D). This implies that in the lower complexity range a greater wavelength prolongation is necessary to facilitate the same decrease in complexity. Also, the absence of boundaries formed by neighbouring rotors diminishes the chance of rotor termination in the lower complexity range. Moreover, we show that the effect of a given agent on arrhythmia complexity depends on the activity of the agent at high frequency activation, which can differ from its effect at low frequencies. Sotalol, for instance had a significant effect on 1 Hz APD, but not on minimal APD, which may explain the inability of sotalol to terminate rotors during VF. In contrast, increasing minimal APD by BaCl₂ and BayK8644 induced a notable reduction in the number of rotors during VF. However, decreasing minimal APD by nitrendipine treatment did not increase average arrhythmia complexity. This can be explained by the minute tendency for the formation of new wavebreaks during sustained VF, despite an increase in effective size of the culture.

Importantly, in earlier in silico work by Ten Tusscher et al., minimal APD was shown to be predictive of arrhythmia complexity.

Figure 6 (A) Typical sequence (12 ms between snapshots) of activation in (from top to bottom) Langendorff-perfused control hearts, 20 μM 2-APB treated hearts, and 20 μM 2-APB treated hearts after introduction of BaCl₂ with corresponding optical action potential signals. (B) Quantification of minimal APD in control hearts and hearts perfused with 500 μmol/L BaCl₂. (C) Assessment of activation frequency and (D) complexity in hearts treated with 20 μM 2-APB before and after introduction of BaCl₂. *P < 0.05 vs. control.
in animal and human hearts. We now showed that minimal APD is not only predictive of arrhythmia complexity, but that prolongation of minimal APD also strongly and effectively reduced arrhythmia complexity.

### 4.2 APD and CV restitution in fibrillation

Other theories involving prevention or termination of VF mostly originate from the ‘multiple wavelet’ and the ‘mother rotor’ theory.16,27 In general, both assume flattening of APD restitution or narrowing of CV restitution should prevent wavebreaks and consequent disorganization of conduction, which should result in termination of VF.16,27 Indeed, several studies confirmed that flattening of APD restitution by, for instance, verapamil or bretylium can convert VF to VT.10,11,14,28 However, the anti-fibrillatory effects of aforementioned substances are generally confirmed by mapping in silico models during or resembling the first minute after VF induction. Yet, Wiggers et al.7 showed that the heart goes through several stages during VF, by which organization and periodicity increase after the first 2 min.5–8,10 Hence, therapies aiming to decrease disorganization of conduction could prove to be effective only in this first phases of fibrillation. The findings in the present study partially explain Wiggers’ stages III and IV of fibrillation because as ischaemia progresses, gap junctional coupling is reduced which may slow VF into a stable, organized, and periodical multi-rotor arrhythmia. Our data on these typical characteristics of sustained VF are consistent with a previous report of sustained (slow) VF in an isolated rabbit heart model of VF.10 Furthermore, we showed that during these phases of VF, interventions that flatten APD or CV restitution in an attempt to terminate VF may be considered inadequate. This is best exemplified by the effect of the I_{CaL} inhibitor nitrendipine on VF after 2-APB treatment, which according to earlier studies should have flattened APD restitution and terminated VF. However, nitrendipine only caused detrimental effects: increasing activation frequency without affecting complexity. Interestingly, activation of I_{CaL} by BayK8644 treatment, did decrease complexity during VF, exemplifying the reversal of effects of I_{CaL} targeting in the early phases through the later phases of VF.

### 4.3 I_{K1} blockade and VF termination

Another approach to revert sustained VF to VT or sinus rhythm could be to destabilize the rotors maintaining VF in an effort to achieve termination of these rotors by collision with a pre-existing boundary.15,29 In several studies, rotor stability was demonstrated to depend on specific ion channel currents, such as I_{K1}.14,15,29–31 We showed that blockade of I_{K1} during VF decreased rotor frequency and the complexity of conduction, ultimately reverting VF back to VT.32 However, rotor stability during VF did not seem to be specifically dependent on the I_{K1} current but on the effect blockade of I_{K1} had on the minimal APD. Therefore, the decrease in complexity could be reproduced with Bayk8644, a substance that increases minimal APD by augmenting I_{CaL} without affecting I_{K1}.23 By altering the stability of these rotors, collision of rotors with pre-existing boundaries, but also novel critical boundaries formed by wavefronts propagated from adjacent rotors, are enforced, significantly reducing the complexity of activation patterns during fibrillation.

Patients suffering from sustained VF are still in a timeframe to be resuscitated without cerebral damage.34 Therefore, the observation that an increase in minimal APD decreases complexity of sustained VF may have important consequences for the treatment of VF, since VF, when driven by a smaller number of rotors, requires less energy for defibrillation.35

### 4.4 Study limitations

In our study, 2-APB treatment was used to induce arrhythmias. This agent is known to block gap junctional communication, but also the IP3 receptor and TRP channels, while causing activation of voltage-independent calcium channels.18,19,36 Of these effects, gap junctional uncoupling has been shown to be related to arrhythmia induction in CMC cultures as a consequence of wavebreaks caused by pre-existent heterogeneity in gap junctional coupling.2,37,38 In addition, voltage-independent calcium influx may also cause pro-arrhythmic features in 2-APB treated hearts.36 As such, the clinical significance of these mechanisms of VF initiation may be very limited. However, as VF can show the same maintenance properties, regardless of the method of initiation,39 this does not hinder the study of VF maintenance and termination as was performed in the present study.

In the ex vivo mapping experiments, complexity was determined by the number of epicardial wavefronts, instead of the number of rotors. However, in line with previous research, we found that during sustained VF, the number of epicardial rotors is minimal, attenuating the significance of the epicardial number of rotors.10 Moreover, this study makes use of a neonatal rat cardiomyocyte culture model as well as adult rat hearts. Rat hearts differ considerably from human hearts in terms of the ion currents determining the action potential morphology. Hence, the conclusions drawn from this study are only conceptually in relation to the mechanisms of VF maintenance and cannot be readily extrapolated to the human or clinical setting.

### 4.5 Conclusions

Incubation of neonatal rat myocardial cultures or Langendorff-perfused adult rat hearts with increasing dosage of 2-APB allows for the systematic study of arrhythmia complexity, ultimately resembling sustained VF. Arrhythmia complexity and activation frequency during fibrillation can be decreased pharmacologically by transient VF destabilization through prolongation of minimal APD in vitro and ex vivo, regardless of ionic mechanism. Accordingly, this study could provide a novel conceptual framework for future anti-arrhythmic drug design as well as an extension in the rationale for treatment options of sustained VF.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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